Functional Analysis of SNPs Variants of BCRP/ABCG2

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Purpose. The aim of the current study was to identify the effect of single nucleotide polymorphisms (SNPs) in breast cancer resistance protein (BCRP/ABCG2) on its localization, expression level, and transport activity.

Methods. The cellular localization was identified using the wild type and seven different SNP variants of BCRP (V12M, Q141K, A149P, R163K, Q166E, P269S, and S441N BCRP) after transfection of their cDNAs in plasmid vector to LLC-PK1 cells. Their expression levels and transport activities were determined using the membrane vesicles from HEK293 cells infected with the recombinant adenoviruses containing these kinds of BCRP cDNAs.

Results. Wild type and six different SNP variants of BCRP other than S441N BCRP were expressed on the apical membrane, whereas S441N BCRP showed intracellular localization. The expression levels of Q141K and S441N BCRP proteins were significantly lower compared with the wild type and the other five variants. Furthermore, the transport activity of E_1S, DHEAS, MTX, and PAH normalized by the expression level of BCRP protein was almost the same for the wild type, V12M, Q141K, A149P, R163K, Q166E, and P269S BCRP.

Conclusions. These results suggest that Q141K SNPs may associate with a lower expression level, and S441N SNPs may affect both the expression level and cellular localization. It is possible that subjects with these polymorphisms may have lower expression level of BCRP protein and, consequently, a reduced ability to export these substrates.

KEY WORDS: adenovirus; BCRP/ABCG2; interindividual difference; SNPs.

INTRODUCTION

ABCG2, also referred to as breast cancer resistance protein (BCRP), mitoxantrone resistance-associated protein (MXR), and placenta-specific ATP-binding cassette transporter (ABCP), is a member of the ATP-binding cassette (ABC) transmembrane transporter family (1–3). BCRP mRNA encodes a 72.6 kDa membrane protein composed of 655 amino acids (1,3,4). It has a single ATP binding domain at the amino terminus (at amino acid 61-270) followed by six transmembrane domains (at amino acids 394-416, 428-450, 478-499, 506-528, 533-555 and 629-651) (1,5). BCRP may form a homodimer to become functionally active (6). In BCRP-expressing cells, the intracellular concentration of substrate anticancer drugs is reduced, suggesting its protective role against drug toxicity (7). In normal human tissues, BCRP is expressed on the apical membrane of enterocytes, trophoblast cells in the placenta, the bile canalicular membrane of hepatocytes, and the apical membrane of lactiferous ducts in the mammary gland (8). Current evidence indicates that BCRP could contribute to the disposition of some substrates (9,10). Moreover, it was recently shown that BCRP also transports some endogenous compounds such as sulfate conjugates (11). It is also reported that some mutations in the open reading frame of BCRP are associated with resistance to some anticancer drugs. For example, an amino acid mutation at position 482 affects the resistance to adriamycin and methotrexate (12,13). Therefore, it is possible that certain kinds of single nucleotide polymorphisms (SNPs) of BCRP may alter its function and, consequently, affect the disposition of substrate drugs.

To date, some pieces of information about BCRP SNPs have been reported. Recently, the SNPs of BCRP in 100 healthy Japanese subjects have been analyzed in 84 cell lines established from clinically dissected human tumors and also in 60 Japanese individuals who were given irinotecan, an anticancer drug, for the treatment of various types of cancer (14). On analyzing the specimens from the 100 Japanese volunteers, 7 kinds of SNPs were identified for the BCRP gene: G34A (V12M), C376T (Q376Stop), C421A (Q141K), G1098A (E366E), G1322A (S441N), T1465C (F489L), and C1515- (AFFVM505-509ASSL Stop). The allele frequencies of these SNPs are 18, 1, 36, 1, 0.5, 0.5, and 0.5%, respectively. In the 84 cell lines, 7 kinds of SNPs were identified and their frequency for G34A (V12M), C376T (Q126Stop), C421A (Q141K), G445C (A149P), G488A (R163K), C805T (P269S), and G1098A (E366E) are 22, 3, 29, 1, 0.6, 0.6 and 2%, respectively. In the present study, we focused on the 7 SNPs, 6 of which were found in Japanese samples and cell samples, and 1 was reported in the NCBI database (rs1061017). We constructed expression systems for the wild type and SNPs variants of BCRP (V12M, Q141K, A149P, R163K, Q166E, P269S, S441N BCRP) and examined whether these SNPs variants of BCRP alter its localization, expression level, and transport activity.

MATERIALS AND METHODS

Materials

[^{3}H]Estrone-3-sulfate (E_1S; 46 Ci/mmol), [^{3}H]dehydroepiandrosterone sulfate (DHEAS; 79.1 Ci/mmol), and [^{3}H]*p*-aminohippuric acid (PAH; 4.51 Ci/mmol) were purchased from PerkinElmer Life Science, Inc. (Boston, MA, USA). [^{3}H]Methotrexate (29 Ci/mmol) was purchased from Ameri-

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ABBREVIATIONS: ABC transporter, ATP-binding cassette transmembrane transporter; Ad, adenovirus; BCRP, breast cancer resistance protein; DHEAS, dehydroepiandrosterone sulfate; E_1S, estrone 3-sulfate; ER, endoplasmic reticulum; K_m, Michaelis constant; MOI, multiplicity of infection; MTX, methotrexate; PAH, *p*-aminohippurate; SNPs, single nucleotide polymorphisms; V_{max}, maximum transport velocity.

can Radiolabeled Chemicals, INC. (St. Louis, MO, USA). Unlabeled E_1S, DHEAS, methotrexate, PAH, and ATP, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals used were commercially available and of reagent grade.

LLC-PK1 and HEK293 cells were cultured in Medium 199 (GIBCO BRL, Gaithersburg, MD, USA) and Dulbecco's modified Eagle's medium (GIBCO BRL), respectively, after addition of 10% fetal bovine serum, and penicillin (100 U/ml) and streptomycin (100 mg/ml).

Construction of BCRP-Containing Expression Vectors and Recombinant Adenovirus

Wild-type BCRP cDNA was purchased from Invitrogen Corp. (Carlsbad, CA, USA) (no. H24176). The complete BCRP cDNA was amplified with the primers containing NheI site and Kozak sequence attached at the 5′-end and that with the ApaI site at the 3′-end by PCR, and then was inserted into pcDNA3.1 vector plasmid, resulting in wt BCRP/pcDNA3.1. Using site-directed mutagenesis, SNP variants of BCRP (V12M, Q141K, A149P, R163K, Q166E, P269S and S441N BCRP) were constructed on pcDNA3.1 vector (SNPs type BCRP/pcDNA3.1). V12M BCRP was amplified with 5′-GTCGAAGTTTTTATCCCAATGTCACAAGGAAACACCAATGGC-3′ and ’-GCCATTGGTGTTTCCTTGTGACATTGGGATAAAAACTTCGAC-3′. Q141K BCRP was amplified with 5′-CGGTGAGAGAAAACTTAAAGTTCTCAGCAG-3′ and ’-GCTGCTGAGAACTTTAAGTTTTCTCTCACCG-3′. A149P BCRP was amplified with 5′-GCAGCTCTTCGGCTTCCAACAACTATGACG-3′ and ’-CGTCATAGTTGTTGGAAGCCGAAGAGCTGC-3′. R163K BCRP was amplified with 5′-CGAACGGATTAACAAGGTCATTCAAGAG-3′ and 5′-CTCTTGAATGACCTTGTTAATCCGTTCG-3′. Q166E BCRP was amplified with 5′-GATTAACAGGGTCATTGAAGAGTTAGGTCT-3′ and 5′-CCAGACCTAACTCTTCAATGACCCTGTTAA-3′. P269S BCRP was amplified with 5′-GACTTATGTTCCACGGGTCTGCTCAGGAGGCCTTGGG-3′ and 5′-CCCAAGGCCTCCTGAGCAGACCCGTGGAACATAAGTC-3′. S441N BCRP was amplified with 5′-CCAACCAGTGTTTCAGCAATGTTTCAGCCGTGGAACTC-3′ and 5′-GAGTTCCACGGCTGAAACATTGCTGAAACACTGGTTGG-3′. From these pcDNA3.1 vectors, BCRP cDNA was subcloned into the NheI and ApaI sites of the pShuttle plasmid vector, transferred into Adeno-XTM Viral DNA using the Adeno-X Expression System (BD Biosciences, Palo Alto, CA, USA), resulting in pAd-wt BCRP. To produce recombinant adenovirus, pAd-wt BCRP was digested with PacI, and transfected to HEK293 cells by FuGENE6 (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. For SNPs type BCRPs, viruses were prepared in the same way, resulting in the production of pAd-SNPs BCRP (pAd-V12M, Q141K, A149P, R163K, Q166E, P269S, and S441N BCRP). Recombinant viruses prepared as described previously (Ad-wt BCRP and Ad-SNPs type BCRP) (15) were purified by CsCl gradient centrifugation, dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol, and stored in aliquots at -80°C. Then, the resulting virus titer was determined as described previously (16).

Immunohistochemical Staining

For immunohistochemical staining, LLC-PK1 cells transfected with wild-type BCRP or SNPs type BCRP in pcDNA3.1 vector were plated at a density of 5×10^5 cells in 12-well dishes, 72 h prior to the experiments. After fixation with 4% (w/v) paraformaldehyde for 10 min and permeabilization in 1% TritonX-100 in PBS for 10 min, cells were incubated with the monoclonal antibody against BCRP (BXP-21) (Kamiya Biomedical Company, Seattle, WA, USA) for 1 h at room temperature. Then, cells were washed three times with PBS and incubated with Goat anti-mouse IgG Alexa 488 (Molecular Probes, Inc., Eugene, OR, USA) diluted 250-fold in PBS for 1 h at room temperature, and mounted in VECTASHIELD Mounitng Medium (Vector Laboratories, Burlingame, CA, USA). The localization of BCRP protein was visualized by confocal lazer microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY, USA).

Preparation of Membrane Vesicles

To prepare membrane vesicles, HEK293 cells were plated at a density of 2×10^6 cells per 15 cm dish. After 72 h, cells were infected with recombinant adenoviruses containing the wild-type and SNPs type BCRP at 2×10^7 pfu per plate. As a negative control, cells were infected with the virus containing GFP cDNA (pAd-GFP). Cells were harvested at 48 h after infection and then the membrane vesicles were isolated using a standard method described previously in detail (17). Briefly, cells were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.4, at 4°C) and stirred gently for 1 h on ice in the presence of 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml aprotinin. The cell lysate was centrifuged at 100,000 × *g* for 30 min at 4°C and the resulting pellet was suspended in 10 ml isotonic TS buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4 at 4°C) and homogenized in a Dounce B homogenizer (glass/glass, tight pestle, 30 strokes). The crude membrane fraction was layered on top of a 38% (w/v) sucrose solution in 5 mM Tris-HEPES, pH 7.4 at 4°C, and centrifuged in a Beckman SW41 rotor centrifuge at 280,000 × *g* for 45 min at 4°C. The turbid layer at the interface was collected, diluted to 23 ml with TS buffer, and centrifuged at 100,000 × *g* for 30 min at 4°C. The resulting pellet was suspended in 400 μl TS buffer. Vesicles were formed by passing the suspension 30 times through a 27-gauge needle using a syringe. The membrane vesicles were finally frozen in liquid nitrogen and stored at -80°C until required. Protein concentrations were determined by the Lowry method.

Western Blot Analysis

For the Western blot analysis, membrane vesicles were dissolved in 3 x SDS sample buffer (New England BioLabs, Beverly, MA, USA), and separated on a 10% SDS-polyacrylamide gel electrophoresis plate with a 4.4% stacking gel. The molecular weight was determined using a prestained protein marker (New England BioLabs). Proteins were transferred electrophoretically to a polyvinylidene diflouride membrane (Pall, East Hills, NY, USA) using a blotter (Bio-Rad Laboratories, Richmond, CA, USA) at 15 V for 1 h The membrane was blocked with PBS containing 5% skimmed milk for 1 h at room temperature. After blocking, the membrane was incubated for 1 h at room temperature in BXP-21

(Kamiya Biomedical Company, Seattle, WA, USA) diluted 100-fold with 5% skimmed milk. Then, the membrane was washed with PBS containing 0.1% Tween-20 and allowed to bind to Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes, Inc. Eugene, OR, USA) which was diluted 5,000-fold with skimmed milk for 1 h to detect BCRP. Subsequently, the fluorescence was measured in a densitometer (Odyssey, ALOKA, Tokyo, Japan). The protein expression levels of each SNPs variant of BCRP were determined by analyzing the band density associated with three different applied amount of the membrane vesicles on the Western blot analysis.

Vesicle Transport Assays

The uptake study of [^{3}H]E_1S, [^{3}H]DHEAS, [^{3}H]MTX, [^{3}H]PAH was performed as reported previously (18). Briefly, the transport medium (10 mM Tris, 250 mM sucrose and 10 mM $MgCl_2$, pH 7.4) contained the ligands, 5 mM ATP and an ATP-regenerating system (10 mM creatine phosphate and 100 mg/ l creatine phosphokinase). An aliquot of transport medium (15 μl) was mixed rapidly with the vesicle suspension (2 μg protein for [^{3}H] E_1S and 10 μg protein for others in 5 μl). The transport reaction was stopped by the addition of 1 ml ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl and 10 mM Tris-HCl (pH 7.4). 900 μl of the stopped reaction mixture was passed through a 0.45 μm HA filter (Millipore Corp., Bedford, MA, USA), and then washed twice with 5 ml stop solution. The radioactivity retained on the filter was measured in a liquid scintillation counter (LS 6000SE, Beckman Instruments, Fullerton, CA, USA) after the addition of scintillation cocktail (Clear-sol I, Nacalai Tesque, Tokyo, Japan). The amount of ligand taken up into vesicles was normalized in terms of the amount of membrane protein. The uptake activity was defined as the amount of ligand divided by the ligand concentration in the medium. The ATP-dependent uptake of ligands was calculated by subtracting the ligand uptake in the absence of ATP from that in its presence.

RESULTS

Localization of Human BCRP in LLC-PK1 Cells

The localization of human BCRP was determined by immunohistochemical staining. Monoclonal antibody against human BCRP (BXP-21) detected a high fluorescence signal at the apical membrane of LLC-PK1 cells after transfection of the wild type BCRP/pc DNA3.1 (Fig. 1). No specific fluorescence signal was observed at the apical membrane or in the cytoplasm after transfection of pcDNA3.1 plasmid vector (data not shown). We also analyzed the cellular localization of BCRP variants. In our experimental system, except for one SNP variant of BCRP (S441N BCRP/pcDNA3.1), all variants showed the same localization as the wild-type BCRP, at the apical membrane of LLC-PK1 cells (Fig. 1). S441N BCRP was expressed intracellularly (Fig. 1).

Expression Level of Human BCRP in HEK293 Cells Using Recombinant Adenoviruses

The expression level of BCRP in the membrane fraction isolated from the BCRP-expressing HEK293 cells was determined by Western blot analysis using an anti-human BCRP monoclonal antibody (BXP-21). As previously reported, BCRP was detected at an approximate molecular weight of 72 kDa (Fig. 2). In contrast, in the membrane vesicles from the Ad-GFP infected cells, no BCRP was detected (Fig. 2). The expression levels of wild-type BCRP and variants were estimated from the band density. Except for two BCRP variants (Q141K and S441N BCRP), the expression levels of each BCRP SNPs were approximately the same as that of the wild-type BCRP (Fig. 2). The expression level of Q141K BCRP was approximately 30–40% of the wild-type BCRP, whereas that of S441N BCRP was much lower, and could not be determined with any accuracy (Fig. 2).

Transport Activity of BCRP

Because we previously reported that [^{3}H]E_1S is taken up into BCRP-expressing membrane vesicles in an ATP-

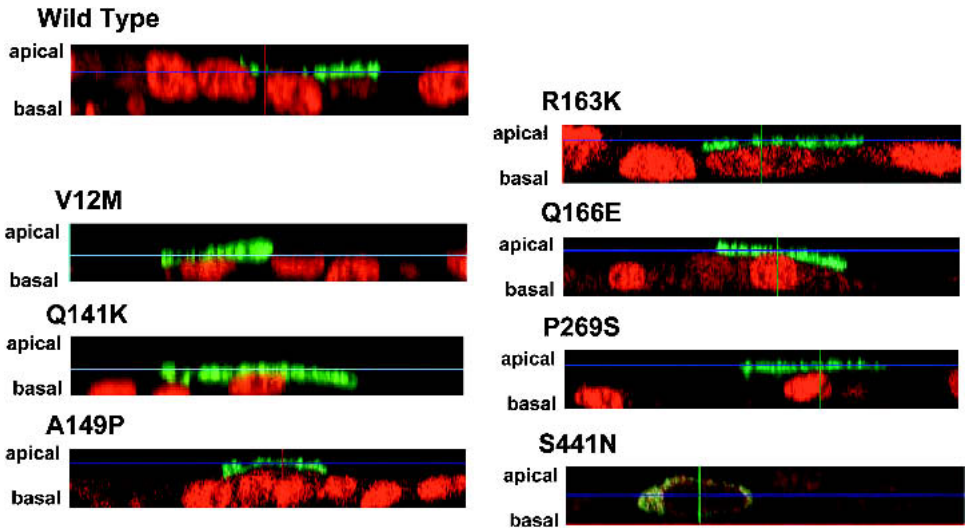


Fig. 1. Localization of BCRP in LLC-PK1 cells. The immunolocalization of BCRP molecules in the LLC-PK1 cells transfected with BCRP cDNA was determined using monoclonal antibody against BCRP (green). Nuclei were stained with PI (red). Figures show the Z-sectioning images, top side of the figure shows the apical membrane, and bottom side is the basal membrane.

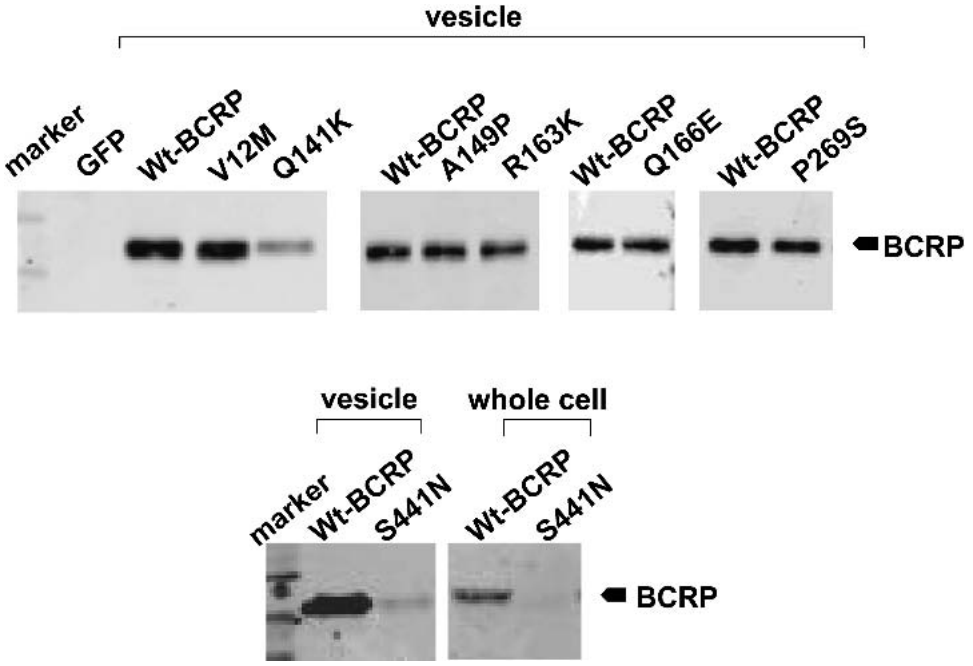


Fig. 2. Expression level of BCRP in membrane vesicles. The expression level of the wild type and SNPs variants of BCRP proteins was determined in the isolated membrane vesicles from HEK293 cells infected with recombinant adenovirus using Western blot analysis. The expression levels of wild type and S441N SNPs BCRP were also determined also in whole cell lysates.

dependent manner (11), the effect of SNPs on the BCRP-mediated transport of [^{3}H]E_1S was examined. In the current study, we used the same batch of transfected cells for measurement of the expression level and transport activity. Ad-wt BCRP expressing membrane vesicles transported [^{3}H]E_1S up to approximately 0.5 nmol min^{-1} mg^{-1} membrane protein, whereas no uptake of [^{3}H]E_1S was observed by the membrane vesicles from Ad-GFP infected cells. Because the amount of [^{3}H]E_1S molecules taken up by 2 μg membrane vesicles at 1 min (1 pmol) is approximately 1/10 of that in the incubation medium (10 pmol), [^{3}H]E_1S molecules were not depleted from the incubation medium. Based on this consideration, it is possible for us to determine the initial uptake velocity from 1 min data. Indeed, we could find that the time profile for the BCRP-mediated uptake of [^{3}H]E_1S was linear up to 2 min.

Except for two SNP variants of BCRP (Q141K and S441N BCRP), the ATP-dependent uptakes per mg membrane protein of SNP variants (V12M, A149P, R163K, Q166E, P269S BCRP) were similar to that of the wild-type BCRP (Fig. 3a). The uptake activity of Q141K BCRP per mg membrane protein was approximately 30–40% of the wild-type BCRP, and that of S441N was almost the same as that of the GFP-infected control cells. These uptake activities were inhibited by the excess amount of E_1S, 100 μM (Fig. 3a).

Then, in order to compare the intrinsic transport activity of the wild-type and SNP variants of BCRP, the uptake determined per mg membrane protein (Fig. 3a) was normalized relative to the expression levels estimated by Western blot analysis (Fig. 2), except for S441N BCRP the expression level of which was extremely low (Fig. 2). As shown in Fig. 3b, the transport activity of other SNP variants of BCRP (V12M, Q141K, A149P, R163K, Q166E, and P269S BCRP) was almost identical to that of the wild-type BCRP.

As far as V12M and Q141K BCRP were concerned, these have a high allele frequency in Japanese and we determined the kinetic parameters for the transport of [^{3}H]E_1S. As shown in Fig. 4, the ATP-dependent uptake of [^{3}H]E_1S was saturable, and the K_m values were 11.6 ± 4.79, 9.07 ± 1.52, and 14.0 ± 7.27 μM, and the V_{max} values were 13.3 ± 3.3, 13.5 ± 1.29, and 4.57 ± 1.58 nmol min^{-1} mg^{-1} protein, for the wild type, V12M, and Q141K BCRP, respectively. In addition to [^{3}H]E_1S, the transport of other BCRP substrates was examined. As previously reported, ATP-dependent uptake of [^{3}H]methotrexate and [^{3}H]DHEAS was observed. The absolute values of their transport activity were about at 7.5 pmol 2 min^{-1} mg^{-1} mebrane protein and 150 pmol 2 min^{-1} mg^{-1} membrane protein, whereas GFP-expressing membrane vesicles tranport them at about 0.5 pmol 2 min^{-1} mg^{-1} membrane protein and 25 pmol 2 min^{-1} mg^{-1} membrane protein, respectively. Moreover, BCRP-mediated transport of [^{3}H]PAH was also detectable, and the activity of this was 10 pmol 2 min^{-1} mg^{-1} membrane protein, whereas GFP-expressing membrane vesicles tranport them at about 0.5 pmol 2 min^{-1} mg^{-1} membrane protein. Figure 5a shows the ATP-dependent uptake of DHEAS, PAH, and MTX per mg membrane protein for the wild-type and SNPs BCRP (V12M, Q141K, A149P, R163K, Q166E, P269S, and S441N BCRP). Although Q141K BCRP exhibited a lower activity than wild type BCRP, no significant transport was observed for S441N BCRP (Fig. 5a). Figure 5b shows the intrinsic transport activities normalized by the expression levels of BCRP protein. There was no significant difference between wild type BCRP and SNPs variants.

DISCUSSION

In the current study, we analyzed the function of BCRP SNPs in terms of their localization, expression level, and

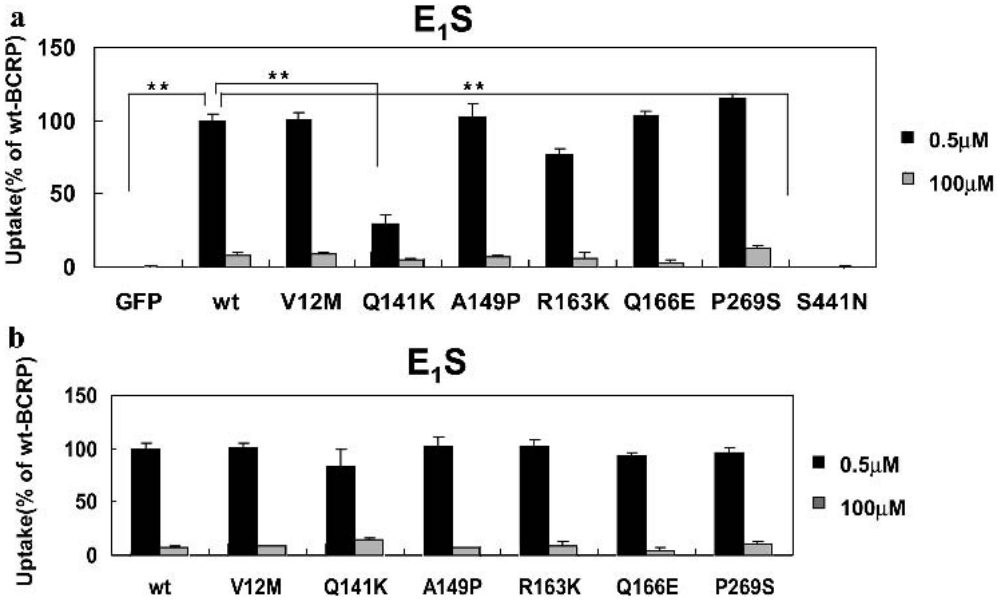


Fig. 3. The transport of E_1S by BCRP variants. The uptake of [^{3}H]E_1S by 2 μg membrane vesicles prepared from BCRP cDNA-infected and GFP cDNA-infected HEK 293 cells was examined for 2 min at 37°C in a medium containing 33 nM [^{3}H]labeled E_1S, tracer (0.5 μM) and excess concentrations (100 μM) of unlabeled E_1S. Panel 3a shows the ATP-dependent uptake of [^{3}H]E_1S by BCRP variants after normalization by the membrane protein level. The uptake was calculated by subtracting the ligand uptake in the absence of ATP from that in its presence. Panel 3b shows the ATP-dependent uptake of [^{3}H]E_1S by BCRP variants after normalization by the BCRP protein levels. For the preparation of Panel 3b, the data in Panel 3a were corrected by taking into account the BCRP protein expression level in each membrane vesicle preparation determined by the Western blot analysis (Fig. 2). Results are given as % of the wild-type BCRP. **Significantly different from wild type BCRP-expressing membrane vesicles by ANOVA followed by Dunnett's test ($p < 0.01$).

transport activity. BCRP expression systems were constructed using recombinant adenoviruses with the expectation of the high expression of the exogenous genes. Compared with the membrane vesicles isolated form the P-388 cells which were infected with BCRP cDNA by the recombinant retroviruses (11), the uptake of E_1S per mg membrane protein determined in the present study was much higher. This expression system may be useful for the analysis of functional changes in SNP variants of BCRP in a sensitive manner.

Our findings suggest that two SNP variants of BCRP may affect the function of BCRP. Q141K BCRP showed a lower expression level, which is approximately 30–40% of that of the wild type. This result is consistent with the previous report that the transfection of Q141K BCRP cDNA to PA317 cells and KB-3-1 human cells also resulted in lower expression levels (19). Recently, investigation of the expression level of BCRP in 99 Japanese placenta samples revealed that people who were homozygous for this mutation showed significantly lower expression levels of BCRP (D. Kobayashi, I. Ieiri, H. Takane, M. Kimura, Y. Norikawa, H. Tohyama, S. Irie, A. Urae, H. Suzuki, H. Kusuhara, N. Terakawa, K. Mine, K. Ohtsubo and Y. Sugiyama, data presented at 18th Annual Meeting of The Japanese Society for the Study of Xenobiotics, Sapporo, October 8–10, 2003). The clinical relevance of this *in vitro* study has major implications from the point of view of the utility of the *in vitro* system for the prediction of individual pharmacokinetic difference.

Although more detailed analysis is required to clarify the mechanism governing the reduced protein expression of Q141K BCRP, immunohistochemical staining revealed that this variant is expressed on the plasma membrane and, therefore, the altered cellular localization may not be related to the reduced protein level. It has been reported that the mRNA level of this variant is similar to that of the wild type in the transfected cells where the reduced protein level was observed (19). In addition, in the human intestine, the expression of BCRP mRNA was similar in subjects with wild type and Q141K BCRP genes, whereas there was no significant correlation between the protein and mRNA levels for BCRP (20). It has also been suggested that linkage disequilibrium is present between a CTCA deletion in the 5′-flanking region (g-19572-19569) and Q141K in Swedish subjects (21). More detailed analysis is required to clarify the mechanism for the reduced protein expression of this BCRP variant and its physiologic significance.

As far as the cellular localization was concerned, S441N BCRP was the only variant which was expressed in the intracellular compartment. We also found that the intracellular localization of S441N BCRP in HEK293 cells after transient expression (data not shown), whereas the wild-type BCRP was expressed on the cell membrane. It is possible that this variant is retained in the endoplasmic reticulum and most of the protein is degraded in the proteosomes as reported for many kinds of membrane proteins (22). Western blot analysis revealed that the expression of S441N is significantly lower than the wild type BCRP. Its expression level was reduced not only in the isolated membrane fraction, but also in the whole cell lysate (Fig. 2). These results suggest that this SNPs may

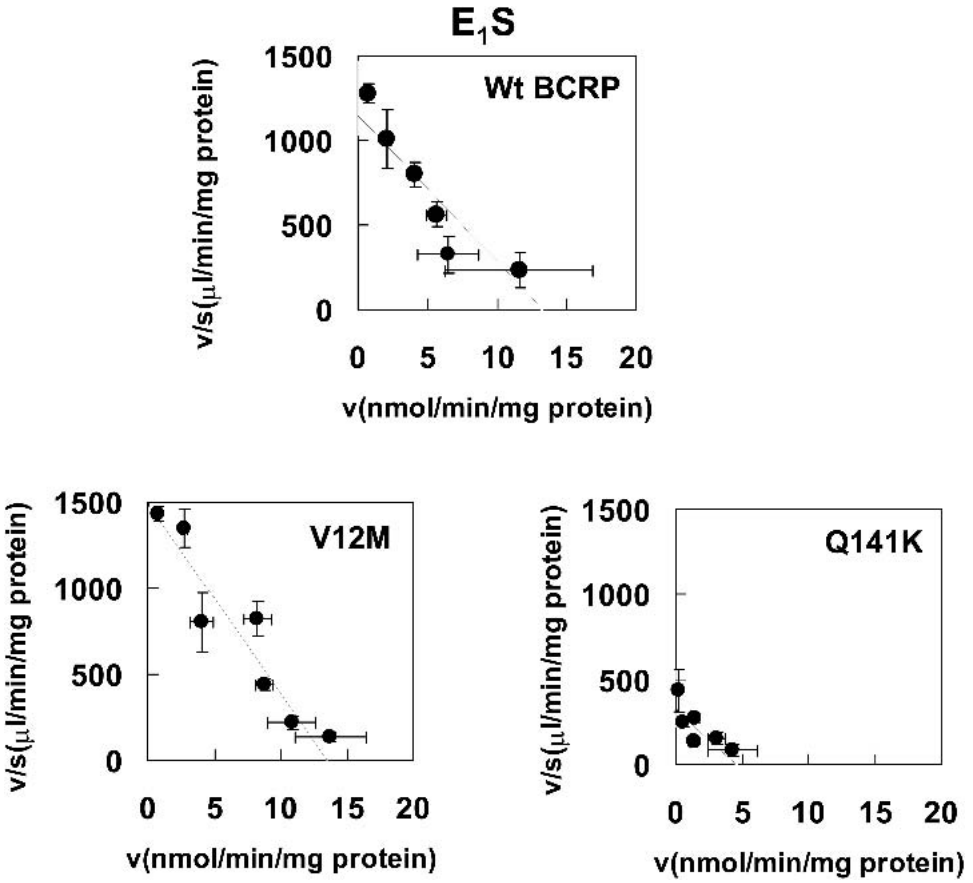
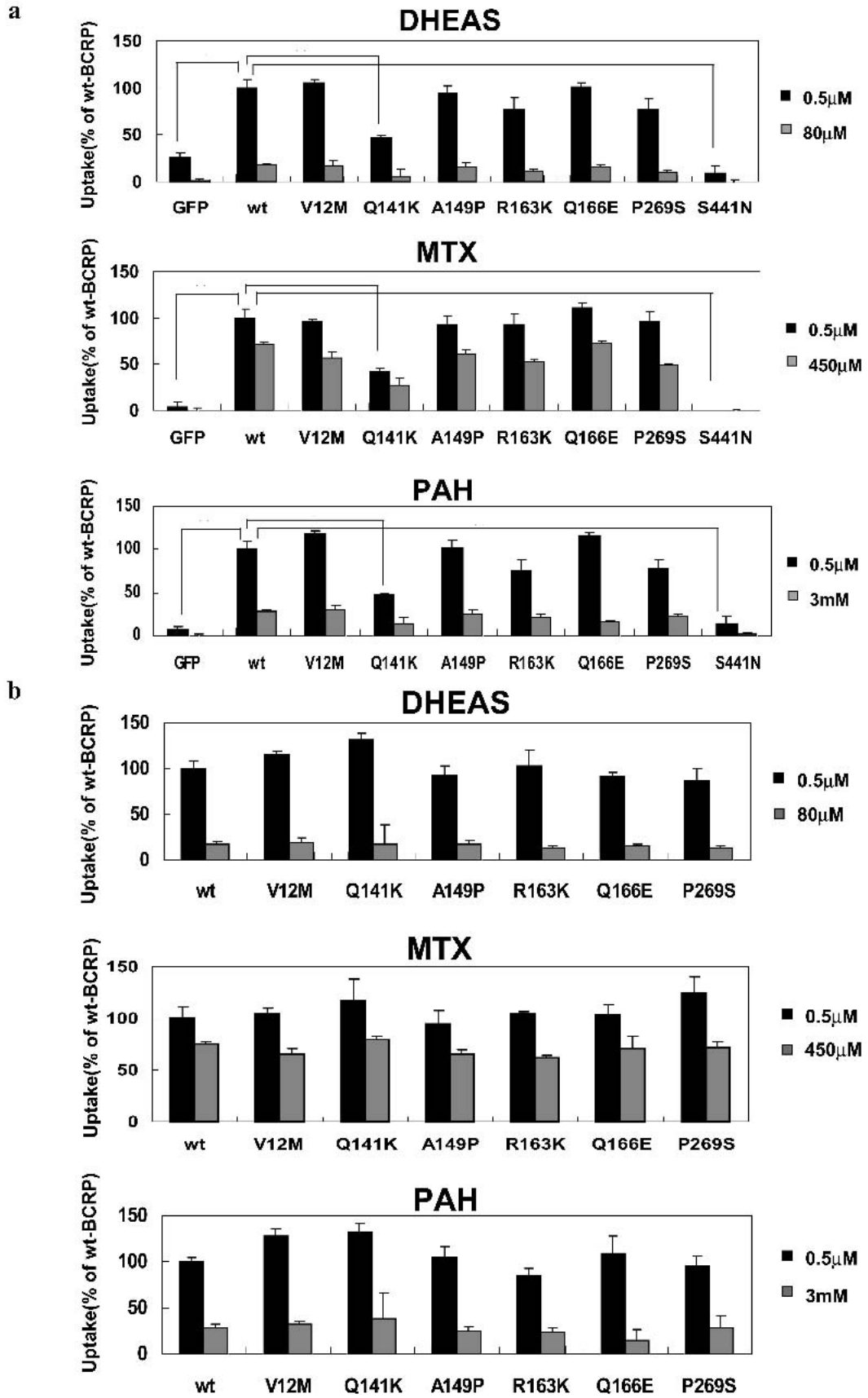


Fig. 4. Saturation of BCRP mediated transport of E_1S. Saturation of [^{3}H]E_1S transport was determined for the wild-type, V12M, and Q141K BCRP. The uptake of [^{3}H]E_1S by 2 μg membrane vesicles prepared from BCRP cDNA-infected HEK 293 cells was examined for 1 min at 37°C in a medium containing 33 nM [^{3}H]labeled E_1S and several concentrations of unlabeled E_1S. The results are given as the Eadie-Hofstee plots. Each point and bar represents the mean ± SE value of triplicate determinations.

affect both cellular localization and expression level. Concerning the cellular localization of SNPs variants of BCrP, Mizuarai *et al.* reported the intracellular localization of V12M BCRP in stably transfected LLC-PK1 cells very recently (23). The finding by Mizuarai *et al.* (23) was in marked contrast to the present finding that V12M BCRP is expressed on the apical membrane of transiently transfected LLC-PK1 cells (Fig. 1). At the present moment, we do not have any good answer to account for the discrepancy. It is possible that the cellular localization of V12M BCRP is affected by the culture conditions of LLC-PK1 cells.

Although it has been reported that the amino acid replacement at the protein of 482 alters the substrate specificity of BCRP (12,13), there has been no report on alterations in the substrate specificity due to SNP variations. In the present study, we examined the transport activity of some organic anions using isolated membrane vesicles expressing the wild type and SNP variants of BCRP. In addition to the previously described substrates such as E_1S, DHEAS and methotrexate (11), we found that PAH is also transported by BCRP. For these compounds, our results indicated that the transport activity per BCRP molecule for 6 kinds of SNP variants (V12M, A149P, R163K, Q166E, P269S, and also Q141K BCRP) is almost the same as that of the wild type BCRP (Figs. 3 and 5). In addition, the extent of the inhibition of the transport of [^{3}H]DHEAS, [^{3}H]methotrexate and [^{3}H]PAH by 80 μM DHEAS, 450 μM methtrexate and 3 mM PAH, respectively (Fig. 5), is accounted for by considering the transport char-

Fig. 5. The transport of DHEAS, MTX, and PAH by BCRP variants. The uptake of each compounds by 10 μg membrane vesicles prepared from BCRP and GFP expressing-HEK 293 cells was examined for 2 min at 37°C in a medium containing 33 nM [^{3}H]labeled compounds, tracer (0.5 μM), and excess concentrations (80 μM, 450 μM, and 3 mM, for DHEAS, MTX, and PAH, respectively) of unlabeled compounds. Panel 5a shows the ATP-dependent uptake of [^{3}H]compounds by BCRP variants after normalization by the membrane protein level. The uptake was calculated by subtracting the ligand uptake in the absence of ATP from that in its presence. Panel 5b shows the ATP-dependent uptake of these compounds by BCRP variants after normalization by the BCRP protein levels. For the preparation of Panel 5b, the uptake data were corrected by taking into account the BCRP protein expression level in each membrane vesicle preparation determined by the Western blot analysis (Fig. 2). Results are given as % of the wild type BCRP. **Significantly different from the wild type BCRP-expressing membrane vesicles by ANOVA followed by Dunnett's test ($p < 0.01$).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DHEAS
Uptake(% of wt-BCRP)
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100
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0
GFP
wt
V12M
Q141K
A149P
R163K
Q166E
P269S
S441N
0.5μM
80μM
MTX
Uptake(% of wt-BCRP)
0.5μM
450μM
PAH
Uptake(% of wt-BCRP)
0.5μM
3mM
b
DHEAS
Uptake(% of wt-BCRP)
wt
V12M
Q141K
A149P
R163K
Q166E
P269S
0.5μM
80μM
MTX
Uptake(% of wt-BCRP)
0.5μM
450μM
PAH
Uptake(% of wt-BCRP)
0.5μM
3mM

acteristics of these compounds; we reported that DHEAS reduced the BCRP-mediated transport of [^{3}H]E_1S with an approximate IC_{50} value of 55 μM (11). The K_m value for BCRP-mediated transport of methotrexate is reported to be 0.68 ~1.34 mM (24,25). We also found that PAH is transported by BCRP with the K_m value of 3~5 mM (unpublished observations). Furthermore, the K_m values for E_1S were similar between the wild type, V12M and Q141K BCRP (Fig. 4).

It has been reported that there is a marked ethnic difference in the frequency of Q141K SNPs, and this is the prevalent allele in the Japanese population (19,21). In the present study, we constructed SNP variants of BCRP based on the information from 100 Japanese placenta specimens, 84 established cancer cell lines, and 60 Japanese individuals who received the administration of irinotecan. In particular, the frequency of the Q141K variant was 29–36% in the Japanese, whereas the frequency in 26 Caucasians subjects was only 8%. Among the 100 Japanese specimens, S441N SNPs were only found in one heterozygous subject and, consequently, their allele frequency was calculated to be only 0.5%. It is possible that the disposition of BCRP substrates is different between the subjects with the wild type BCRP and those with the SNPs variants of BCRP. One of the most important substrates for BCRP is pheophorbide a, a dietary catabolite of chlorophyll. It has been shown that the plasma concentration of pheophorbide a is increased in the Bcrp1 (–/–) mice, resulting in the occurrence of severe phototoxic ear lesions (10). Since pheophorbide a is also transported by human BCRP (10), it is likely that Q141K and S441N SNPs may be involved in the phototoxicity and protoporphyria induced by the intake of chlorophyll.

Concerning the disposition of antitumor drugs, it has also been reported that Bcrp1 (–/–) hematopoietic stem cells are sensitive to mitoxantrone-induced toxicity (26). These data suggest that the ability to protect stem cells from some genotoxic xenobiotics might be lower in subjects who have Q141K and S441N SNPs in BCRP gene. It has also been suggested that the oral absorption of topotecan is restricted by the intestinal expression of BCRP (9). This suggestion is based on the findings that, after the oral administration of both topotecan and the inhibitor of Mdr1 and Bcrp1, GF120918, the bioavailability of this antitumor drug in plasma was dramatically increased, not only in normal mice but also in Mdr1 (–/–) mice (9). It has also been reported that, in human jejunal biopsies, mRNA expression of BCRP is higher than that of MDR1 mRNA (27), suggesting the importance of BCRP in drug absorption. It has also been shown that BCRP plays an important role in placenta (9). In mdr1a/1b (–/–) mice, administration of GF120918 resulted in the higher topotecan levels in fetuses and maternal plasma. Moreover, SNPs of BCRP may also be involved in the intestinal toxicity of SN-38, an active metabolite of irinotecan. Since BCRP also transports SN-38 (28), it is possible that subjects who have Q141K or S441N SNPs variants of BCRP are more sensitive to SN-38.

In conclusion, we have shown that two kinds of SNP variants of BCRP (Q141K and S441N BCRP) are associated with the reduced expression. In particular, S441N variation is associated with the altered cellular localization. Since the allele frequency of Q141K in Japanese subjects is as high as 29-36%, it is possible that the interindividual variations in *in vivo* disposition of BCRP substrates may result from the genotype of BCRP.

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