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In Vitro Transport Activity and Trafficking of MRP2/*ABCC2* Polymorphic Variants

Xia Wen[1], **Melanie S. Joy**[2], and **Lauren M. Aleksunes**[1,3]

[1]Department of Pharmacology and Toxicology, Rutgers University Ernest Mario School of Pharmacy, Piscataway, NJ 08854, USA

[2]Department of Pharmaceutical Sciences, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Aurora, CO 80045, USA

[3]Environmental and Occupational Health Sciences Institute, Rutgers University, Piscataway, NJ 08854, USA

Abstract

Purpose—Multidrug resistance-associated protein 2 (MRP2/*ABCC2*) is an efflux pump that removes drugs and chemicals from cells. We sought to characterize the expression, trafficking and *in vitro* activity of seven single nucleotide polymorphisms (SNPs) in the *ABCC2* gene.

Methods—*ABCC2* SNPs were generated using site-directed mutagenesis and stably expressed in Flp-In HEK293 cells, which allows targeted insertion of transgenes within the genome. Total and cell surface expression of MRP2 as well as accumulation of substrates (calcein AM and 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate, CDCF) were quantified in cells or inverted membrane vesicles expressing wild-type (WT) or variant forms.

Results—The cell surface expression of the C-24T-, G1249A-, G3542T-, T3563A-, C3972T- and G4544A-MRP2 variants was similar to WT-MRP2. While expression was similar, transport of calcein AM was enhanced in cells expressing the G3542T-, T3563A-, C3972T-, and G4544A-MRP2 variants. By comparison, cells expressing the C2366T-MRP2 variant had 40-50% lower surface expression, which increased the accumulation of calcein AM up to 3-fold. Accumulation of CDCF in inverted membrane vesicles expressing the C2366T-MRP2 variant was also reduced by 50%. In addition, the G1249A-MRP2 variant had decreased transport of CDCF.

Conclusions—Taken together, these data demonstrate that genetic variability in the *ABCC2* gene influences the *in vitro* expression, trafficking, and transport activity of MRP2.

Keywords

MRP2; *ABCC2*; transporter; SNP; variant

Corresponding Author: Lauren M. Aleksunes, Pharm.D., Ph.D., Department of Pharmacology and Toxicology, Rutgers, the State University of New Jersey, Ernest Mario School of Pharmacy, 170 Frelinghuysen Road, Piscataway, NJ 08854, Phone: +1 (848) 445-5518, Fax: +1 (732) 445-0119, aleksunes@eohsi.rutgers.edu.

Introduction

Human multidrug resistance-associated protein 2 (MRP2, *ABCC2*) is an efflux transporter that belongs to the ATP-binding cassette transporter, subfamily C. The proposed structure for MRP2 includes 17 transmembrane helices with three membrane-spanning domains (MSD) and two highly conserved nucleotide binding domains (NBD) (Fig 1). The MRP2 protein localizes to the apical membrane of polarized cells, including hepatocytes, proximal tubule cells and enterocytes where it facilitates the excretion of drugs and chemicals. In particular, MRP2 mediates the ATP-dependent efflux of various drugs and chemicals, including glucuronide, sulfate and glutathione conjugates (reviewed in 1, 2).

Genetic variants in transporter proteins may alter the pharmacokinetics and subsequent pharmacological and toxicological effects of drugs (3-5). For MRP2/*ABCC2,* both mutations and single nucleotide polymorphisms (SNPs) have been described. Missense, nonsense, and splice-site mutations in *ABCC2* cause truncated and dysfunctional MRP2 proteins leading to the genetic disorder, Dubin-Johnson syndrome (6). Individuals afflicted with Dubin-Johnson syndrome exhibit benign conjugated hyperbilirubinemia despite normal liver functioning (7, 8). Beyond these mutations, SNPs in the *ABCC2* gene can alter the *in vitro* activity and/or clinical phenotypes of patients in the absence of overt changes in conjugated bilirubin clearance (5, 9, 10). To date, a number of *ABCC2* variants have been identified with differing allele frequencies across ethnic and racial groups (9, 11-14) (Fig. 1, Table 1).

Prior studies have yielded somewhat inconsistent results regarding the extent to which *ABCC2* SNPs alter MRP2 functioning. One example is the G1249A-MRP2 variant. In a clinical report, it was suggested that individuals expressing the G1249A-MRP2 variant have enhanced MRP2 efflux activity in enterocytes (5). This was postulated as a mechanism to explain the reduced absorption of the β_1-selective blocker and MRP2 substrate, talinolol, in subjects expressing the G1249A-MRP2 variant. In fact, data from *in vitro* systems have demonstrated mixed findings. Recent work suggests that the G1249A-MRP2 variant has higher MRP2-stimulated ATPase activity that is associated with greater efflux of the substrate sorafenib (15). In other work, G1249A-MRP2 was shown to decrease the apparent affinity for glutathione and glucuronide-conjugated substrates (16). However, in yet another study, G1249A-MRP2 had no effect on the transport of the MRP2 substrates, 17β-estradiol-D-glucuronide, leukotriene C4 and 2,4-dinitrophenyl-*S*-glutathione, when expressed in membrane vesicles isolated from LLC-PK1 kidney cells (9). It is possible that these divergent findings result from the utilization of different experimental models. Also, MRP2 may possess multiple orientations for substrates to interact within the substrate binding cavity which could help to explain these results. Nonetheless, additional work is needed to understand the effect of *ABCC2* SNPs on MRP2 functioning using a controlled system that allows for the similar integration of variants into the genome and direct comparisons between SNPs.

In the present study, we aimed to clarify the influence of seven *ABCC2* SNPs on the expression and function of the MRP2 transporter *in vitro* using the Flp-In transfection system. The Flp recombinase allows controlled integration and expression of transfected

genes in Human Embryonic Kidney (HEK) 293 cells at a specific genomic location called the Flp Recombination Target site (FRT) (17). Stably transfected cells included wild-type (WT-MRP2) and C-24T- (in the 5′-UTR), G1249A- (V417I), C2366T- (S789F), G3542T- (R1181L), T3563A- (V1188E), C3972T- (I1324I) and G4544A-MRP2 (C1515Y). These variants were selected based upon their allele frequencies within populations, associations with clinical phenotypes, locations within the MRP2 protein (MSD, NBD), and/or prior *in vitro* studies suggesting they impact MRP2 functioning (9, 11, 12). The stably transfected Flp-In cells were used to determine whether SNPs in the *ABCC2* gene influenced 1) the total and cell surface expression of MRP2 protein, 2) the intracellular accumulation of the substrate calcein AM (18) and 3) the uptake of a second substrate 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCF) into inverted plasma membrane vesicles (19).

Materials and Methods

Reagents and Chemicals

Lipofectamine LTX and PLUS reagents, hygromycin B and zeocin were purchased from ThermoFisher Scientific (Waltham, MA). Unless specified, all other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Site-Directed Mutagenesis

The full-length human *ABCC2* WT cDNA clone was obtained by digestion of a pCMV6-NEO plasmid containing *ABCC2* cDNA (Origene, Rockville, MD) using the restriction enzymes SalI and NotI (New England Biolabs, Ipswich, MA). After purification and sequencing, *ABCC2* WT cDNA was used as a template to generate variant cDNA clones (C-24T, G1249A, C2366T, G3542T, T3563A, C3972T and G4544A, Table 1) by using a QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). All clones and mutagenesis were confirmed by DNA sequencing at Genewiz (South Plainfield, NJ). Primer sequences for generating *ABCC2* variants were provided in Supplemental Table 1.

Cell Culture and Transfection

Flp-In 293 cells (ThermoFisher Scientific) were grown at 37°C with 5% CO_2 in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, glutamine (2 mM) and zeocin (100 μg/ml). *ABCC2* WT and variant cDNAs were inserted into the pcDNA5/FRT vector and transfected into Flp-In 293 cells containing an integrated FRT site. In detail, Flp-In 293 cells (7×10^5/well) were seeded in 6-well plates and allowed to adhere overnight. The opti-MEM I reduced serum medium (ThermoFisher Scientific) was used for the transfection of pcDNA5/FRT/MRP2 (WT or variants) or pcDNA5/FRT (empty vector, EV), pOG44 and Lipofectamine LTX. Stable cell lines were generated using hygromycin B (200 μg/ml) for selection. Colonies were collected and screened for MRP2 expression by Western blot analysis.

Calcein AM Accumulation

The fluorescent substrate calcein AM was used to characterize the transport activity of WT-MRP2 and variants in Flp-In 293 cells (18, 20). In brief, cell suspensions (prepared in

medium, 500,000 cells/ml) were loaded with calcein AM (0.5, 2 or 10 μM) for 30 min at 37°C, 5% CO_2 (*uptake period*). After washing with culture medium, cells were incubated in culture medium for 60 min to allow calcein AM efflux from the cells (*efflux period*). Following washing and centrifugation, the cell pellet was suspended in phosphate-buffered saline (PBS) and relative fluorescence units (RFU) for each cell was quantified on a Cellometer Vision cell counter using filter cube VB-535-402 (excitation/emission: 535/402 nm) (Nexcelom Bioscience LLC, Lawrence, MA).

Cell Surface Protein Isolation and Western Blot Analysis

Localization of WT-MRP2 and variants into the plasma membrane was assessed using the Pierce Cell Surface Protein Isolation kit (ThermoFisher Scientific). A cell-impermeable, cleavable biotinylation reagent (Sulfo-NHS-SS-Biotin) was used to label exposed primary amines of proteins on the surface of cells. The labeled cells were then harvested, lysed and purified using a NeutrAvidin agarose resin. To prepare total cell lysates, cells were placed in lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton-100 and 1% protease inhibitor cocktail. The protein concentration of cell surface preparations or cell lysates was determined by Pierce 660 nm Protein Assay or the Bicinchoninic Acid (BCA) protein assay kit (ThermoFisher Scientific), respectively. For Western blot analysis, cell surface fractions, cell lysates or vesicles isolated from WT-MRP2 and variants cells (15 μg of protein) were separated by SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane at 4°C overnight. After blocking with 5% nonfat dry milk in PBS with 0.5% of Tween 20 (PBS/T), membranes were incubated with primary antibody against MRP2 (M_2III-5, 1:600) (Enzo Life Sciences, Farmingdale, NY) followed by incubation with anti-mouse secondary antibodies (1:2000, Sigma) for 1 to 2 h. The SuperSignal West Dura chemiluminescent substrate (ThermoFisher Scientific) was applied to membranes prior to detection of luminescence using a FluorChem E system (ProteinSimple, San Jose, CA). Target protein band intensities were semi-quantified and normalized to β-ACTIN (Primary ab8227, 1:2000, Secondary 1:2000) or Na^+/K^+ ATPase (Primary ab76020, 1:20,000, Secnodary 1:2000) (Abcam, Cambridge, MA) levels.

MRP2 Protein Localization by Indirect Immunofluorescence Analysis

Indirect immunofluorescence staining was used to assess human MRP2 localization. Cells (2×10^5) were seeded in chamber slides overnight. After fixation in 4% paraformaldehyde, cells were blocked with 5% goat serum in 0.1% PBS-Triton X for 1 h and then incubated with an anti-MRP2 antibody (M_2III-6, 1:150, Enzo Life Sciences) overnight. Sections were washed and incubated with a goat anti-mouse IgG Alexa 488 antibody (*green*, 1:1000, ThermoFisher Scientific) for 1 h. Images were acquired using a Leica TCS SP5 DMI6000CS inverted confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL). Negative controls without primary antibody were included to ensure minimal nonspecific staining. Images are shown at 63× magnification.

RNA Isolation and mRNA Quantification

Total RNA from Flp-In 293 cell expressing MRP2 WT and variants was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The

concentration of total RNA was quantified by UV spectrophotometry at 260/280 nm using a Nanodrop spectrophotometer 2000 (Thermo Fisher Scientific, Wilmington, DE). The messenger RNA (mRNA) expression of human MRP2 was quantified by qPCR assay using Sybr Green as detection of amplified products in the ABI 7900HT PCR system (Applied Biosystems, Carlesbad, CA). Ct values were converted to ΔΔCt by comparing to a reference gene GAPDH. The following primers (forward and reverse, respectively) were used for MRP2 (5′-AGCCATGCAGTTTTCTGAGGCCT-3′ and 5′-TGGTGCCCTTGATGGTGATGTGC-3′), GAPDH (5′-GACATCAAGAAGGTGGTGAA-3′ and 5′-CGTTGTCATACCAGGAAATG-3′). MRP2 primer sequences did not overlap with the SNP variant sites.

Isolation of Membrane Vesicles

Membrane vesicles were isolated from Flp-In 293 WT-MRP2 and variants cells as previously described (21). Briefly, cells were harvested by centrifugation at 3500 × *g* for 20 min. Cell pellets were then suspended in ice-cold hypotonic buffer (0.3 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 0.1 mM EDTA and 1% protease inhibitor), mixed at 4°C for 20 min and centrifuged at 100,000 × *g* for 30 min. The obtained pellet was homogenized using a tight-fitting Dounce homogenizer for 25 strokes in ice-cold TS buffer (10 mM Tris-HEPES, 250 mM sucrose, pH7.4) containing 1% protease inhibitor. After centrifugation at 4000 × *g* at 4°C for 20 min, the supernatant was centrifuged at 100,000 × *g* at 4°C for 60 min. The membrane pellet was resuspended in ice-cold TS buffer and passed through a 27-gauge needle 25 times to obtain vesicles. Protein concentrations were measured using the BCA protein assay kit (ThermoFisher Scientific). MRP2 protein expression was quantified by Western blotting analysis as described above.

CDCF Transport into Membrane Vesicles

To quantify transport in membrane vesicles, vesicles (50 μg) were incubated with CDCF (2.5, 10 and 50 μM) in reaction buffer (50 mM MOP-Tris, 70 mM KCl, 7.5 mM $MgCl_2$) including 200 mM glutathione and 10 mM MgATP at 37°C for 10 min. Reactions were stopped by adding ice-cold buffer (40 mM MOPS-Tris, 70 mM KCl) and transferred to a 96-well multiscreen filter plate (Millipore, Billerica, MA). After washing, vacuum filtration, and solubilization with 50% methanol, CDCF fluorescence was read at an excitation wavelength of 504 nm and emission wavelength of 529 nm in a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA).

Data Analysis

Quantitative results were expressed as mean ± S.E. and analyzed using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA). Student's t test was used for comparison of two groups. One-way ANOVA with a Tukey's multiple comparisons test was used to compare groups of 3 or more. Data were log transformed prior to statistical analysis if they were not normally distributed. Statistical significance was set at $p < 0.05$.

Results

Expression and activity of the MRP2 transporter using the Flp-In system

MRP2 protein was not detected in empty vector-containing cells (Figs. 2A and B). Transfection of the human *ABCC2*-containing plasmid into Flp-In 293 cells led to MRP2 protein expression in both cell lysates and at the plasma membrane (Figs. 2A and B). Accordingly, accumulation of the MRP2 substrate, calcein AM, was 4-fold lower in cells expressing WT-MRP2 compared to empty vector-containing cells (Fig. 2C).

Intracellular accumulation of calcein AM in wild-type MRP2 and variant cells

Calcein AM is a fluorescent substrate of MRPs and multidrug resistance protein 1 (18). In the present study, calcein AM was used to evaluate MRP2 transport activity in Flp-In 293 WT-MRP2 and variant cells, as MRP2 was the only efflux transporter expressed. Compared to WT-MRP2 cells, accumulation of calcein AM (0.5 μM) was significantly increased by 1.5-fold in the C2366T-MRP2 cells, while accumulation was 20-30% lower in cells expressing the G3542T-, C3972T- and G4544A-MRP2 variants (Fig. 3A). Further analysis was performed across a range of calcein AM concentrations (0.5, 2, 10 μM) (Figs. 3B). The C2366T-MRP2 variant demonstrated greater calcein AM accumulation at 0.5 and 2 μM (Fig. 3B). By comparison, calcein AM accumulation was lower in the G3542T-, T3563A-, C3972T-, and G4544A-MRP2 variants compared to WT-MRP2 (Figs. 3B).

Protein expression and localization of MRP2 in wild-type and variant cells

Compared to cells expressing WT-MRP2, total protein expression was significantly lower in cells expressing C2366T-MRP2 (decreased by 50%) and higher in T3563A-, C3972T- and G4544A-MRP2 variants (30-40% increase, Fig. 4A). Trafficking of MRP2 to the cell surface was measured using the biotinylation of amine groups on the exterior of the plasma membrane. As shown in Fig. 4B, protein expression at the cell surface was reduced 60% for the C2366T-MRP2 variant compared to WT-MRP2 cells. Slight, but insignificant, changes of 10-20% were observed in the cell surface expression of MRP2 in cells with other variants. Of note, after normalizing calcein AM transport to MRP2 expression in the plasma membrane, the extent of intracellular accumulation of calcein AM was similar between WT-MRP2 and C2366T-MRP2 variant cells, suggesting that the dysfunction of calcein AM transport in C2366T-MRP2 variant resulted from an overall decrease in MRP2 protein expression.

As protein expression was consistently decreased in C2366T-MRP2 cell lysates and cell surface fractions, the subcellular localization and protein expression of MRP2 were also further assessed by indirect immunofluorescence (Fig. 5). Detection of MRP2 protein in WT-MRP2 and C2366T-MRP2 variant cells (*green)* revealed reduced protein expression in the variant cells that was consistent with the lower expression levels seen in cell lysates and at the cell surface. The staining of MRP2 protein in cells expressing the other MRP2 variants, including G1249A-, G3542T-, T3563A-, C3972T- and G4544A-MRP2 was also performed (Fig. 5). MRP2 was consistently expressed in the plasma membrane of all variant cells (*green*). The intracellular intensity of fluorescent staining tended to be higher in T3563A-, C3972T- and G4544A-MRP2 variants compared to cells expressing WT-MRP2,

which was consistent with the elevated MRP2 protein expression observed in cell lysates (Fig. 4A). For these variants, the increase in MRP2 expression may represent greater protein accumulation in the intracellular compartment, rather than at the cellular surface (Fig. 4B).

Because glutathione (GSH) is an important co-substrate that can alter MRP2 activity, we also measured cellular levels. Intracellular GSH concentrations in WT-MRP2 and variant cells were similar (Supplementary Fig. 1).

MRP2 expression and CDCF transport in wild-type and variant inverted membrane vesicles

The ability to detect differences in transport activity between MRP2 variants may be dependent upon the selection of substrate. Therefore, the transport of CDCF, another substrate of MRP2 (19), into inverted plasma membrane vesicles isolated from WT-MRP2 and variant cells was also performed. Compared to vesicles containing WT-MRP2, vesicles obtained from cells expressing the C2366T-MRP2 variant exhibited less MRP2 protein (Fig. 6A). In addition, decreased MRP2 protein expression was observed in vesicles containing the G3542T- and G1249A-MRP2 variants. CDCF transport was lower in vesicles expressing the G1249A- and C2366T-MRP2 variants compared to the vesicles with MRP2 WT (Figs. 6B and C). Following normalization of transport rates to MRP2 protein expression in the vesicles, there was no difference in the intrinsic activity of the C2366T-MRP2 variant, further suggesting that the change of MRP2 transport activity in inverted membrane vesicles with the C2366T-MRP2 variant was related to an overall decrease in protein expression. However, this was not the case for the G1249A-MRP2 variant. The transport of CDCF to G1249A vesicles was still approximately 30-50% lower compared to WT-MRP2 after normalizing to its MRP2 protein content, indicating a reduced functional ability of the G1249A-MRP2 variant to transport CDCF (Fig. 6C).

Discussion

In the present study, we characterized the protein expression, trafficking, and transport activity of seven MRP2 variants in intact cells and inverted membrane vesicles. In Flp-In 293 cells expressing WT-MRP2 and variants, transport of calcein AM was decreased in cells expressing the C2366T-MRP2 variant, while it was increased in G3542T-, T3563A-, C3972T-, and G4544A-MRP2 variant cells. The gain-of-function for the G3542T-, T3563A-, C3972T-, and G4544A-MRP2 variants likely resulted from differences in transporter activity compared to wild-type cells since the cell surface expression of each variant was unchanged. By comparison, the reduced efflux of calcein AM likely resulted from lower expression of C2366T-MRP2 in the plasma membrane rather than changes in intrinsic activity. Supporting this finding, the uptake of CDCF was also decreased in inverted membrane vesicles from cells expressing the C2366T-MRP2 variant. Interestingly, the G1249A-MRP2 variant exhibited decreased CDCF transport in membrane vesicles whereas no change was seen in the cellular extrusion of calcein AM. These divergent results may represent a difference in substrate interactions with G1249A-MRP2, a nonsynonymous variant found in MSD1 on the cytoplasmic loop between transmembranes 7 and 8. It is known that MRP2 possesses at least two drug binding sites, including a site where the substrate is transported and a second site that can modulate transport (22). While four basic amino acids not studied in this paper have

been identified as key residues for MRP2 efflux (23), it is possible that SNPs in additional locations can modify transport of some substrates. Taken together, the data suggest that genetic variability in the MRP2 efflux transporter can influence not only its expression, but also its transport activity in a substrate-dependent manner.

MRP2 is responsible for the efflux of GSH as well as GSH conjugates of drugs and chemicals (24, 25). GSH serves as a cofactor that enables MRP-mediated efflux (26, 27). Importantly, we found that the intracellular GSH concentrations were similar between cells expressing WT-MRP2 and the variants (Supplemental Fig 1). While this means that GSH was sufficiently available to serve as a cofactor, the possibility remains that the interaction of GSH with MRP2 was altered in the variant cells. Prior work has pointed to cytoplasmic loop 3 (between transmembrane 5 and 6) as an important region for interaction with GSH (28); however, there is the potential that other portions of the MRP2 protein may also be involved. GSH was included as a cofactor for the inverted vesicle assays, however, other studies suggest that may not be necessary in order to transport CDCF by MRP2 (29, 30). Because of this, the availability and/or interaction of GSH with variant forms of MRP2 may not explain the differences in functional activity that was observed between variants.

Our data are largely consistent with prior work investigating the functional consequences of MRP2 SNPs on *in vitro* transport activity. Compared to experimentation with other intact cell lines or plasma membrane vesicles, similar results were observed using the Flp-In recombination system. In vesicles obtained from LLC-PK1 kidney cells expressing C2366T-MRP2, the protein expression of MRP2 was significantly lower compared to its expression in WT-MRP2 (9) (Fig 4). This corresponded with reduced transport of leukotriene C4, estradiol-3-glucuronide, estradiol-17β-glucuronide and tauroursodeoxycholic acid in *Sf9*-derived membrane vesicles containing C2366T-MRP2 (16). By contrast, fibrosarcoma-related Rht14-10 cells expressing the G3542T-MRP2 variant showed decreased efflux of glutathione-monochlorobimane, which was associated with lower total MRP2 protein expression (11). In the current study, we observed only a trend for decreased MRP2 protein expression at the surface of G3542T-MRP2 cells that was not statistically significant. However, cells expressing G3542T-MRP2 exhibited increased transport of calcein AM compared to WT-MRP2. Similar enhancement of transport activity for the G3542T-MRP2 variant has been observed in cells treated with the substrate glutathione-methylfluorescein despite lower MRP2 protein expression (11). It is likely that altered transport activity by MRP2 variants may be substrate-dependent and influenced by the location and nature of the amino acid change in the MRP2 protein.

To date, multiple clinical studies have reported associations between genetic variation in the *ABCC2* gene and drug efficacy or adverse drug reactions (5, 31). For example, patients expressing the *ABCC2* variant C-24T have reduced clearance of methotrexate, mycophenolic acid and irinotecan (32-35). However, the *in vitro* findings in the current study did not correspond with these clinical data. This discrepancy points to alternate regulatory mechanisms *in vivo* that are masked in an overexpression system.

In addition to associations between the C-24T-MRP2 variant and altered pharmacokinetics, studies have also investigated the impact of the G1249A-MRP2 SNP on protein expression

in human tissues. In human placentas, carriers of the variant allele (1249AA and 1249GA) had reduced enrichment of MRP2 mRNA early in gestation (36). Conversely, G1249A-MRP2 has been associated with higher MRP2 protein levels in human liver tissues (37). In the current study, the G1249A variant was not associated with significant changes for MRP2 mRNA (Supplemental Fig. 2), protein expression or calcein AM efflux in intact cells. However, in inverted membrane vesicles, lower uptake of CDCF by MRP2 was observed in cells expressing the G1249A-MRP2 variant compared to WT-MRP2s. Thus, further work is needed to understand the tissue-specific influences imparted by polymorphic variants on MRP2 expression in addition to functional changes.

The mechanisms by which genetic variants can influence transporter expression are an avenue of significant research. Several factors, including indirect regulation of DNA-protein binding properties, mRNA secondary structure, mRNA stability, protein expression-posttranscriptional alterations (10), stability of the protein in endoplasmic reticulum, susceptibility to ubiquitin-mediated proteasomal degradation (38), interaction with microRNAs (39) and direct regulation to transport activity, have been suggested as mechanisms for altered expression and/or activity of transporter variants. For example, altered protein expression for the G3542T-MRP2 variant was linked to altered synthesis or stability of the *ABCC2* SNP mRNA in fibrosarcoma cells (11). The G4544A-MRP2 variant exhibited impaired ATPase activity and resulted in higher cellular accumulation of lopinavir, calcein and carboxyfluorescein diacetate in HEK293 cells, consistent with reduced transport efficiency (31). In a previous study, the decrease in MRP2 protein levels in LLC-PK1 cells expressing the C2366T-MRP2 variant was related to impaired maturation and trafficking of MRP2 from the endoplasmic reticulum to the Golgi complex and ultimately to the increased degradation of the protein (8, 9). As a result, there are multiple mechanisms by which genetic variants can affect MRP2 trafficking, expression, and activity leading to both tissue- and substrate-dependent differences that affect the overall pharmacokinetics of drugs.

Conclusion

In conclusion, the C2366T-MRP2 variant had lower total and cell surface MRP2 expression as well as decreased efflux of calcein AM compared to WT-MRP2. The accumulation of CDCF in the C2366T-MRP2 variant inverted membrane vesicles was also reduced compared to vesicles containing WT-MRP2. The G1249A-MRP2 variant exhibited decreased CDCF transport. By contrast, the transport of calcein AM was enhanced in cells expressing the G3542T-, T3563A-, C3972T-, and G4544A-MRP2 variants. These data indicate that genetic variability in the *ABCC2* gene influences the *in vitro* expression, trafficking, and transport activity of MRP2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BCA	bicinchoninic acid
CDCF	5(6)-carboxy-2′,7′-dichlorofluorescein diacetate
EV	empty vector
Flp-In 293 cells	Flp-In human embryonic kidney 293 cells
MRP2	multidrug resistance-associated protein 2
MSD	membrance-spanning domain
NBD	nucleotide-binding domain
RFU	relative fluorescence units
SNPs	single nucleotide polymorphisms
WT	wild-type

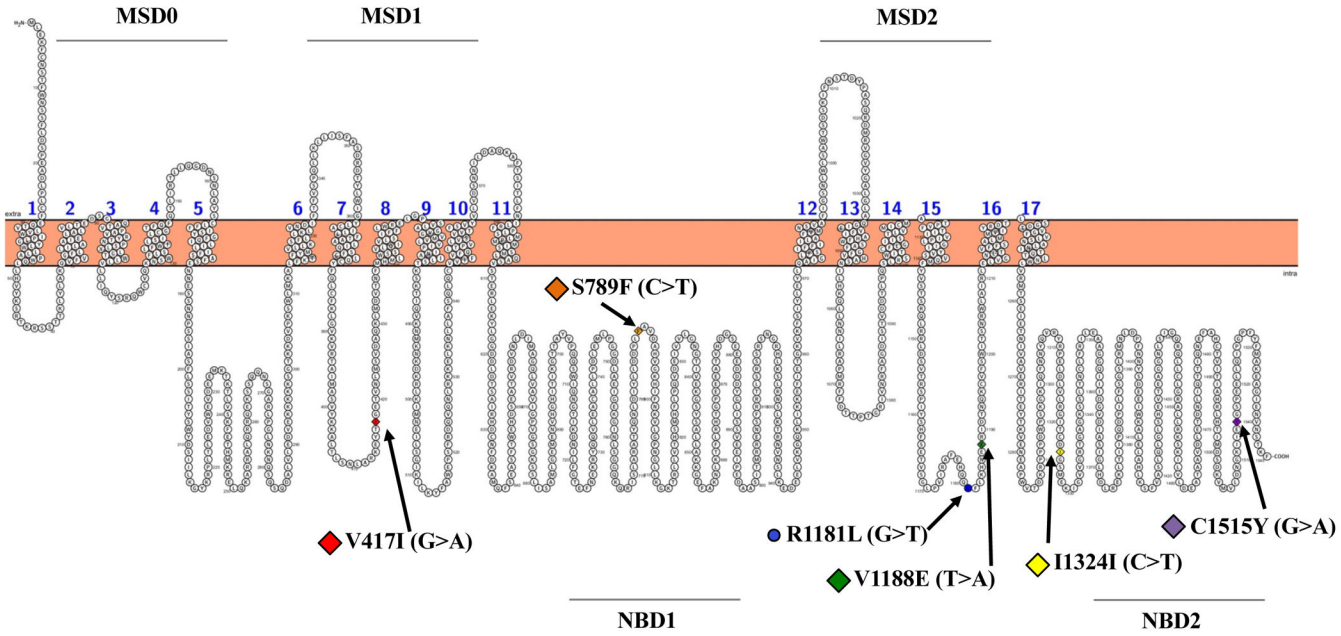

Figure 1. Localization of variants in a predicted topology model of the human MRP2 transporter

The full-length model of the MRP2 protein was generated using the open source tool Protter (http://wlab.ethz.ch/protter/start/). The C-24T variant is found in the 5′-untranslated region of the *ABCC2* promoter and not shown in the figure. MSD: membrane-spanning domain; NBD: nucleotide-binding domain.

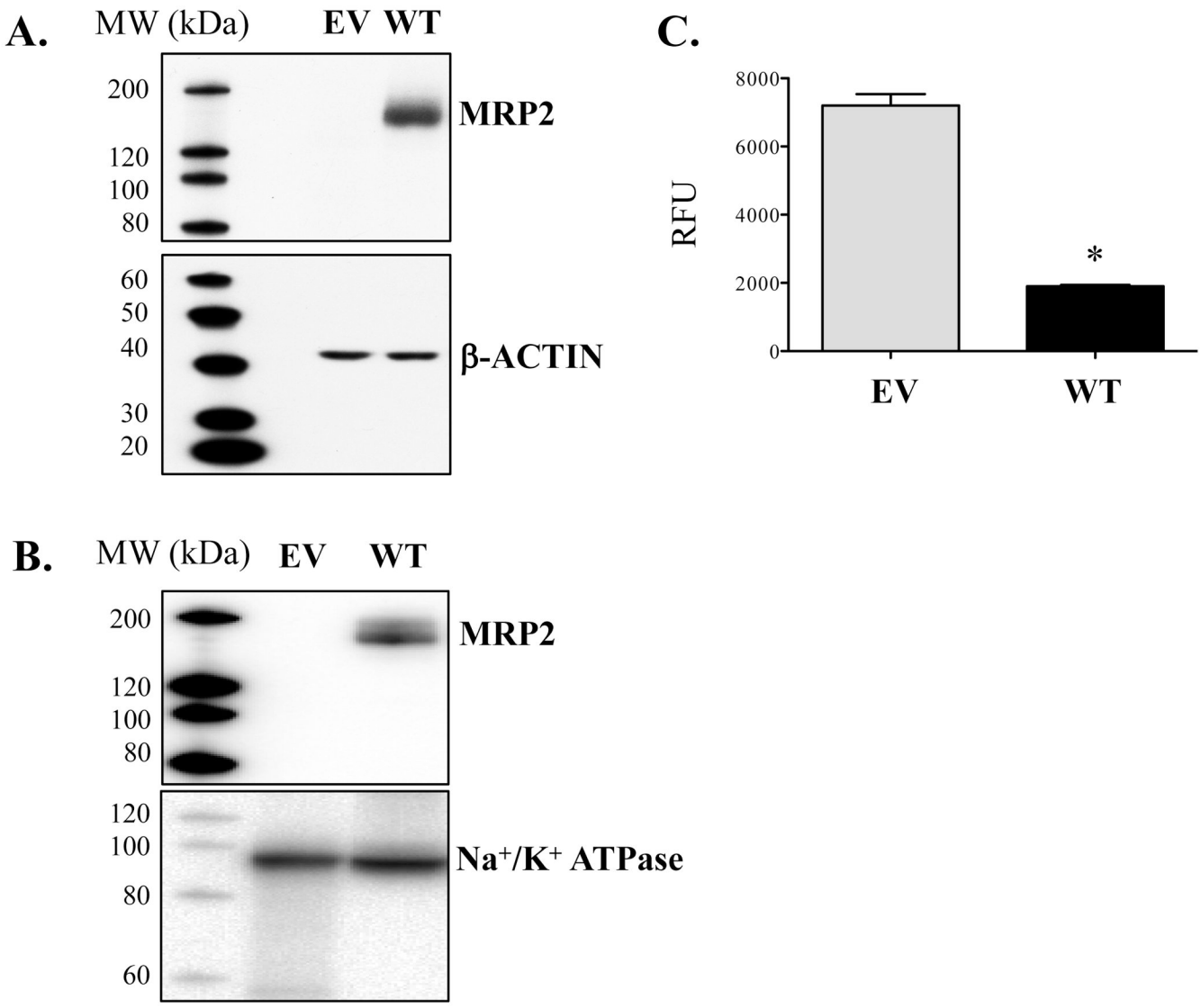

Figure 2. Expression and activity of the MRP2 transporter using the Flp-In system

Flp-In 293 cells were stably transfected with empty vector (EV) or full-length human *ABCC2* plasmids (wild-type, WT). Protein expression of MRP2 in cell lysates (A) and biotinylated membrane fractions (B) was detected by Western blot analysis. β-ACTIN and Na^+/K^+ ATPase were used as loading controls for total and cell surface expression, respectively. (C) EV and WT-MRP2 cells were treated with calcein AM (0.5 μM) for 30 min (*uptake period*), washed, and then incubated with fresh culture media for 60 min (*efflux period*). Intracellular accumulation of calcein AM was determined at the end of the efflux period using a fluorescent cell counter. Data are expressed as relative fluorescence units (RFU) and presented as mean ± SE (n=4). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to EV.

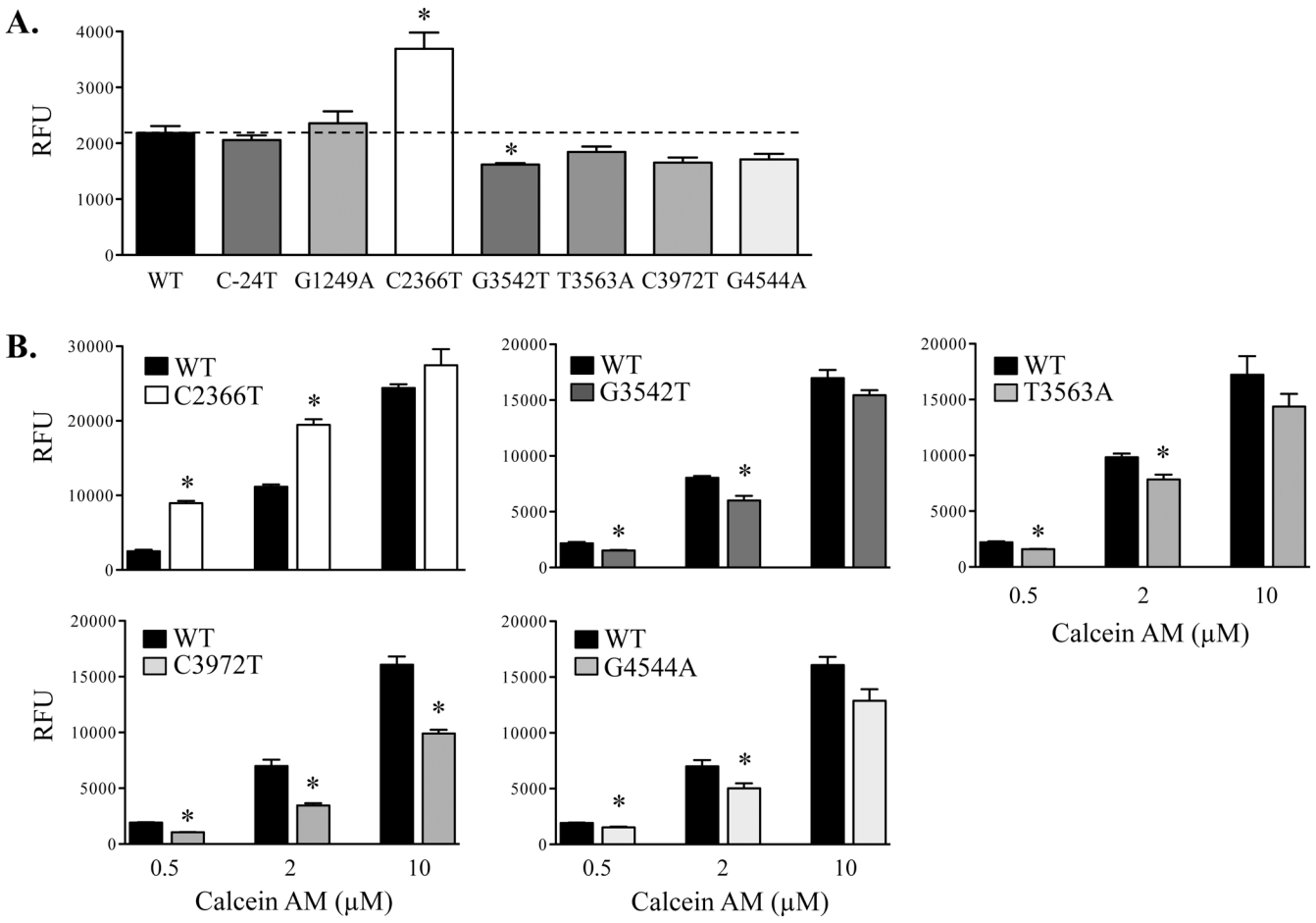

Figure 3. Transport of calcein AM in MRP2 wild-type and variant cells

Flp-In 293 cells stably expressing *ABCC2* wild-type (WT) or variant plasmids were exposed to calcein AM for 30 min (*uptake period*), washed, and then incubated with fresh culture medium for 60 min (*efflux period*). Intracellular calcein AM accumulation was determined at the end of the efflux period using a fluorescent counter. (A) WT-MRP2 and variant cells were exposed to calcein AM (0.5 μM). (B) WT-MRP2 and variants were exposed to calcein AM (0.5, 2, and 10 μM). Data are expressed as relative fluorescence units (RFU) and presented as mean ± SE (n=4-6). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to WT.

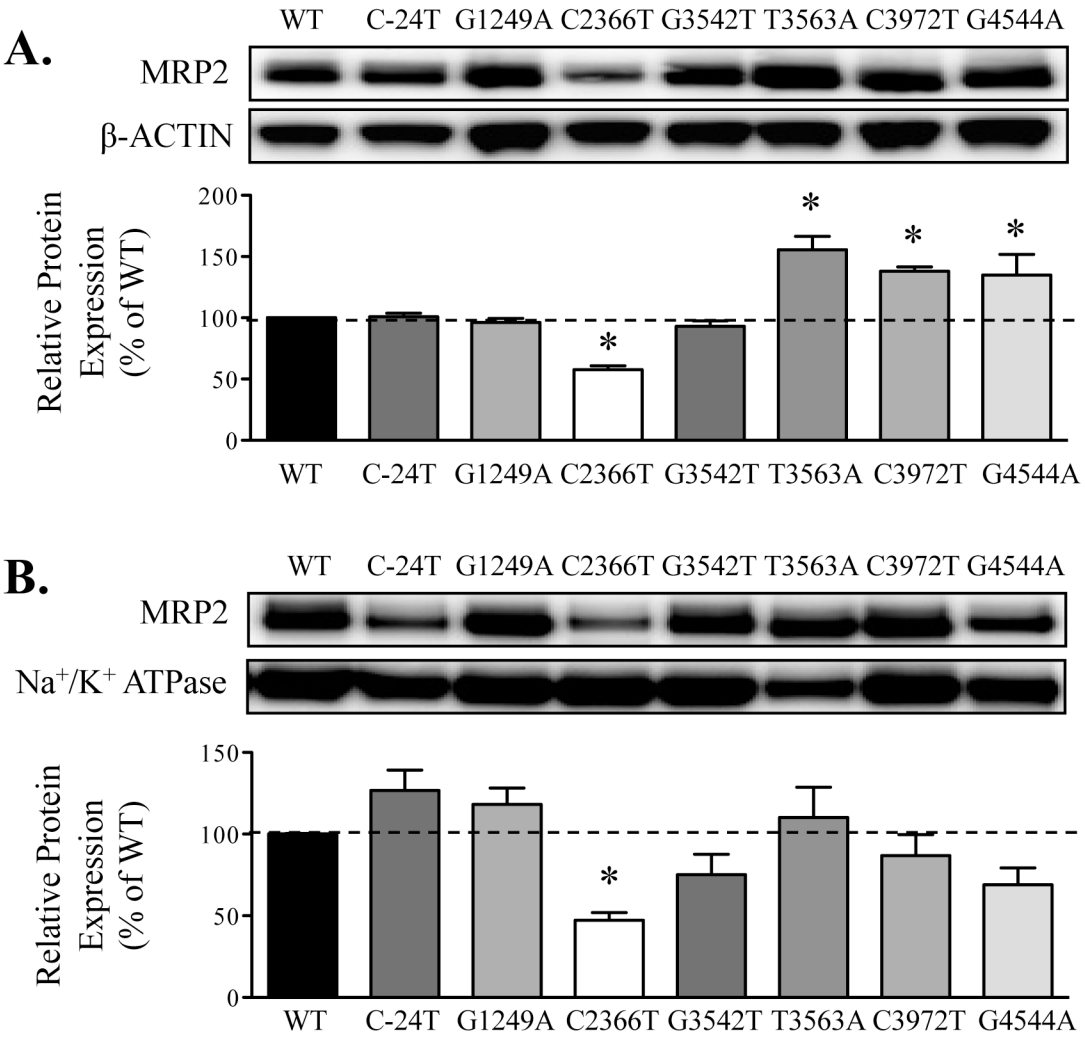

Figure 4. Protein expression and localization of MRP2 in wild-type and variant cells

Flp-In 293 cells were stably transfected with *ABCC2* wild-type (WT) or variant plasmids. Protein expression of MRP2 in cell lysates (A) and biotinylated membrane fractions (B) was detected by Western blot analysis. β-ACTIN and Na^+/K^+ ATPase were used as loading controls for total and cell surface expression, respectively. Data are expressed as relative protein expression and presented as mean ± SE (n=3). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to WT.

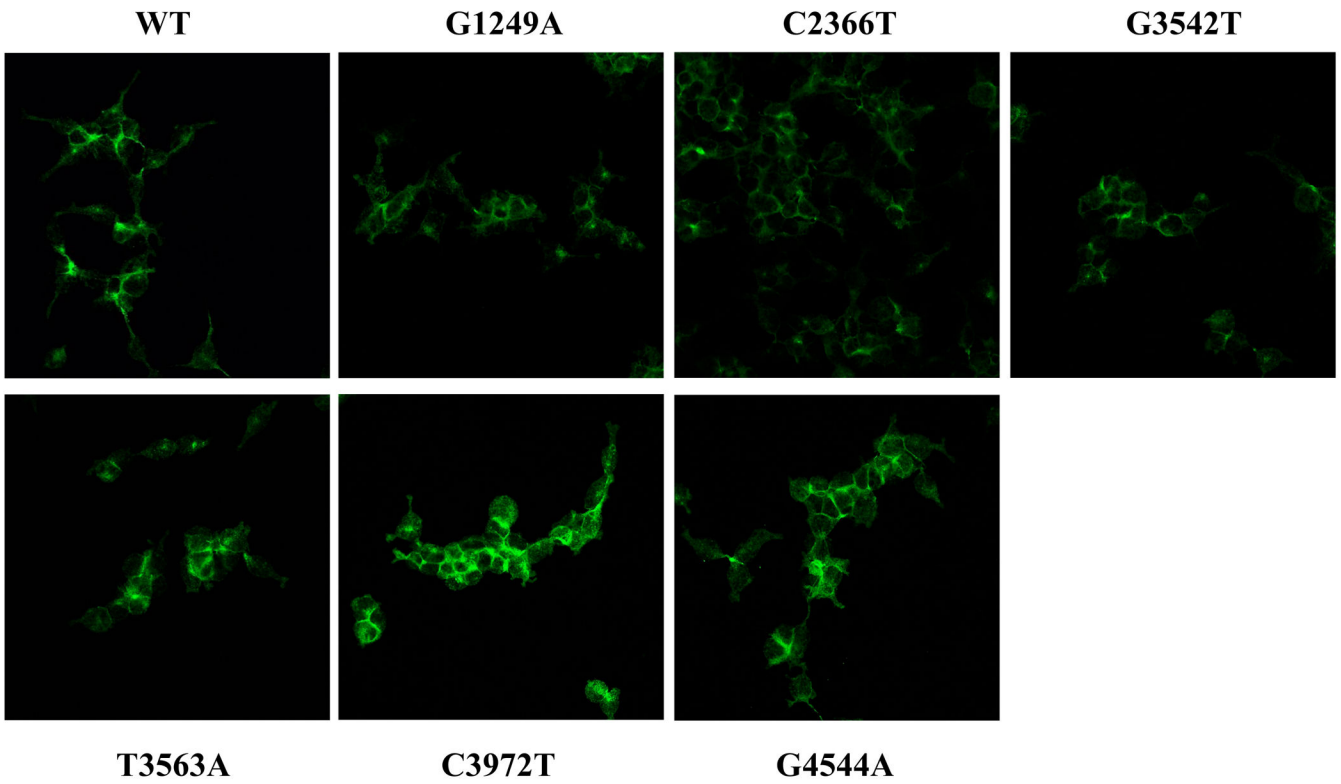

Figure 5. Localization of MRP2 protein in wild-type and variant cells

Flp-In 293 cells were stably transfected with *ABCC2* wild-type (WT) or variant plasmids. Staining of MRP2 protein in WT, G1249A, C2366T, G3542T, T3563A, C3972T, and G4544A variant cells was performed using indirect immunofluorescence analysis. Cells were incubated with an anti-MRP2 antibody (M_2III-6) (*green*). Images are shown at 63× magnification.

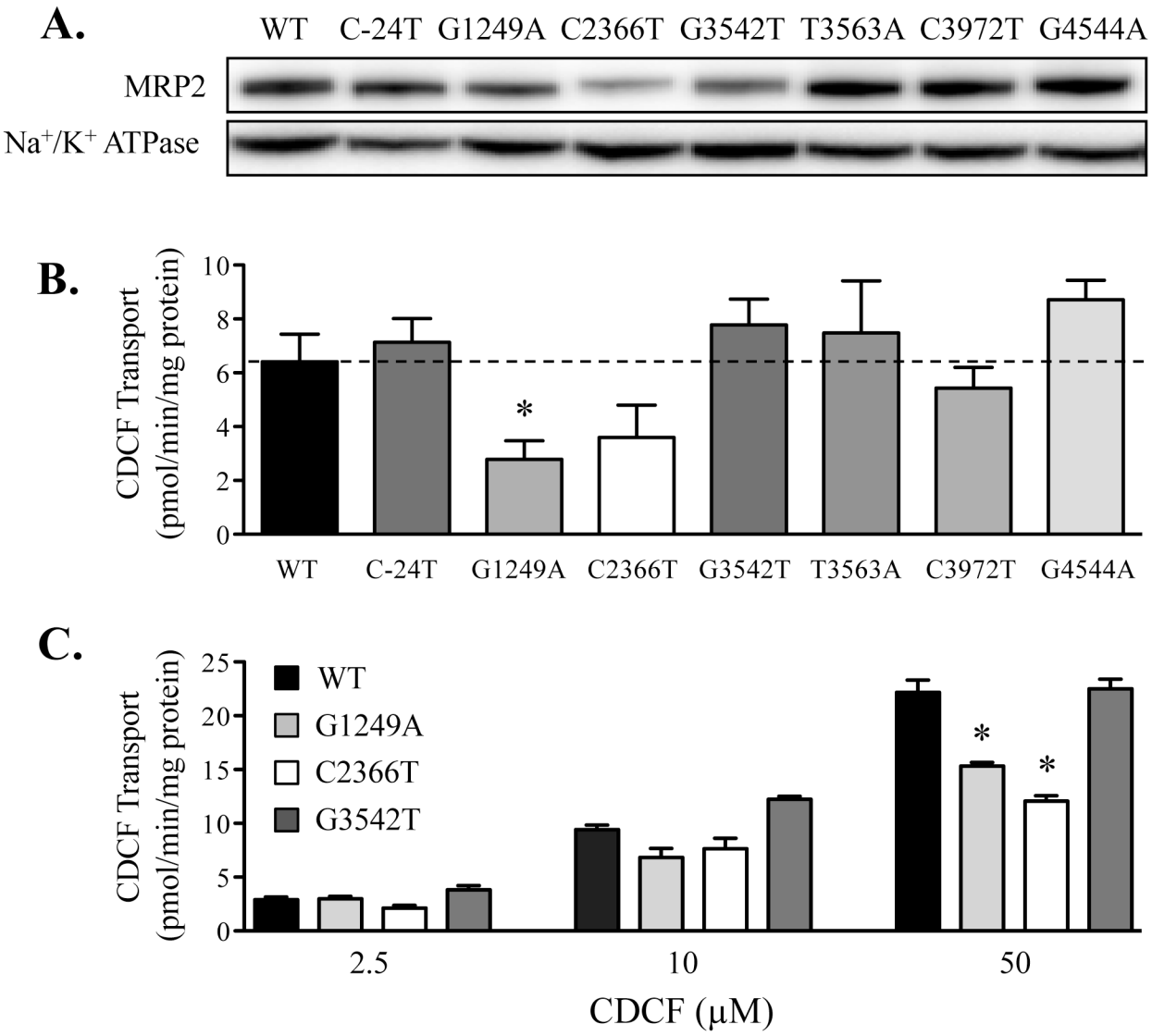

Figure 6. Expression of MRP2 protein and CDCF transport in membrane vesicles isolated from wild-type and variant cells

Flp-In 293 cells were stably transfected with *ABCC2* wild-type (WT) or variant plasmids. (A) Protein expression of MRP2 in membrane vesicles isolated from cells was detected by Western blot analysis. Na^+/K^+ ATPase was used as a loading control. (B) CDCF (10 μM) transport in membrane vesicles isolated from cells expressing WT-MRP2 and variants. Transport of CDCF (10 μM) in empty vector-transfected vesicles was 4.2 pmol/min/mg protein. (C) CDCF (2.5, 10 and 50 μM) transport in membrane vesicles isolated from cells expressing WT-MRP2 and variants G1249A, C2366T, G3542T. Transport was conducted using vesicles (50 μg) incubated with CDCF at 37°C for 10 min. Data are presented as mean ± SE (n=3-6). Asterisks (*) represent statistically significant differences ($p < 0.05$) compared to WT.

Table 1

Selected Genetic Polymorphisms in the Human *ABCC2* Gene

db SNP id[a]	Variant mRNA position[b]	Nucleotide change	Amino acid translation[c]	Exon	Population[d]/Chromosome Sample Count	Allele Frequency in HapMap[e]
rs717620	C-24T	C>T	5′-UTR	-	CEU/226	T: 0.18; C: 0.82
					HCB/86	T: 0.22; C: 0.78
					JPT/172	T: 0.19; C: 0.81
					YRI/226	T: 0.03; C: 0.97
rs2273697	G1249A	G>A	V417I	10	CEU/226	A: 0.24; G: 0.76
					HCB/86	A: 0.07; G: 0.93
					JPT/172	A: 0.13; G: 0.87
					YRI/226	A: 0.22; G: 0.78
rs56220353	C2366T	C>T	S789F	18	Not available	
rs8187692	G3542T	G>T	R1181L	25	CEU/120	T: 0.00; G: 1.00
					HCB/90	T: 0.01; G: 0.99
					JPT/172	T: 0.01; G: 0.99
					YRI/226	T: 0.14; G: 0.86
rs17216324	T3563A	T>A	V1188E	25	Not available	
rs3740066	C3972T	C>T	I1324I	28	CEU/120	T: 0.34; C: 0.66
					HCB/90	T: 0.27; C: 0.73
					JPT/88	T: 0.28; C: 0.72
					YRI/120	T: 0.27; C: 0.73
rs8187710	G4544A	G>A	C1515Y	32	CEU/226	A: 0.05; G: 0.95
					HCB/90	A: 0.00; G: 1.00
					JPT/90	A: 0.00; G: 1.00
					YRI/226	A: 0.12; G: 0.88

[a] Single Nucleotide Polymorphism Database (http://www.ncbi.nlm.nih.gov/snp)

[b] NM_000392

[c] NP_000383

[d] CEU: European; HCB: Han Chinese; JPT: Japanese; YRI: Sub-Saharan African

[e] HapMap data obtained from the Single Nucleotide Polymorphism database (dbSNP)