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ORIGINAL ARTICLE

**Kuniaki Morisaki · Robert W. Robey
Csilla Özvegy-Laczka · Yasumasa Honjo
Orsolya Polgar · Kenneth Steadman
Balázs Sarkadi · Susan E. Bates**

Single nucleotide polymorphisms modify the transporter activity of ABCG2

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Abstract Single nucleotide polymorphism (SNP) analyses of the *ABCG2* gene have revealed three nonsynonymous SNPs resulting in the amino acid changes at V12M, Q141K and D620N. To determine whether the SNPs have an effect on drug transport, human embryonic kidney cells (HEK-293) were stably transfected with full length *ABCG2* coding wild-type or SNP variants of ABCG2. In 4-day cytotoxicity assays with mitoxantrone, topotecan, SN-38 or diflomotecan, cells transfected with wild-type R482 ABCG2 showed IC_{50} values up to 1.2-fold to 5-fold higher than cells expressing comparable levels of Q141K ABCG2, suggesting that the Q141K SNP affects drug transport. FTC-inhibitable mitoxantrone efflux normalized to ABCG2 surface expression as assayed by the anti-ABCG2 antibody 5D3 was significantly lower in cells transfected with Q141K ABCG2 than in those transfected with wild-type R482 ABCG2 ($P = 0.0048$). Values for V12M and D620N ABCG2 were comparable to those for wild-type R482 ABCG2. The vanadate-sensitive ATPase activity of ABCG2 was assayed in Sf9 insect cells infected with wild-type or SNP variants of ABCG2. Basal ATPase activity in cells transfected with Q141K ABCG2 was 1.8-fold lower than in cells transfected with wild-type ABCG2, but was comparable among cells expressing wild-type, V12M or D620N ABCG2. Confocal studies of ABCG2 localization revealed higher intracellular staining in the Q141K transfectants than in cells transfected with wild-type or V12M ABCG2. Decreased transport of Hoechst 33342 was observed in Sf9 cells expressing V12M ABCG2; however, this was not true in HEK-293 cells expressing V12M ABCG2. These results suggest that the Q141K SNP affects the transport efficiency of ABCG2 and may result in altered pharmacokinetics or drug-resistance profiles in clinical oncology.

Keywords Nucleotides · Polymorphism · ABCG2

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K. Morisaki · R. W. Robey · Y. Honjo · O. Polgar
K. Steadman · S. E. Bates (✉)
Cancer Therapeutics Branch, Center for Cancer Research,
National Cancer Institute, National Institutes of Health,
9000 Rockville Pike, Bldg 10 Rm 12C103,
Bethesda, MD 20892, USA
E-mail: sebates@helix.nih.gov
Tel.: +1-301-402-1357
Fax: +1-301-402-1608

C. Özvegy-Laczka · B. Sarkadi
National Medical Center, Institute of Hematology
and Immunology, Membrane Research Group,
Hungarian Academy of Sciences, 1113 Budapest, Hungary

Introduction

The multidrug resistance phenotype of tumor cells is believed to be one of the mechanisms by which cancer cells elude the cytotoxic effects of chemotherapeutic agents. ATP-binding cassette (ABC) transporters, such as P-glycoprotein (Pgp) and the multidrug resistance-associated protein (MRP1), have been shown to confer resistance to a variety of chemotherapeutic agents by transporting drug substrates from cells in an energy-dependent manner [8, 15]. Overexpression of these transporters in cancer cells thus has the potential to limit the efficacy of anticancer agents. The human ABC half-transporter ABCG2 has also been identified as a protein responsible for conferring the multidrug resistance phenotype [3, 13, 29]. ABCG2 overexpression has been detected in cell lines selected for resistance to mitoxantrone, the camptothecins topotecan and SN-38, anthracyclines, and flavopiridol [9, 27, 32, 39, 40, 47].

The importance of ABCG2 extends beyond its potential role in drug resistance in oncology. ABCG2 has been shown to affect the oral absorption of

chemotherapeutic agents that are ABCG2 substrates and may serve to protect the developing fetus from toxic agents [21, 24]. Additionally, emerging evidence suggests that ABCG2 forms part of the blood-brain barrier, again supporting the theory of a protective role for ABCG2, much like that suggested for Pgp [11, 12].

We have previously reported that mutations at amino acid 482 in ABCG2 profoundly affect the substrate specificity of the protein [18]. Cells transfected with wild-type *ABCG2* with an arginine at amino acid 482 are resistant to mitoxantrone, SN-38 and topotecan, while cells transfected with either of the mutant *ABCG2* genes encoding a glycine or threonine at amino acid 482 are additionally resistant to the anthracyclines and rhodamine 123. Cells transfected with the mutant *ABCG2* have also been found to exhibit greater resistance to mitoxantrone than cells transfected with wild-type *ABCG2* [38]. Methotrexate resistance has also been shown to depend on the amino acid at position 482, as cells expressing wild-type ABCG2 are resistant to the drug while cells expressing the mutant protein are not [10, 46]. Similarly, in drug-selected MEF3.8 and KOT52 mouse fibroblast cell lines, mutation of the arginine at amino acid 482 to a serine or methionine in mouse *Abcg2* results in a gain of function in the ability of the protein to transport the anthracyclines and rhodamine 123, much like the human ABCG2 mutations [2]. Wang et al. have also identified an R482M mutation in a doxorubicin-resistant human T-cell line [47].

The search for single nucleotide polymorphisms (SNPs) has become of increasing importance as it has been recognized that SNPs may contribute to altered protein expression and/or function. Among ABC transporters, SNPs have been linked with altered protein expression levels and altered substrate specificity. A synonymous C3435T SNP (despite no alteration in protein sequence) in Pgp has been linked to reduced levels of intestinal Pgp expression as well as altered pharmacokinetics of Pgp substrates, such as digoxin, rhodamine 123, and fexofenadine [14, 16, 17]. It has been speculated that this SNP is linked with an as-yet-unidentified SNP in a promoter or regulatory region.

We previously sequenced the *ABCG2* gene in 90 genomic DNA samples representing a global genetic diversity and identified three nonsynonymous SNPs —34G → A, substituting a valine for methionine (V12M); 421C → A, substituting a glutamine for lysine (Q141K); and 1858C → A, substituting an aspartic acid for asparagine (D620N)—in the coding region of *ABCG2* [18]. All are in Hardy-Weinberg equilibrium. The SNPs at amino acid 12 and 141 occur at an allelic frequency of 12.2% and 6.9%, respectively. In a study of SNPs in ABCG2 in the general Japanese population, the V12M and Q141K SNPs were observed at higher allelic frequencies (17.2% and 26.6%, respectively) [19]. Similarly, in other studies, the V12M and Q141K SNPs have also been found to be the most frequent polymorphisms in various ethnic and racial groups including Caucasian, Asian, and Swedish populations [3, 48]. Because of their considerable frequency, it is important to determine if these SNPs could affect ABCG2 expression and/or its function as a drug transporter.

Materials and methods

Establishment of stable transfectants

Human embryonic kidney cells (HEK-293) were stably transfected with a PC DNA 3.1 vector (Invitrogen, Carlsbad, Calif.) containing full-length *ABCG2* encoding wild-type (R482), mutant (R482T, R482G), or SNP variants (V12M, Q141K, or D620N) of ABCG2. The SNP variants contained the wild-type amino acid (arginine) at position 482. Expression of ABCG2 in the transfectants was enforced by selection in G418 (Invitrogen). Clones were screened for ABCG2 expression by examining surface expression with the anti-ABCG2 antibody, 5D3 (eBioscience, San Diego, Calif.). Stable transfectants were maintained in EMEM (ATCC, Manassas, Va.) supplemented with 10% FBS, penicillin and streptomycin with G418 at a concentration of 2 mg/ml. The ABCG2 sequence was confirmed in all clones used in this study. The wild-type and mutant ABCG2-transfected cells have been characterized previously [38].

RNA isolation, Northern blot analysis and probe labeling

RNA was isolated using RNA STAT-60 according to the manufacturer's instructions (Tel Test, Friendswood, Tx.). Northern blot labeling was performed as previously described using a riboprobe generated from the first 662 bp of ABCG2 subcloned into a pCRII-TOPO vector (Invitrogen) [38]. Labeling was accomplished using the Riboprobe In Vitro Transcription System following the manufacturer's instructions (Promega, Madison, Wis.). Blots were washed in 5× SSC for 15 min at room temperature, followed by two washes in 1× SSC/0.1% SSC preheated to 42°C for 15 min each time. Autoradiographs were obtained after 24 h at −70°C.

Immunoblot analysis

Microsomal membrane fractions were subjected to electrophoresis and transferred to PVDF membranes as previously described [26]. Blots were probed with the monoclonal anti-ABCG2 antibody BXP-21 (Kamiya Biomedical, Seattle, Wash.) [28].

Cytotoxicity assays

Sulforhodamine B 4-day cytotoxicity assays were performed [43]. Cells were plated in flat-bottomed 96-well plates at a density of 2500 cells per well and allowed

to attach for 24 h at 37°C in an atmosphere containing 5% CO_2. Chemotherapeutic agents at various concentrations were added to the cells and the plates were allowed to incubate for 96 h at 37°C in an atmosphere containing 5% CO_2. Cells were subsequently fixed in 50% trichloroacetic acid and stained with sulforhodamine B solution (0.4% sulforhodamine B w/v in 1% acetic acid). Optical densities were read on a Bio-Rad plate reader at an absorbance of 540 nm. Each concentration was tested in quadruplicate and controls were performed in replicates of eight. Relative resistance values were obtained by dividing the IC_{50} value of each drug for the ABCG2-transfected clone by the IC_{50} value for the empty vector pcDNA3-10 clone, and *P* values were determined using a Student's *t*-test.

Flow cytometry

Flow cytometry assays were performed as previously described [37]. Briefly, cells were trypsinized, and incubated for 30 min at 37°C in an atmosphere containing 5% CO_2 and in complete medium (phenol red-free IMEM with 10% fetal calf serum) containing 20 μ*M* mitoxantrone or 5 μg/ml Hoechst 33342 with or without 10 μ*M* of the ABCG2 inhibitor fumitremorgin C (FTC). FTC has been previously shown to block ABCG2-mediated drug transport [35, 36]. Cells were then washed once in cold complete medium and incubated for 1 h at 37°C in substrate-free medium continuing with or without FTC to generate the FTC/Efflux and Efflux histograms, respectively. Subsequently, cells were washed with cold DPBS and placed on ice in the dark until analyzed. The difference in mean channel numbers between the FTC/Efflux and Efflux histograms, termed FTC-inhibitable efflux or FTC/Efflux−Efflux, was calculated for each transfected clone, and at least two separate experiments were performed.

To determine cell surface expression of ABCG2, cells were trypsinized and resuspended in 2% BSA/DPBS to which was added the phycoerythrin-conjugated anti-ABCG2 antibody 5D3 (eBioscience, San Diego, Calif.) or phycoerythrin-conjugated mouse IgG negative control antibody (eBioscience). The cells were incubated with antibody for 30 min at room temperature and washed with DPBS. Surface expression of ABCG2 was calculated as the difference in mean channel number between the 5D3 antibody histogram and the negative control antibody histograms. At least two separate determinations were performed for each clone.

Mitoxantrone fluorescence was detected on a FACSort flow cytometer (Becton Dickinson, San Jose, Calif.) equipped with a 635 nm red diode laser and a 561 nm bandpass filter, and phycoerythrin fluorescence was detected with a 488 nm argon laser and 585 nm bandpass filter. A FACSVantage flow cytometer with a 360 n *M* UV laser was used to measure intracellular Hoechst 33342 fluorescence. For all samples, at least 10,000 events were collected. Debris was eliminated by gating on forward versus side scatter and dead cells were excluded based on propidium iodide staining.

Determination of ABCG2 ATPase activity in Sf9 insect cells

Recombinant baculoviruses containing ABCG2 cDNAs encoding the wild-type, mutant and SNP variants were generated and Sf9 insect cells were infected and cultured as previously described [31, 33, 34]. Cells were also infected with mutant R482G ABCG2 encoding a K86M mutation that has been shown to abolish the function of the resulting protein [34]. ATPase activity was measured by colorimetric detection of inorganic phosphate liberation [31]. The potent FTC analog Ko143 [45] (a kind gift from Drs. J. Allen and G. Koomen, Division of Experimental Therapy, The Netherlands Cancer Institute, and Laboratory of Organic Chemistry, University of Amsterdam, Amsterdam, The Netherlands) was used to inhibit the ATPase activity of ABCG2. A Student's *t*-test was used to determine *P* values.

Confocal microscopy

For confocal laser scanning microscopy, cells were plated onto eight-well chamber slides (Nalge Nunc, Rochester, N.Y.) at a density of 0.5×10^5 cells/well and cultured for 2 days. After three brief washes with Dulbecco's phosphate-buffered saline (DPBS), cells were fixed for 5 min at room temperature with 4% paraformaldehyde (Sigma, St. Louis, Mo.) in DPBS. After five washes with DPBS, samples were further fixed and permeabilized with prechilled (−20°C) methanol (Reanal, Budapest, Hungary) for 5 min at room temperature. After five additional washes with DPBS, cells were blocked for 1 h at room temperature in DPBS-based blocking buffer containing 2 mg/ml bovine serum albumin, 1% fish gelatin, 0.1% Triton-X 100, and 5% goat serum (all Sigma, St. Louis, Mo.). Samples were then incubated with a 1:100 dilution of the mouse monoclonal anti-ABCG2 antibody, BXP-21 (Kamiya Biomedical) for 1 h at room temperature. After subsequent washes, cells were incubated for 1 h at room temperature with a 1:250 dilution of Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, Ore.). After repeated washes, the staining was analyzed with an Olympus IX70 laser scanning microscope.

Transport of Hoechst 33342 in Sf9 insect cells

Hoechst 33342 dye fluorescence was measured in a fluorescence spectrophotometer (Perkin Elmer LS 50B, Perkin Elmer/Applied Biosystems, Foster City, Calif.) at 350 nm (excitation)/460 nm (emission), using 5×10^5 insect cells in 2 ml HPMI solution (120 m *M* NaCl,

5 m *M* KCl, 400 μ*M* $MgCl_2$, 40 μ*M* $CaCl_2$, 10 m *M* Hepes, 10 m *M* $NaHCO_3$). This dye becomes fluorescent only in a complex with DNA, and the increase in cellular fluorescence reflects dye influx into the cells. Cells were preincubated at 37°C in HPMI for 4 min, and further incubated with 2 μ*M* Hoechst 33342 for 10 min. Subsequently, the inhibitor Ko143 (1 μ*M*) was added to the cells. The initial increase in fluorescence observed is due to rapid dye uptake and nuclear staining in dead cells, while further cellular dye uptake is reflected by an increase in fluorescence. At the end of each experiment, for standardization, full cellular staining was obtained by the addition of 8 μ*M* digitonin, which disrupts the integrity of the cell membrane [34]. A Student's *t*-test was used to determine *P* values.

Results

Establishment of stable transfectants

We had previously generated stable transfectants containing empty vector, wild-type (R482) or mutant (R482G, R482T) ABCG2 [38]. For the current study, we generated ABCG2 transfectants with three nonsynonymous SNPs. Additionally, we generated an ABCG2 transfectant with the first 11 amino acids deleted and the V12M SNP (1_33del34G → A, 1_11delV12M) in order to examine whether the substituted methionine at amino acid 12 can serve as the first codon for a functional ABCG2. Clones were initially screened using the anti-ABCG2 antibody 5D3 and, from the positive clones obtained, 12 clones transfected with V12M, Q141K, D620N, or 1_11delV12M were selected for further study: V12M-12, -13 and -14; Q141K-5, -8, -13 and -16; D620N-2, -3 and -23; and 1_11delV12M-2 and -8. Northern blot and immunoblot analysis of some of the transfectants were subsequently performed.

By Northern blot and immunoblot analysis, D620N-3 and D620N-23 showed relatively low expression levels (Fig. 1a, b). Although Northern blot analysis demonstrated higher expression of ABCG2 mRNA in wild-type (482R) ABCG2-transfected clones than in V12M-13 and Q141K-8 clones, immunoblot analysis showed generally comparable (within two- to threefold) ABCG2 protein expression in 482R, V12M-13, and Q141K-8 transfectants. As shown in Fig. 1a, ABCG2 expression was also observed in the 1_11delV12M clones, despite deletion of the first 11 amino acids.

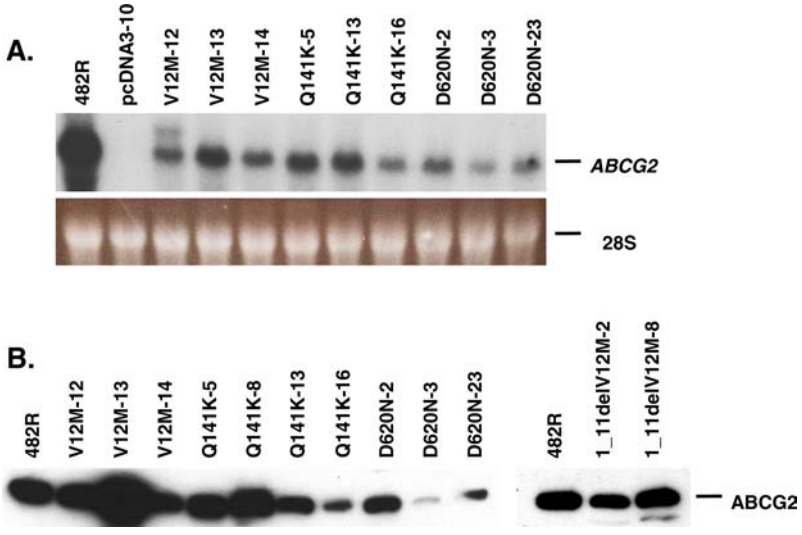


Fig. 1 Expression of ABCG2 in stable transfectants. **a** Northern blot analysis of ABCG2 expression in representative HEK-293 cells transfected with wild-type, V12M, Q141K, or D620N ABCG2. Total RNA (20 μg) from each transfectant was electrophoresed and transferred to a PVDF membrane. The membrane was hybridized with a riboprobe generated from the first 662 bp of ABCG2. **b** Western blot analysis of ABCG2 expression in selected ABCG2-transfected clones. Membrane protein from each clone (30 μg) was separated by SDS/PAGE, transferred to a PVDF membrane, and probed with the monoclonal anti-ABCG2 antibody BXP-21

Differential resistance among cells transfected with wild-type, V12M and Q141K ABCG2

To determine whether the SNPs affected the transport activity of ABCG2, 4-day cytotoxicity assays were performed with the ABCG2 substrates mitoxantrone, topotecan, SN-38, and diflomotecan (BN80915) on ABCG2-transfected HEK-293 cells expressing comparable amounts of ABCG2. Diflomotecan is a camptothecin derivative, similar to SN-38, which has recently been shown to be a weak ABCG2 substrate [5]. Comparable surface expression of ABCG2 in the 482R-2, Q141K-5, Q141K-8 and V12M-13 clones was confirmed using the 5D3 antibody, as shown in Fig. 2a. This is in contrast to the immunoblot data that showed somewhat higher levels of ABCG2 in the V12M-13 clone. The difference in mean channel numbers between the negative control histogram (solid line) and the 5D3 antibody histogram (dashed line) was approximately equal for all

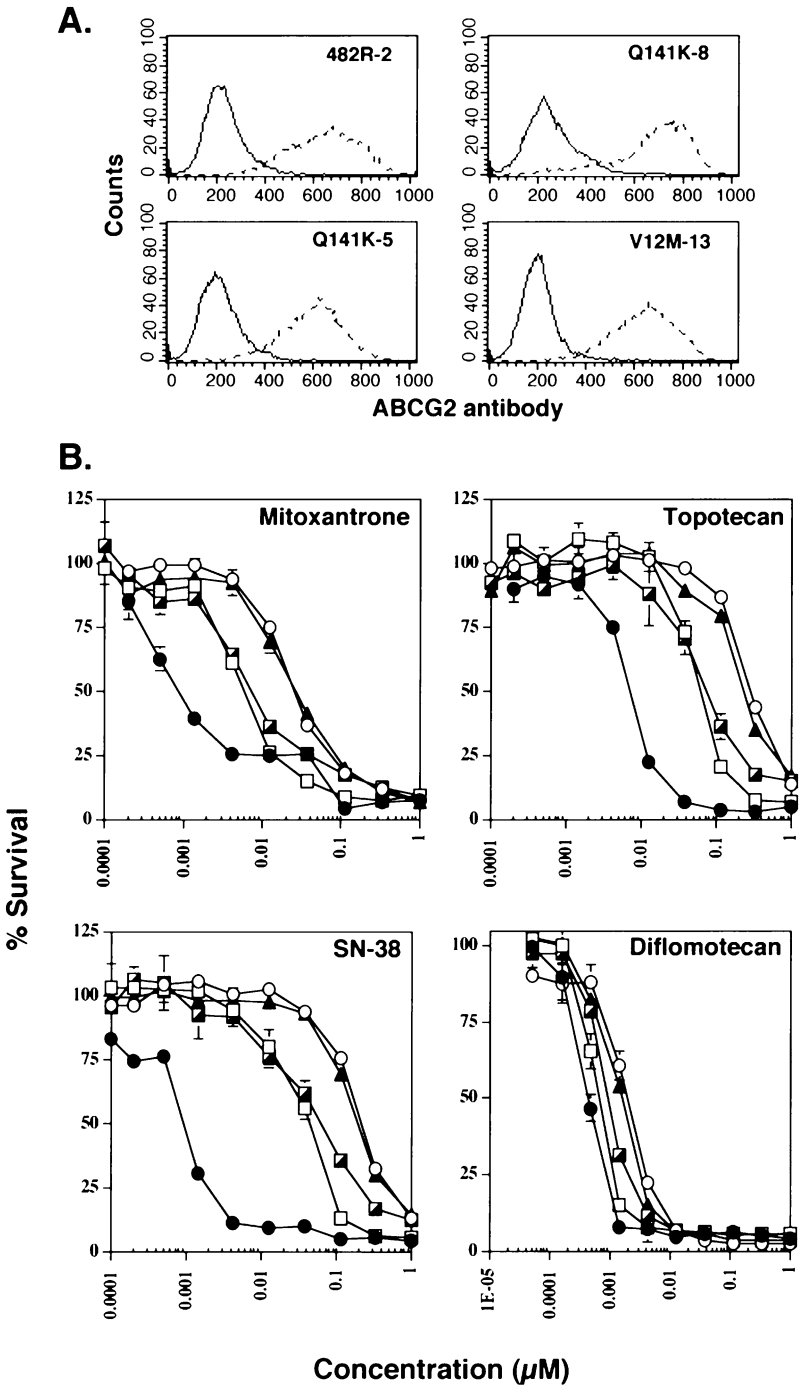


Fig. 2 Impact of SNPs on ABCG2-mediated resistance. **a** ABCG2-transfected HEK-293 cells were incubated for 30 min in phycoerythrin-labeled negative control antibody (*solid line*) or 5D3 antibody (*dashed line*). **b** Cytotoxicity assays were performed with mitoxantrone, topotecan, SN-38, or diflomotecan on HEK-293 cells transfected with empty vector (*filled circles*), or the 482R-2 (*open circles*), V12M-13 (*filled triangles*), Q141K-5 (*open squares*), and Q141K-8 (*hatched squares*) clones from **a**. Representative results are shown

the clones. As summarized in Table 1, compared with empty vector-transfected pcDNA3-10 cells, all ABCG2 transfectants showed resistance to mitoxantrone, topotecan, SN-38 and diflomotecan. Except for the Q141K-5 clone with diflomotecan, P values for this comparison were < 0.05. This is related to the fact that ABCG2 does not confer substantial resistance to diflomotecan [5]. The V12M-13 and 482R-2 clones were comparably resistant to mitoxantrone, topotecan, SN-38 and diflomotecan, whereas the Q141K-5 and -8 clones had IC_{50} values that were 3-fold to 5-fold lower for mitoxantrone and topotecan, 2-fold to 3.4-fold lower for SN-38, and 1.2-fold to 2.3-fold lower for diflomotecan. Representative results for mitoxantrone, topotecan, SN-38 and diflomotecan are shown in Fig. 2b. These results suggest that the Q141K SNP has functional consequences in the resulting ABCG2 protein.

Correlation between FTC-inhibitable mitoxantrone efflux and surface expression in ABCG2 transfectants

To confirm the impaired transport of mitoxantrone in cells expressing Q141K ABCG2, we selected two to six

Table 1 Relative resistance (*RR*) of ABCG2-transfected cells to ABCG2 substrates. RR values were obtained by dividing the IC_{50} value of each drug for the ABCG2-transfected clone by the IC_{50} value for the pcDNA3-10 clone. At least three independent experiments were performed. In all cases $P < 0.05$ except for the Q141K-5 clone with diflomotecan where $P = 0.09$ (IC_{50} values are in nanomoles)

Clone	Mitoxantrone		Topotecan		SN-38		Diflomotecan	
	IC_{50}	RR	IC_{50}	RR	IC_{50}	RR	IC_{50}	RR
pcDNA3-10	0.6 ± 0.3	–	8.7 ± 1.2	–	1.2 ± 0.7	–	0.3 ± 0.2	–
482R-2	34 ± 5.4	57	275 ± 150	32	123 ± 68	103	1.4 ± 0.6	5
V12M-13	44 ± 15	73	250 ± 71	29	100 ± 1	83	1 ± 0.1	3
Q141K-5	7.4 ± 1.8	12	55 ± 7	6	36 ± 11	30	0.6 ± 0.07	2
Q141K-8	10.8 ± 5.3	18	90 ± 14	10	66 ± 11	55	0.9 ± 0.1	3

clones each of ABCG2-transfected cells expressing R482, R482T, R482G, V12M, Q141K or D620N ABCG2. The cells were incubated in 20 μ*M* mitoxantrone with or without 10 μ*M* of the ABCG2 inhibitor FTC for 30 min. The cells were then washed and incubated for 60 min in mitoxantrone-free medium continuing with or without FTC and assayed by flow cytometry to generate the representative histograms in the right column of Fig. 3. Cells were also incubated with 5D3 antibody or negative control antibody for 30 min to generate the representative histograms in the left column of Fig. 3. All cells were shown to transport mitoxantrone to some degree. Interestingly, clones expressing 1_11delV12M ABCG2 transported mitoxantrone, suggesting that the first 11 amino acids are not necessary for protein function.

FTC-inhibitable mitoxantrone efflux, the differences in mean channel numbers between the FTC/Efflux and Efflux histograms, was plotted against ABCG2 surface expression for a series of clones transfected with wild-type, mutant or variant ABCG2 as seen in Fig. 4a. Surface ABCG2 expression was determined from the difference between the 5D3 histogram and the negative control histogram for each clone. Each clone was assayed at least twice and a total of 28 distinct clones were examined. Mitoxantrone efflux values and surface ABCG2 expression values were then plotted for five wild-type ABCG2 transfected clones expressing varying levels of ABCG2 and the regression line shown in Fig. 4a was drawn through these points. Nearly all the values for the efflux and expression for cells transfected with mutant R482G and R482T ABCG2 fell above the regression line for wild-type ABCG2, confirming a gain-of-function in the transport of mitoxantrone in these cells. Efflux and expression values for cells transfected with V12M and D620N ABCG2 fell close to the line, while values for cells transfected with Q141K ABCG2 fell predominantly below the line.

To determine the significance of these differences in transport, mitoxantrone efflux values were divided by ABCG2 surface expression values for each of the clones to generate an efflux per unit expression value and the results are presented in the box-plot analysis shown in Fig. 4b. Among the transfectants, Q141K variants showed significantly lower values compared to the transfectants with wild-type ABCG2 and the other SNP variants, V12M and D620N ($P = 0.0048$, 0.0005, and 0.0126, respectively), suggesting that Q141K ABCG2 transports mitoxantrone less efficiently than wild-type ABCG2. Although V12M and D620N variants showed somewhat higher efficiency of mitoxantrone transport than 482R, no statistically significant difference was found. Again, we confirmed earlier results showing increased transport of mitoxantrone in mutant ABCG2 [38]. Efflux per unit expression values in cells transfected with mutant 482G and 482T ABCG2 were significantly higher than for wild-type ABCG2 ($P = 0.0003$ R482G; $P = 0.0013$ R482T).

Effect of SNP variants on the ATPase activity of ABCG2

It has been reported that human ABCG2 can be expressed in its biologically active form in Sf9 insect cells, and the ATPase activity of wild-type and mutant ABCG2 has been determined using this model [33, 34]. We next examined the ATPase activity of V12M, Q141K, and D620N variants using this system. Sf9 cells were infected with recombinant baculoviruses carrying the respective human *ABCG2* cDNAs and comparable ABCG2 expression was verified by immunoblot analysis of membrane fractions (Fig. 5a). The membrane fractions containing wild-type ABCG2 and SNP variant proteins were then assayed for ATPase activity. Despite similar levels of ABCG2 expression, the basal ATPase activity was significantly lower in the case of the Q141K variant than in wild-type ABCG2, whereas in the other SNP variants, the ATPase activity was similar to that observed in wild-type ABCG2 (Fig. 5b).

Next, the drug-stimulated ATPase activity of ABCG2 was examined in Sf9 membrane fractions. We found no significant stimulation of ATPase activity by mitoxantrone in membrane protein from cells infected with any of the ABCG2 proteins, consistent with previous results [34]. The lower basal ATPase activity in the Q141K variant persisted in the presence of mitoxantrone (Fig. 5b). Ko143, an analogue of FTC, markedly inhibited ATPase activity in protein obtained from cells infected with any of the ABCG2 variants. At 1 μ*M*

Ko143, ATPase activity in membrane protein from cells expressing any of the ABCG2 proteins was almost completely abrogated. The impact of Hoechst 33342 on ATPase activity was examined. It has been previously reported that Hoechst 33342, a known ABCG2 substrate, decreases the ATPase activity of wild-type or mutant ABCG2 in a concentration-dependent manner [34]. Similarly, ATPase activity in membranes containing wild-type ABCG2 or the SNP variants decreased with the addition of Hoechst 33342. Finally, flavopiridol has been previously reported to be a substrate for ABCG2, with cells selected in flavopiridol developing ABCG2 overexpression as a mechanism of resistance [39]. When flavopiridol was added in increasing amounts, a modest reduction in ATPase activity was noted in protein isolated from cells infected with any of the ABCG2 variants (data not shown).

Effect of SNPs on cellular localization of ABCG2

To determine if the SNPs affected membrane localization of ABCG2, we performed immunofluorescence studies on HEK-293 cells stably transfected with wild-type, V12M or Q141K ABCG2. As seen in Fig. 6a, HEK-293 cells expressing wild-type ABCG2 demonstrated predominantly membrane staining with the anti-ABCG2 antibody BXP-21. Similarly, cells expressing V12M ABCG2 also had ABCG2 localized primarily to the cell surface (Fig. 6b). In contrast, HEK-293 cells expressing Q141K ABCG2 demonstrated high intracellular staining with the BXP-21 antibody, as well as cell surface staining (Fig. 6c). These results, despite the selection of clones expressing comparable levels of cell surface ABCG2, suggest impaired membrane trafficking or incorrect membrane insertion of Q141K ABCG2.

Hoechst dye transport in intact Sf9 cells and ABCG2-transfected HEK-293 cells

The fluorescent dye Hoechst 33342 has been shown to be a substrate of ABCG2 [23, 34, 38, 42, 51]. To examine whether the nonsynonymous SNPs in ABCG2 affect the transport of this compound, Hoechst 33342 dye transport was measured in intact Sf9 cells expressing wild-type, V12M, Q141K, or D620N ABCG2, as well as the nonfunctional mutant, R482G/K86M. Immunoblot analysis of protein obtained from the infected cells is shown in Fig. 7a.

Hoechst 33342 transport was comparable in cells expressing wild-type, Q141K or D620N ABCG2. In contrast, Hoechst 33342 transport was significantly lower in cells expressing V12M ABCG2 following normalization to the higher ABCG2 expression in these cells, suggesting less efficient Hoechst 33342 transport by V12M ABCG2 (Fig. 7b). Hoechst 33342 fluorescence was higher in all infected cells when performed in the presence of 1 μ*M* Ko143. In Sf9 cells expressing the R482G/K86M ATP binding-site mutant, Hoechst 33342 uptake was comparable to the level of Hoechst 33342 accumulation found in the Ko143-inhibited cells.

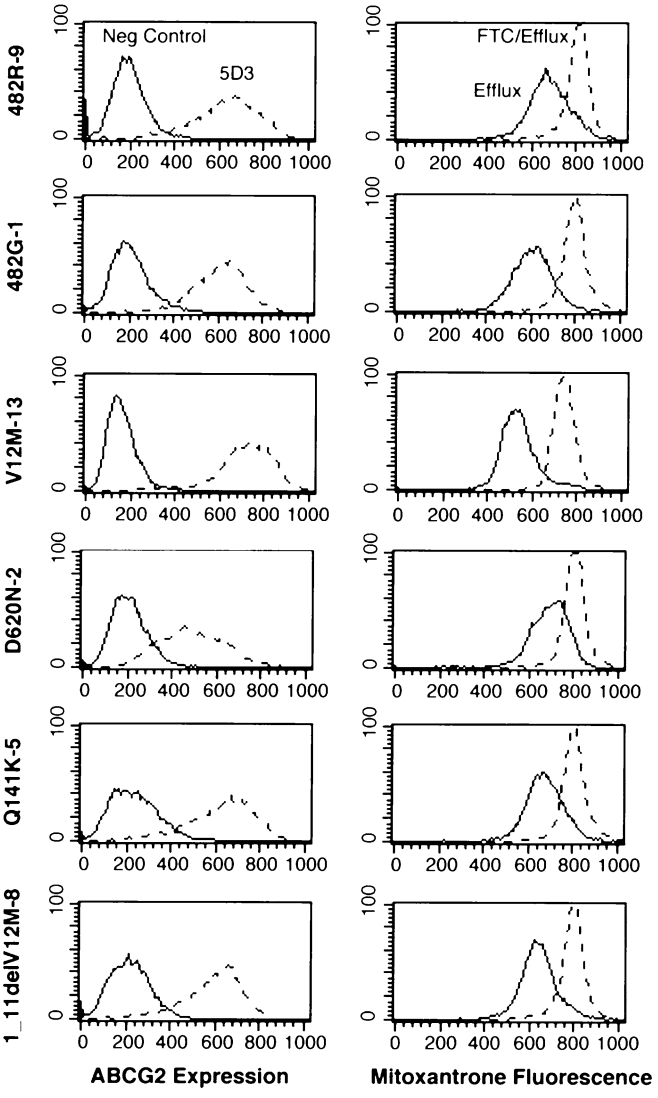


Fig. 3 FTC-inhibitable mitoxantrone efflux and surface expression of ABCG2 in transfected cells. *Left column* Cells were incubated for 30 min with either phycoerythrin-labeled negative control antibody (*solid lines*) or the phycoerythrin-labeled anti-ABCG2 antibody 5D3 (*dashed lines*) according to the manufacturer's instructions. *Right column* Transfected cells were incubated in mitoxantrone with or without 10 μ*M* FTC for 30 min at 37°C, washed, then allowed to efflux for 1 h at 37°C in substrate-free medium continuing with (*dashed lines*, FTC/Efflux histogram) or without (*solid lines*, Efflux histogram) FTC. Representative histograms for 482R-9, 482G-1, V12M-13, D620N-2, Q141K-5, and 1_11delV12M-8 are shown

To evaluate the possibility that Hoechst 33342 transport was impaired in human cells expressing V12M ABCG2, we performed Hoechst accumulation studies with HEK-293 cells transfected with the wild-type or SNP variants of ABCG2. Cells were incubated in 5 μg/ml Hoechst 33342 in the presence or absence of 10 μ*M* FTC for 30 min, washed, and allowed to efflux in

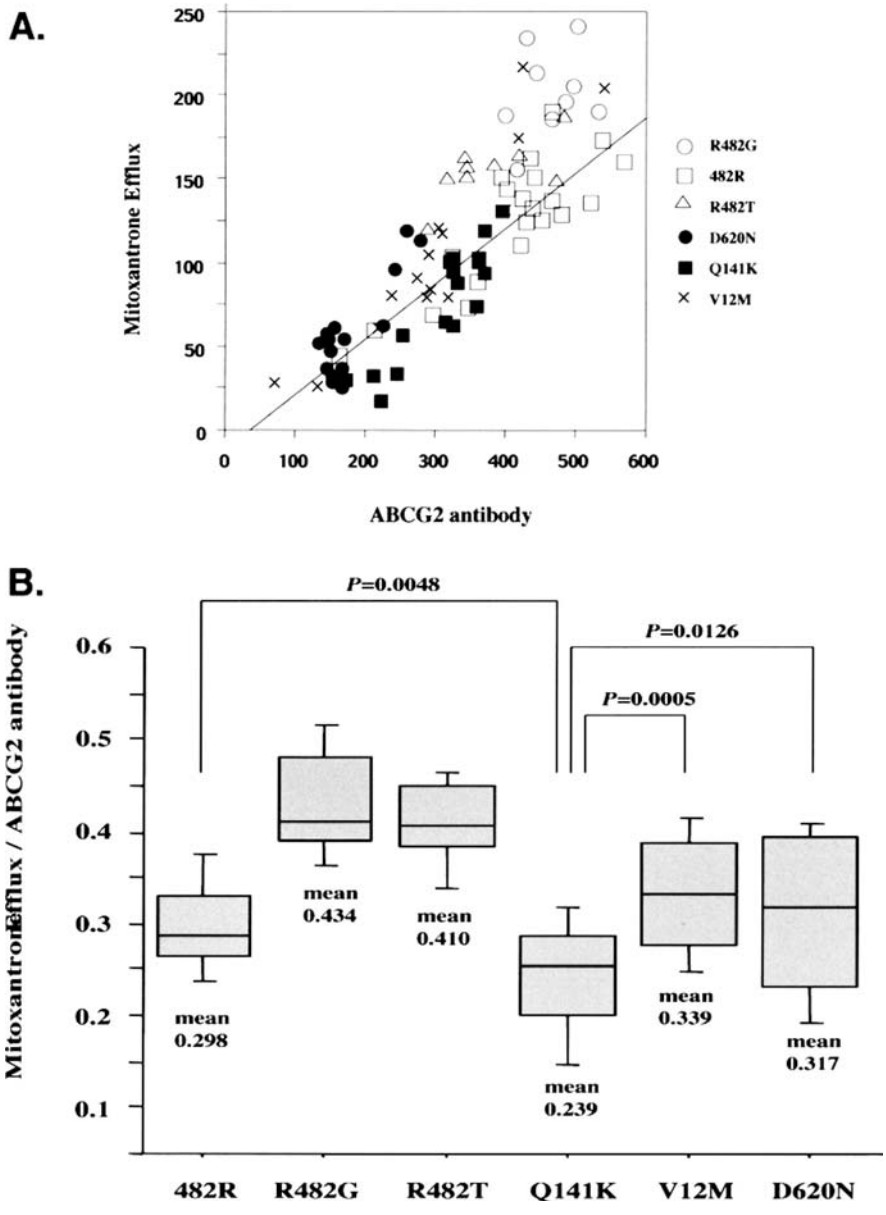


Fig. 4 FTC-inhibitable mitoxantrone efflux and ABCG2 surface expression in ABCG2 transfected clones. **a** Scatter plot analysis. The difference in mean channel number between the FTC/Efflux and Efflux histograms for mitoxantrone was calculated for each clone studied and plotted against ABCG2 expression values determined by surface expression of ABCG2. The regression line shown is drawn through values obtained for five wild-type transfected clones. **b** Box-plot analysis. FTC-inhibitable mitoxantrone efflux values, the difference in mean channel number between the FTC/Efflux and Efflux histograms for mitoxantrone, were divided by ABCG2 surface expression values as determined by the 5D3 antibody to yield an efflux per surface expression value. Values from the experiment in **a** were obtained for ABCG2-transfected HEK-293 clones expressing varying levels of 482R, R482G, R482T, V12M, Q141K, and D620N ABCG2 and a box plot was generated. The box contains the central 50% of the data; the upper edge of the box indicates the 75th percentile of the data set and the lower edge indicates the 25th percentile. The line in the box indicates the median value of the data and the bars represent the minimum and maximum data values

substrate-free medium for 60 min continuing with or without FTC. Cells expressing comparable amounts of protein as determined by surface staining were selected for study (Fig. 7c, left column). No significant difference in FTC-inhibitable Hoechst efflux was observed between cells expressing wild-type (R482), V12M or Q141K ABCG2 (Fig. 7c, right column). Cytotoxicity assays with Hoechst 33342 confirmed marked resistance conferred by ABCG2, with an IC_{50} for the 482R-2 clone of 16.3 ± 15.8 μM and for the V12M-13 clone of 56.7 ± 5.8 μM. Since the IC_{50} for empty vector-transfected cells was 26.3 ± 15.8 n M, this translates to a relative resistance of 621 for 482R-2 and 2155 for V12M-13. These data are not consistent with impaired transport of Hoechst 33342 by V12M ABCG2 and are in contrast to the results obtained with intact Sf9 insect cells.

Discussion

We and others have recently identified several polymorphisms in *ABCG2*, including three nonsynonymous SNPs resulting in amino acid substitution in the coding region of *ABCG2*: V12M, Q141K, D620N [4, 19, 20, 50]. To investigate the possible functional involvement of these nonsynonymous SNPs, we transfected HEK-293 cells with vectors containing *ABCG2* encoding wild-type, mutant, or SNP variants of ABCG2. Our results suggest that the Q141K SNP affects the transport efficiency of ABCG2.

The importance of SNPs has become increasingly apparent in the study of pharmacogenomics. Among those identified in ABC transporters, MDR1 polymorphisms have been investigated most widely. Hoffmeyer

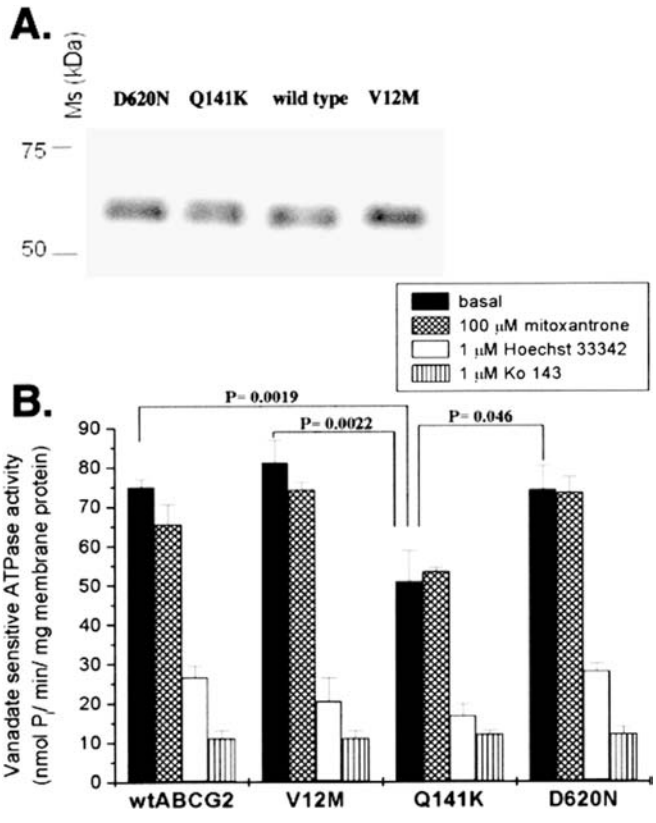


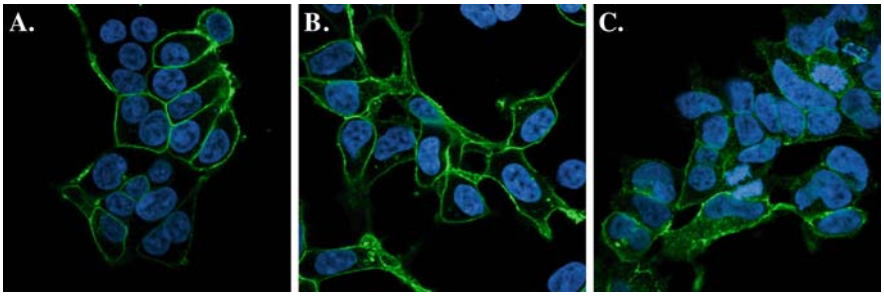
Fig. 5 ATPase activity in Sf9 insect cells infected with ABCG2-bearing baculovirus. **a** Immunoblot detection of human wild-type, D620N, Q141K and V12M ABCG2 expressed in Sf9 insect cells. Membranes of Sf9 cells (1.5 μg total protein from V12M/Sf9, Q141K/Sf9 and D620N/Sf9, 1.0 μg from wild-type ABCG2/Sf9, and 1.2 μg from *β*-galactosidase/Sf9) dissolved in disaggregation buffer were subjected to electrophoresis on 7.5% Laemmli-type gels and blotted onto PVDF membranes, followed by immunodetection with the BXP-21 antibody. **b** ATPase activity measured in membranes of Sf9 cells expressing the wild-type, V12M, Q141K, and D620N variants of human ABCG2. ATPase activity of membranes of isolated Sf9 cells was determined by measuring vanadate-sensitive inorganic phosphate liberation, using 3.3 m *M* Mg ATP. Values are the means ± SD of at least four measurements, performed in two or three different membrane preparations. ATPase activity was measured in the absence of added compounds (*basal*), and with 100 μ*M* mitoxantrone, 1 μ*M* Hoechst 33342 or 1μ *M* Ko 143

et al. reported the identification and distribution of 15 SNPs in systemic screening for MDR-1 polymorphisms [17]. In correlating the presence of MDR-1 polymorphisms and the levels of intestinal MDR1 expression, they demonstrated that the C3435T polymorphism in exon 26, which caused no amino acid substitution, is associated with a significantly reduced MDR1 expression. This result, presumably due to linkage with a regulatory region, has been associated with increased bioavailability of Pgp substrate drugs in several studies, although conflicting reports have appeared [41]. One possible explanation for the discrepancies observed is the endogenous expression of other ABC transporters that also transport Pgp substrates [49]. Indeed, it has been reported that even low endogenous levels of ABC transporters contribute to drug resistance [1]. Alternatively, compensatory increases in expression of other ABC transporters may obscure functional consequences of SNPs. This was highlighted recently by Cisternino et al. who demonstrated increased ABCG2 expression levels in Pgp-deficient mice [11]. The impact of the C3435T polymorphism or other SNP affecting transporter function could thus be variable, depending upon the substrate involved.

Imai et al. have previously reported that the Q141K variant is associated with decreased protein expression in transfected cells and therefore results in increased sensitivity to chemotherapeutic agents [20]. In contrast, we did not observe decreased expression of ABCG2 in HEK-293 cells transfected with Q141K ABCG2. Supporting our findings, Zamber et al. have found no correlation between the Q141K SNP and level of expression of ABCG2 protein [50]. The Q141K SNP does not appear to prevent expression of the protein on the cell surface. However, confocal data suggesting that a larger proportion of protein is intracellular implies that processing may be less efficient.

Four-day cytotoxicity assays demonstrated that, among HEK-293 cells transfected with wild-type, V12M, or Q141K ABCG2, those expressing Q141K ABCG2 had IC_{50} values for mitoxantrone, topotecan, SN-38 and diflomotecan that were as much as fivefold lower than those for cells expressing comparable levels

Fig. 6 Membrane localization of ABCG2 in HEK-293 transfectants. HEK-293 cells transfected with wild-type (482R-2) (**a**), V12M (V12M-13) (**b**), or Q141K (Q141K-8) (**c**) ABCG2 were fixed, permeabilized, and then incubated with the mouse monoclonal anti-ABCG2 antibody, BXP-21. After washing, cells were incubated with Alexa Fluor 488-labeled secondary antibody and subjected to confocal laser scanning microscopy



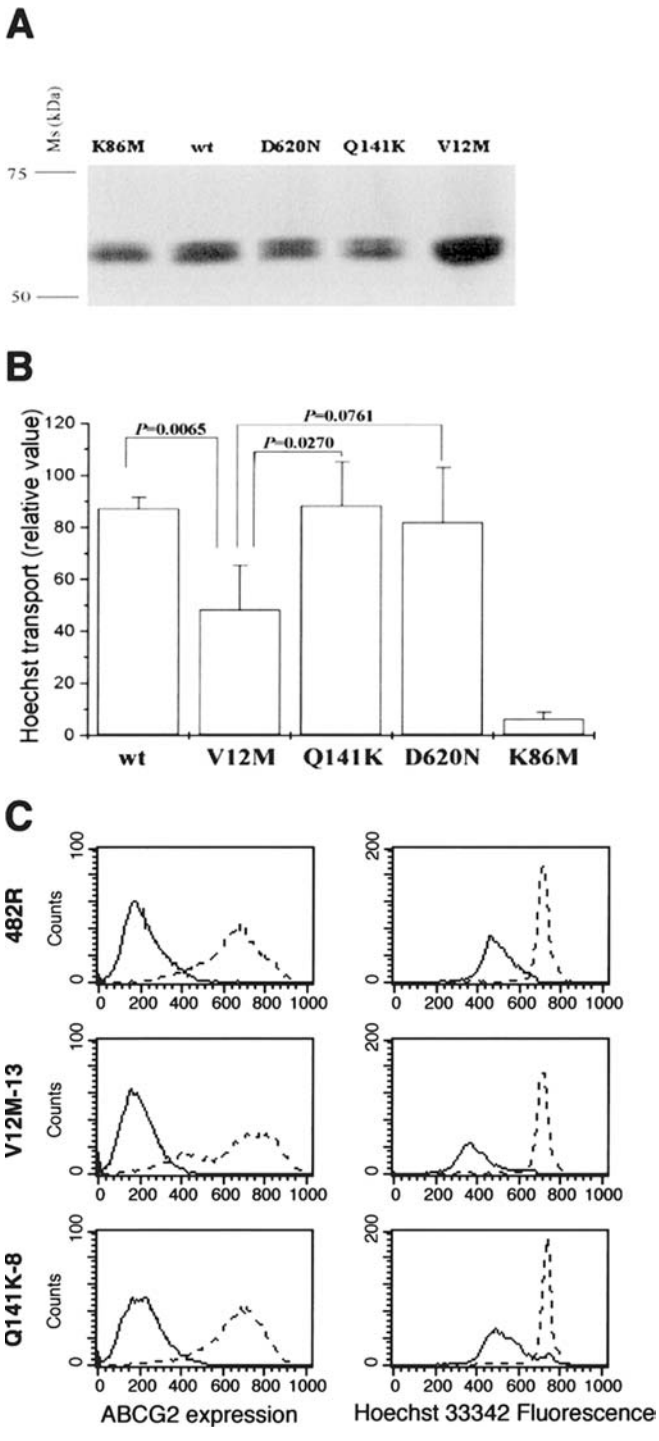


Fig. 7 Hoechst 33342 transport in Sf9 and HEK-293 cells. **a** ABCG2 expression in intact Sf9 cells used for Hoechst 33342 transport assays. Total protein (10 μg) from Sf9 cells expressing wild-type ABCG2 and its variants were electrophoretically separated and transferred to nitrocellulose. The membrane was incubated with the BXP-21 antibody. **b** Hoechst 33342 transport in Sf9 cells expressing wild-type ABCG2 and its variants. **c** Transport of Hoechst 33342 in ABCG2-transfected HEK-293 cells. Cells were incubated for 30 min in medium containing 5 μg/ml Hoechst 33342 with or without 10 μ*M* FTC, washed, then allowed to efflux for 60 min in substrate-free medium continuing with (*dotted line*) or without (*solid line*) FTC

of wild-type or V12M ABCG2. When surface ABCG2 expression was used to normalize the FTC-inhibitable mitoxantrone efflux, we found that HEK-293 cells expressing Q141K ABCG2 transported mitoxantrone less efficiently than cells expressing wild-type, V12M or D620N ABCG2. These findings are in agreement with those of Mizuarai et al., who found that polarized LLC/PK1 cells transfected with Q141K ABCG2 are more sensitive to mitoxantrone, topotecan and an indolocarbazole topoisomerase I inhibitor, all of which are known ABCG2 substrates [30].

However, in contrast to the results of Mizuarai et al., we did not observe enhanced sensitivity to ABCG2 substrate drugs in cells transfected with V12M ABCG2. While the reason for this discrepancy is not clear, it may be explained by the model system used. It is possible that ABCG2 is underglycosylated when expressed in LLC/PK1 cells, leading to impaired function of the protein, as in Sf9 cells, where ABCG2 is known to be expressed in an underglycosylated form [33]. Indeed, we noted impaired Hoechst 33342 transport in Sf9 cells expressing V12M ABCG2. This was in contrast to cytotoxicity assays with transfected human cells that displayed comparable resistance to Hoechst 33342 in cells expressing wild-type or V12M ABCG2. It is of note that Imai et al., using a human expression system, did not find increased sensitivity to mitoxantrone in PC3 cells transfected with V12M ABCG2 compared to cells transfected with wild-type [20]. These contrasting results suggest that the glycosylation status of the protein may be significant for the V12M SNP.

The Q141K SNP has also recently been shown to significantly affect the pharmacokinetics of diflomotecan. Diflomotecan has been recently reported by our group to be a weak substrate of ABCG2 [5]. Despite the relatively poor transport of diflomotecan by ABCG2, Sparreboom et al. have reported that, in patients heterozygous for the Q141K SNP, plasma diflomotecan levels are approximately threefold higher than in patients expressing the wild-type allele [44]. Diflomotecan levels were not found to be affected by variants in *ABCB1*, *ABCC2*, *CYP3A4* or *CYP3A5* genes. These results suggest that the Q141K SNP may alter the pharmacokinetic profile of other ABCG2 substrate drugs.

Basal ATPase activity was determined to be 1.8-fold lower in membrane protein isolated from Sf9 insect cells infected with recombinant baculoviruses containing full-length Q141K ABCG2 compared to protein from cells expressing wild-type or the other SNP variant ABCG2 forms. Our results are in agreement with those of Mizuarai et al. who reported that the ATPase activity of Q141K ABCG2 is 1.3-fold lower than wild-type in the Sf9 system [30].

Various polymorphisms have been detected among the genes encoding ABC transporters other than MDR1 and ABCG2 [22]. The ABC half-transporters, ABCG5 and ABCG8, identified by Berge et al. and Lee et al. [6,

25], are located on chromosome 2p as two adjacent, oppositely oriented genes, and are highly expressed in liver and intestine. The protein products are thought to be involved in sterol metabolism by regulating intestinal absorption and biliary excretion [6, 25]. Several mutations in ABCG5 and ABCG8 including a missense mutation at a conserved residue in ABCG5, a frameshift mutation in ABCG5, and a splicing mutation in ABCG8, have been identified in patients with sitosterolemia, a rare autosomal recessive disorder that causes decreased biliary excretion and increased intestinal absorption of neutral sterols [6, 25]. On the other hand, the association of polymorphisms in ABCG5 and ABCG8 with interindividual variation in plasma concentrations of sterols has also been demonstrated [7, 48]. Weggemans et al. examined the association between the C1950G (Gln640Glu) polymorphism in ABCG5 and blood cholesterol concentrations in 486 subjects and responsiveness to dietary cholesterol in 99 participants [48]. They suggested that a homozygous variant allele at this position may result in increased concentrations of serum cholesterol.

In summary, we evaluated the impact of SNPs on ABCG2 substrate specificity and function. Consistent with other reports, we found that the Q141K polymorphism impaired the activity of the ABCG2 protein. This was observed by cytotoxicity assay, drug accumulation assayed by flow cytometry, and by ATPase assay. In contrast to results in non-mammalian systems, no impairment of the V12M variant was observed in the HEK-293 transfectants. Taken together, the results presented here support the hypothesis that the Q141K variant has the potential to alter the pharmacokinetics of ABCG2 substrates. Whether the presence of the Q141K polymorphism in clinical tumors could promote chemosensitivity is unknown, but remains a question for further study.

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