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Research Paper

The Identification of Two Germ-line Mutations in the *Human Breast Cancer Resistance Protein* Gene that Result in the Expression of a Low/Non-functional Protein

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Purpose. We examined the effects of the nine nonsynonymous germ-line mutations/SNPs in the *breast cancer resistance protein (BCRP/ABCG2)* gene on the expression and function of the protein.
Materials and Methods. We generated cDNAs for each of these mutants (G151T, C458T, C496G, A616C, T623C, T742C, T1291C, A1768T, and G1858A *BCRP*) and compared the effects of their exogenous expression in PA317 cells with a wild-type control.
Results. PA/F208S cells (T623C *BCRP*-transfectants) expressed marginal levels of a BCRP protein species (65 kDa), which is slightly smaller than wild-type (70 kDa), but this mutant did not appear on the cell surface or confer drug resistance. PA/F431L cells (T1291C *BCRP*-transfectants) were found to express both 70 kDa and 65 kDa BCRP protein products. In addition, although PA/F431L cells expressed 70 kDa BCRP at comparable levels to PA/WT cells, they showed only marginal resistance to SN-38. PA/T153M cells (C458T *BCRP*-transfectants) and PA/D620N cells (G1858A *BCRP*-transfectants) expressed lower amounts of BCRP and showed lower levels of resistance to SN-38 compared with PA/WT cells.
Conclusions. We have shown that T623C *BCRP* encodes a non-functional BCRP and that T1291C *BCRP* encodes a low-functional BCRP. Hence, these mutations may affect the pharmacokinetics of BCRP substrates in patients harboring these alleles.

KEY WORDS: BCRP/ABCG2; drug resistance; SN-38; SNPs.

INTRODUCTION

ATP binding cassette (ABC) transporters, such as P-glycoprotein (P-gp) and MRP1, are responsible for the acquisition of multidrug resistance in cancer cells (1–3). These transporters pump out various structurally unrelated anticancer drugs in an ATP-dependent manner. Breast cancer resistance protein (BCRP/ABCG2) is a half-molecule ABC transporter harboring an N-terminal ATP binding domain and a C-terminal transmembrane domain that mediates resistance to SN-38 (an active metabolite of irinotecan), mitoxantron, and topotecan (4–8). We previously reported that BCRP forms a homodimer via Cys-603 interactions and that these homodimeric complexes function as an efflux pump for anticancer agents (9,10). We have also reported earlier that BCRP exports sulfated estrogens, suggesting that there is a physiological role of BCRP for the tissue distribution and excretion of steroid hormones (11). BCRP is also widely expressed in normal human tissues such as the placenta, intestine, kidney, liver, prostate, ovary, testis, and hematopoietic stem cells (5,12,13). BCRP is assumed to play a role in the protective functions of the maternal-placental barrier, blood-testis barrier and hematopoietic stem cells against toxic substances and metabolites (14,15). BCRP has also shown to be expressed in the mammary gland during lactation and, it seems to be responsible for the active secretion of the BCRP substrates into milk (16).

In our previous study, we identified three nonsynonymous SNPs within the *BCRP* gene, G34A substituting Met for Val-12 (V12M), C376T substituting a stop codon for Gln-126 (Q126Stop), and C421A substituting Lys for Gln-141 (Q141K). G34A *BCRP* cDNA-transfected cells were found to express similar amounts of BCRP protein and further showed similar levels of SN-38 resistance compared with wild-type *BCRP* cDNA-transfected cells. In contrast C376T *BCRP* cDNA encodes a nonfunctional protein and C421A *BCRP* cDNA-transfected cells expressed a lower amount of BCRP protein and showed lower resistance to SN-38 than wild-type *BCRP* transfected cells (17). More-

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ABBREVIATIONS: ABC transporter, ATP-binding cassette transmembrane transporter; BCRP, breast cancer resistance protein; DHFR, dihydrofolate reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRES, internal ribosome entry site; SNPs, single nucleotide polymorphisms.

Table I. Frequencies of Germ-line Mutations/SNPs Within The *BCRP* Gene

Variation		Frequency (%)	Number	Population	Reference
Nucleotide	Amino acid				
G34A	V12M	19	29	Japanese	17
G151T	G51C	0.1[a]	350	Japanese	
C376T	Q126Stop	1.2	124	Japanese	17
C421A	Q141K	26.6	124	Japanese	17
C458T	T153M	3.3	30	Cell line	32
C496G	Q166E	0.3[a]	200	Japanese	
A616C	I206L	20	10	Hispanic	33
T623C	F208S	0.3[a]	200	Japanese	
T742C	S248P	0.5[a]	200	Japanese	
T1291C	F431L	0.6[b]	260	Japanese	34
A1768T	N590Y	1.1	88	Caucasians	33
G1858A	D620N	1.1	90	unknown	35

[a] Determined in this study.
[b] Determined in this study and in previous reports (frequencies and patient numbers are combined).

over, as C421A *BCRP* expresses low levels of protein, it was reported to affect the pharmacokinetics of patients harboring this allele who had been treated with an intracavernous administration of difromotecan (18). C421A *BCRP* was also reported to affect the pharmacokinetics of rosuvastain in healthy Chinese males (19).

In our present study, we generated nine *BCRP* cDNAs, each carrying a nonsynonymous germ-line mutation/SNP that has been already published or reported in a database. The effects of these different mutations on BCRP expression and function were examined in cells exogenously expressing these cDNAs.

MATERIALS AND METHODS

BCRP Expression Vectors

We generated nine cDNAs corresponding to the *BCRP* germ-line mutations/SNPs, G151T, C458T, C496G, A616C, T623C, T742C, T1291C, A1768T, G1858A [Table I (17), (32–35)]. G34A and C421A *BCRP* cDNAs were also used as controls. These *BCRP* germ-line mutations/SNPs have been described previously, some are in publication and the others are in the database of National Center for Biotechnology Information. For the transfection of these *BCRP* cDNA species, we generated bicistronic constructs using the pHa-IRES-DHFR vector. Mutant *BCRP* cDNAs were then prepared using the Mutan-Super Express Km site-directed mutagenesis system (Takara, Ohtsu, Japan) as directed by the manufacturer's instructions. Either wild-type or germ-line mutation/SNP-containing *BCRP* cDNAs without any other mutations were subsequently inserted into the pHa-IRES-DHFR bicistronic retrovirus vector that carries *DHFR* cDNA.

Cell Culture Conditions and Establishment of *BCRP* Transfectants

Murine fibroblast PA317 cells were cultured in Dulbecco's modified eagle medium supplemented with 7% fetal bovine serum at 37°C in a humidified 5% CO_2 environment. For the establishment of both wild-type and mutant *BCRP* transfectants, PA317 cells were transfected with pHa-BCRP-IRES-DHFR constructs containing either wild-type, G34A, G151T, C421A, C458T, A616C, T623C, T742C, T1291C, A1768T, or G1858A *BCRP* cDNA using a MBS Mammalian Transfection Kit (Stratagene, La Jolla, CA). The cells were selected with 120 ng/mL of methotrexate, and the resulting mixed populations of resistant cells were designated as PA/WT, PA/V12M, PA/G51C, PA/Q141K, PA/T153M, PA/I206L, PA/F208S, PA/S248P, PA/F431L, PA/N590Y and PA/D620N, respectively. The PA/F208S clones and PA/F431L clones were obtained by limiting dilution.

Cell Growth Inhibition Assay

Anticancer agent resistance levels in both the parental PA317 cells and in the various BCRP transfectants were

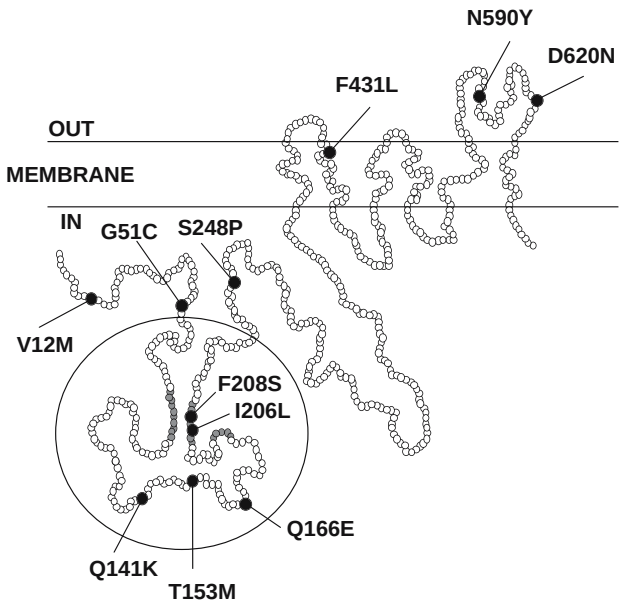
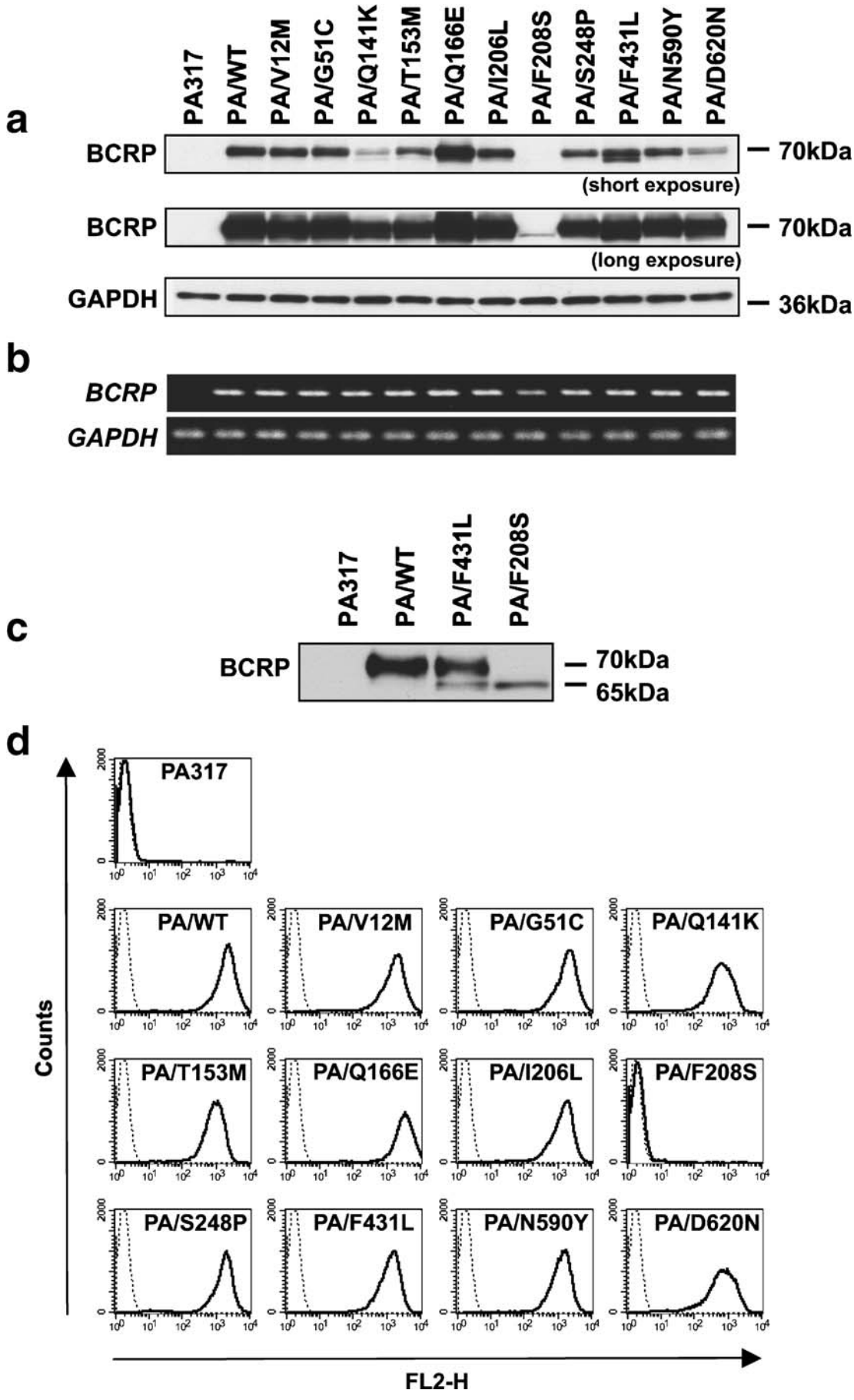


Fig. 1. Schematic representation of the breast cancer resistance protein and locations of the germ-line mutations/SNPs analyzed in this study.


a
PA317
PA/WT
PA/V12M
PA/G51C
PA/Q141K
PA/T153M
PA/Q166E
PA/I206L
PA/F208S
PA/S248P
PA/F431L
PA/N590Y
PA/D620N
BCRP
70kDa
(short exposure)
BCRP
70kDa
(long exposure)
GAPDH
36kDa
b
BCRP
GAPDH
c
PA317
PA/WT
PA/F431L
PA/F208S
BCRP
70kDa
65kDa
d
Counts
PA317
PA/WT
PA/V12M
PA/G51C
PA/Q141K
PA/T153M
PA/Q166E
PA/I206L
PA/F208S
PA/S248P
PA/F431L
PA/N590Y
PA/D620N
FL2-H

evaluated by cell growth inhibition assays after incubation of the cells for 5 days at 37°C in the absence or presence of various concentrations of SN-38 (Yakult Honsha, Tokyo). Cell numbers were determined with a Coulter counter (Sysmex, Kobe, Japan). IC_{50} values (drug dose causing 50% inhibition of cell growth) were determined from growth inhibition curves.

Western Blotting

Cell lysates were obtained as described previously (10). Cell lysates from the *BCRP* transfectants were resolved by SDS-PAGE and then electro-transferred onto a nitrocellulose membrane. The membrane was incubated with 1 μg/mL of anti-BCRP polyclonal antibody 3488 (9) and a GAPDH mouse monoclonal antibody as an internal control, followed by washing and treatment with peroxidase-conjugated sheep anti-rabbit and anti-mouse secondary antibody (Amersham, Buckinghamshire, UK), respectively. The membrane-bound antibodies were visualized with the Enhanced Chemiluminescence (ECL) Plus Western blotting detection system (Amersham).

Fluorescence-activated Cell Sorting (FACS) Analysis of BCRP Expression

The expression levels of human BCRP on the cell surfaces of various *BCRP* transfectants were examined by FACS analysis using a human-specific anti-BCRP monoclonal antibody (eBiosciences, San, Diego, CA), that was raised against a cell surface epitope of BCRP. The cells were incubated with or without a biotinylated anti human ABCG2 (20 μg/mL) for 30 min on ice, followed by washing and incubation with R-phycoerythrin-conjugated streptavidin (400 μg/mL; BD Biosciences, Franklin Lakes, NJ) (20) for 30 min on ice. Fluorescence staining levels were measured using FACS Calibur (BD Biosciences).

Semi-quantitative Reverse Transcriptase Chain Reaction (RT-PCR) Analysis

The isolation of total RNA and subsequent RT-PCR analysis was performed using an RNeasy kit (Qiagen, Valencia, CA) and an RNA LA PCR kit (Takara), respectively, according to the manufacturer's instructions. First-strand *BCRP* cDNA was synthesized from 0.3 μg of total RNA and a 824 bp *BCRP* cDNA fragment was amplified by PCR with the forward and reverse primers, 5′-GATATCAATGATACAGGGTT-3′ and 5′-TGTCCAATAGAATATTCCCC-3′, respectively. The PCR conditions were as follows: 95°C for 5 min, followed by 18–24 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, and a final extension for 7 min at 72°C. As an internal control, the amplification of *GAPDH* cDNA (551 bp fragment) was carried out using the same procedure.

Table II. SN-38 Resistance Levels of PA317 Transfectants[a]

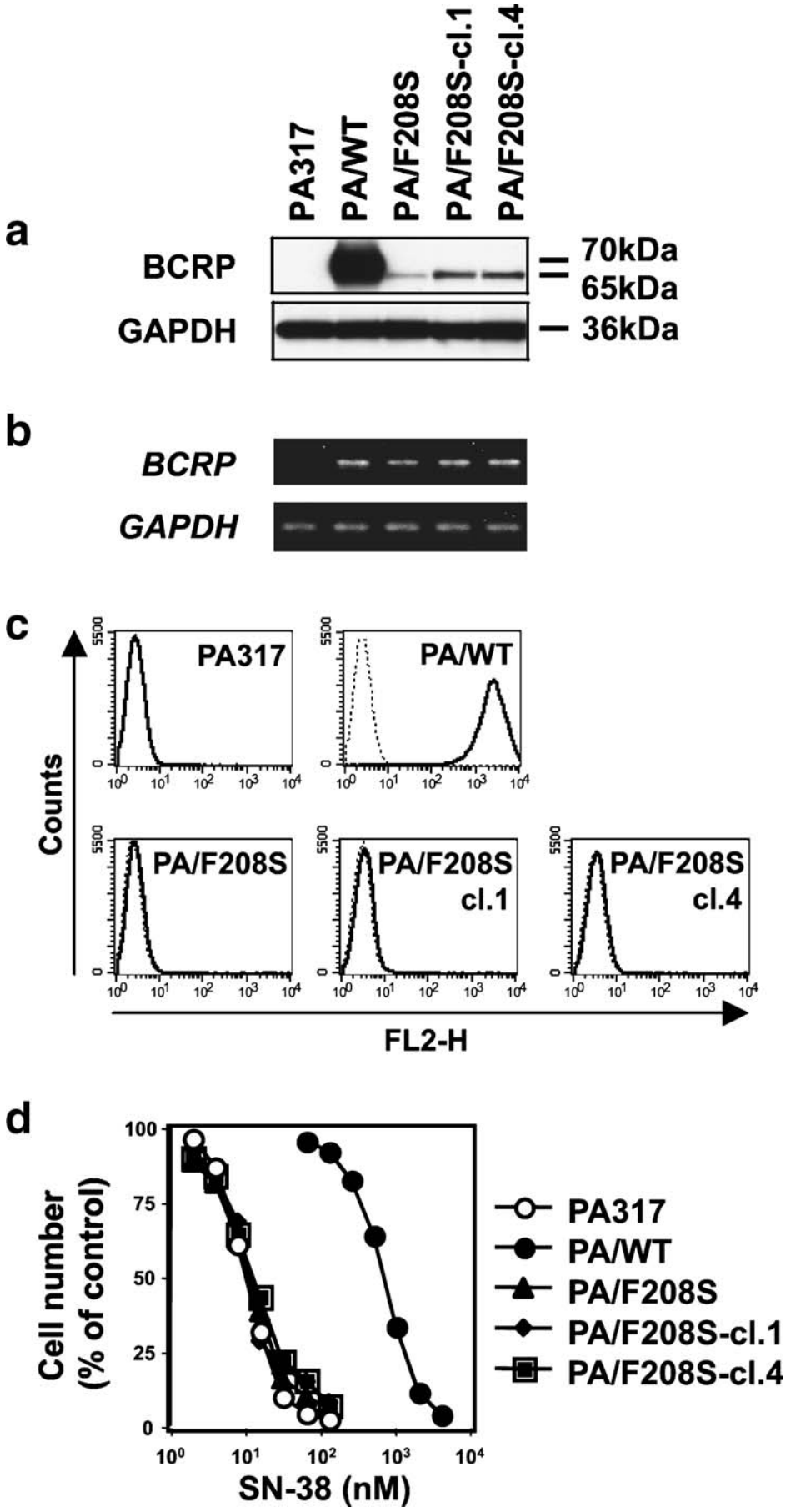
Cell type	IC_{50} (nmol/L)	Degree of resistance
PA317	11 ± 0.2	1
PA/WT	550 ± 16	50
PA/V12M	490 ± 13	45
PA/Q141K	110 ± 5.9	10
PA/T153M	260 ± 15	24
PA/Q166E	680 ± 40	62
PA/F208S	10 ± 0.7	1
PA/F431L	34 ± 0.9	3
PA/D620N	190 ± 5.7	17

[a] Cells were cultured for 5 days with various concentrations of SN-38. Cell numbers were then measured using a Coulter counter, and the IC_{50} values were determined. The degree of drug resistance is calculated as the IC_{50} ratio of resistant cells divided by that of the parental cells. The data are represented as the mean ± SD from triplicate determinations.

Sequencing Analysis of the *BCRP* Gene

Peripheral blood nucleated cells were obtained from both healthy volunteers and cancer patients of Japanese nationality, after obtaining written informed consent, to undertake genetic analysis from each of these individuals. Exon 2 of the *BCRP* gene, which covers the 151st nucleotide of *BCRP* cDNA, was amplified by PCR with the primer set, forward; 5′-GCAATCTCATTTATCTGGACTA-3′ and reverse; 5′-TGTGAGGTTCACTGTAGGTAAA-3′. Exon 5 of the *BCRP* gene, which covers the 496th nucleotide of *BCRP* cDNA was amplified by PCR with the primer set, forward; 5′-CCTTAGTTATGTTATCTTTGTG-3′ and reverse; 5′-GAAACTTCTGAATCAGAGTCAT-3′. Exon 6 of the *BCRP* gene, which covers the 623rd nucleotide of *BCRP* cDNA was amplified by PCR with the primer set, forward; 5′-GCTCACCAAATGATAATGACT-3′ and reverse; 5′-TGGGACATAGTAGTGATAAGA-3; Exon 7 of the *BCRP* gene, which covers the 742nd nucleotide of *BCRP* cDNA was amplified by PCR with the primer set, forward; 5′-GAGCAAACAATCTAAAGGCAA-3′ and reverse; 5′-ACCCAAAGACCAAACAGCACT-3′. Exon 11 of the *BCRP* gene, which covers the 1291st nucleotide of *BCRP* cDNA was amplified by PCR with the primer set, forward; 5′-CTGTCTAAGAATGCTGAGTTG-3′ and revere; 5′-ATCAGTCTAACCAATAGCCCC-3′. The resulting PCR products were directly sequenced using the following primers, which were designed from the respective intronic sequences; 5′-AACTTACTATTGCTTTTCTGTC-3′ (from

◀ **Fig. 2.** BCRP protein and mRNA expression in PA317 transfectants. **a**, Western blot analysis of BCRP in each of the *BCRP* transfectants. Protein samples (20 μg) were subjected to western blotting using the rabbit anti-BCRP polyclonal antibody (3488) or a mouse anti-GAPDH monoclonal antibody. The short and long exposures indicated were of 5 min and 15 min duration on X-ray film, respectively. **b**, Semi-quantitative RT-PCR of *BCRP* mRNA in the PA317 transfectants. The *BCRP* (824 bp) and *GAPDH* (551 bp) transcripts were amplified by RT-PCR from 0.3 μg of total RNA. **c**, Western blot analysis of BCRP in PA317, PA/WT, PA/F431L, and PA/F208S cells as described above. **d**, BCRP cell surface expression analysis in the PA317 transfectants by FACS. Parental PA317 cells and corresponding *BCRP* transfectants were harvested and then incubated with (*bold line*) or without (*dotted line*) a biotinylated anti-human BCRP monoclonal antibody 5D3, followed by treatment with R-phycoerythrin-conjugated streptavidin. After washing, the fluorescence intensities were measured using FACS Calibur.


a
PA317
PA/WT
PA/F208S
PA/F208S-cl.1
PA/F208S-cl.4
BCRP
GAPDH
70kDa
65kDa
36kDa
b
BCRP
GAPDH
c
Counts
PA317
PA/WT
PA/F208S
PA/F208S cl.1
PA/F208S cl.4
FL2-H
d
Cell number (% of control)
SN-38 (nM)
PA317
PA/WT
PA/F208S
PA/F208S-cl.1
PA/F208S-cl.4

−46 to −25 upstream of exon 2), 5′-CTAAACAGTCATGGTCTTAGAAA-3′(from −68 to −46 upstream of exon 5), 5′-AAATGATAATGACTGGTTGTT-3′(from −52 to −32 upstream of exon 6), 5′-AAGAATAGAGTATTTTACTGAGA-3′(from −75 to −53 upstream of exon 7), 5′-CTAAGAATGCTGAGTTGACTG-3′(from −50 to −30 upstream of exon 11).

RESULTS

Expression of BCRP in PA317 Transfectants

The germ-line mutations and resulting amino acid substitutions examined in this study were as follows; G151T (G51C), C458T (T153M), C496G (Q166E), A616C (I206L), T623C (F208S), T742C (S248P), T1291C (F431L), A1768T (N590Y) and G1858A (D620N). G51C, T153M, Q166E, I206L, F208S and S248P are located in the intracellular domain of the protein (Fig. 1 and Table I). F431L, N590Y and D620N are located within the transmembrane domain (Fig. 1 and Table I).

BCRP expression levels in each of the PA317 transfectants were then examined by western blotting. The wild-type *BCRP* transfectants (PA/WT) express a 70-kDa BCRP species (Fig. 2a). Similar to previous findings (14), PA/V12M cells were observed to express similar amounts of BCRP compared with PA/WT cells, whereas PA/Q141K cells expressed significantly lower amounts of BCRP than PA/WT (Fig. 2a). Among the 11 mutant BCRP transfectants under study, PA/F208S cells were found to express the lowest levels of BCRP, corresponding to a 65-kDa protein (Fig. 2a and c). PA/F431L expressed BCRP products of two distinct molecular sizes, 70-kDa and 65-kDa (Fig. 2a and c). PA/T153M and PA/D620N transfectants expressed lower amounts of BCRP than PA/WT cells, but these levels were higher than those in the PA/Q141K cells (Fig. 2a). PA/Q166E cells expressed higher amounts of BCRP (70-kDa) than PA/WT cells (Fig. 2a). The remaining transfectants PA/G51C, PA/I206L, PA/S248P, and PA/N590Y expressed BCRP at levels that were comparable to PA/WT cells (Fig. 2a).

The BCRP mRNA expression levels in each of the transfectants were analyzed by RT-PCR. As shown in Fig. 2b, the 11 mutant *BCRP* transfectants expressed *BCRP* transcript levels that were comparable to PA/WT cells (Fig. 2b).

◀ **Fig. 3.** BCRP protein and mRNA expression in PA/F208S clones. **a**, Western blot analysis of BCRP in PA/208S clones. Protein samples (20 μg) were subjected to western blotting using either a rabbit anti-BCRP polyclonal antibody 3488 or a mouse anti-GAPDH monoclonal antibody. **b**, Semi-quantitative RT-PCR analysis of *BCRP* mRNA in the indicated PA/F208S clones. The *BCRP* (824 bp) and *GAPDH* (551 bp) transcripts were amplified by RT-PCR from 0.3 μg of total RNA. **c**, BCRP cell surface expression analysis of PA/F208S clones by FACS as described for Fig. 2d. **d**, Drug resistance levels for the PA/F208S clones. PA317 (*open circle*), PA/WT (*closed circle*), PA/F208S (*closed triangle*), PA/F208S clone 1 (*closed lozenge*), and clone 4 (*closed square*) cells were cultured for 5 days with various concentrations of SN-38. Cell numbers were determined using a Coulter counter. Data are represented by the mean ± SD from triplicate experiments.

Cell Surface BCRP Expression in the Mutant *BCRP* Transfectants

The expression levels of BCRP on the cell surfaces of each of the transfectants were examined by FACS and were undetectable in either the PA/F208S or parental PA317 cells (Fig. 2d). PA/Q141K, PA/T153M and PA/D620N cells expressed lower amounts of BCRP on their cell surfaces than PA/WT cells (Fig. 2d). These results were consistent with the immunoblotting analysis (Fig. 2a). The cell surface expression of BCRP in PA/Q166E cells was slightly higher compared with PA/WT cells (Fig. 2d). Each of the other transfectants (PA/G51C, PA/I206L, PA/S248P, PA/F431L, and PA/N590Y cells) showed similar cell surface BCRP expression levels to PA/WT (Fig. 2d).

Drug Resistance of Mutant *BCRP* Transfectants

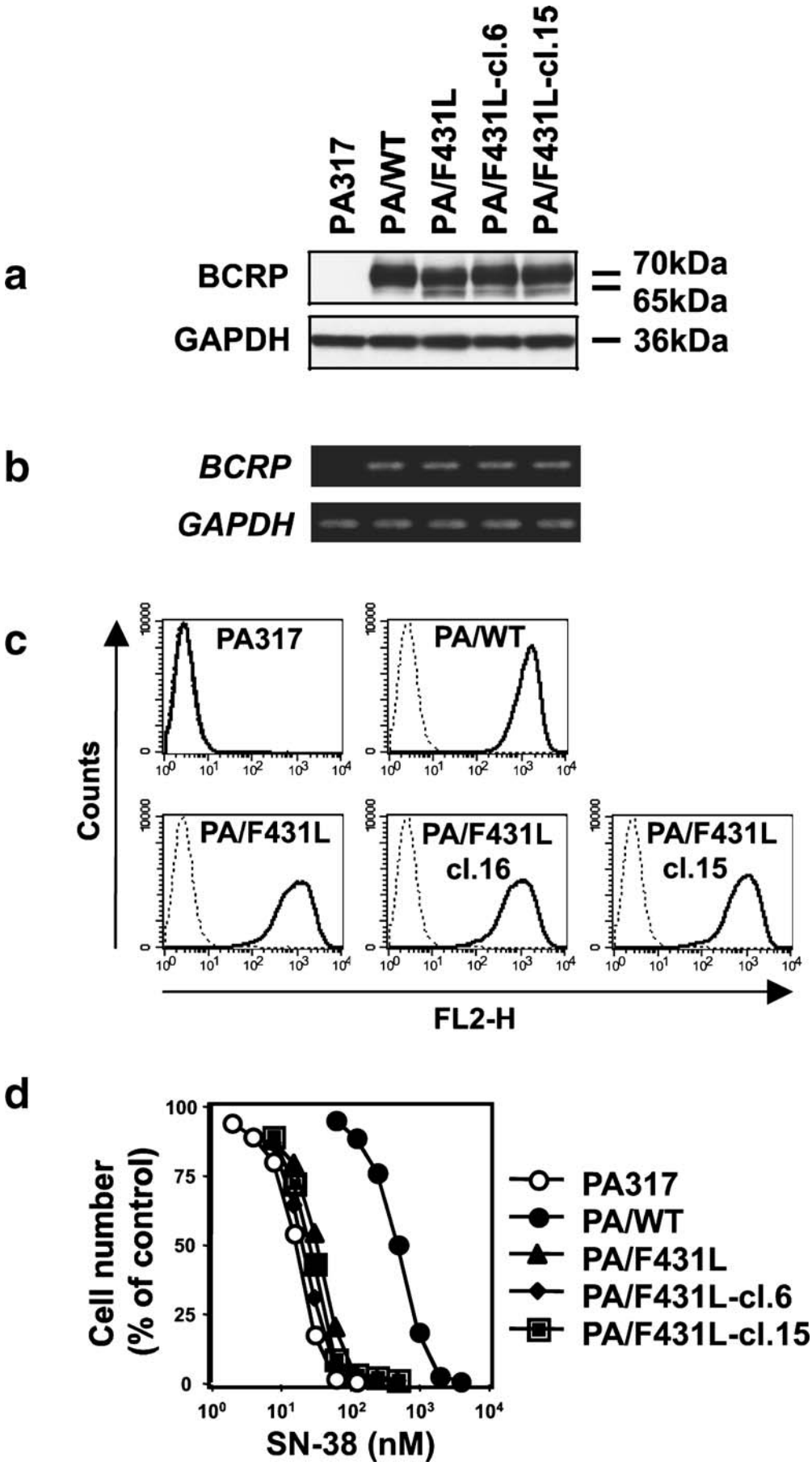
The resistance of each of the *BCPR* transfectants to SN-38 was analyzed by cell growth inhibition assay. PA/WT cells showed a 50-fold higher resistance to SN-38 than the parental PA317 cells (Table II). PA/F208S cells showed a similar level of SN-38 sensitivity to PA317 cells (Table II). PA/F431L cells showed 3-fold higher resistance to SN-38 than PA317 cells but PA/F431L cells were found to be 15-fold more sensitive to this agent than PA/WT cells (Table II). PA/Q141K, PA/T153M, and PA/D620N cells showed 10–24-fold higher resistance levels to SN-38 compared with the parental cells (Table II). However, these cells were 2–5 times more sensitive to SN-38 when compared with PA/WT cells (Table II). Additional transfectants (PA/G51C, PA/Q166E, PA/I206L, PA/S248P, and PA/N590Y cells) showed no change in their drug resistance profiles to SN-38 compared with PA/WT cells (Table II).

Analyses of PA/F208S Subclones

We isolated two independent clones from the population of PA/F208S cells, (PA/F208S-cl.1 and -cl.4) that expressed higher levels of 65-kDa BCRP protein than PA/F208S cells by western blot (Fig. 3a), but the cell surface expression of BCRP were not detectable in these clones by FACS analysis (Fig. 3c). In addition, these clones did not show SN-38 resistance (Fig. 3d).

Analyses of PA/F431L Clones

PA/F431L cells expressed two species of BCRP of molecular weights 70- and 65-kDa (Fig. 2a). To confirm whether these two versions of the protein were derived from a single gene, we isolated independent PA/F431L subclones, PA/F431L-cl.6 and -cl.15. As shown in Fig. 4a, both of these clones simultaneously expressed the 70- and 65-kDa BCRP species, similar to the original mass population of PA/F431L cells. FACS analysis further revealed that these clones also showed similar BCRP expression levels on their cell surfaces to PA/WT and PA/F431L cells (Fig. 4c). Moreover, these clones showed no change in their exogenous *BCRP* mRNA levels compared with PA/WT and PA/F431L (Fig. 4b) but showed only marginal resistance to SN-38 treatment (Fig. 4d).


a
PA317
PA/WT
PA/F431L
PA/F431L-cl.6
PA/F431L-cl.15
BCRP
GAPDH
70kDa
65kDa
36kDa
b
BCRP
GAPDH
c
PA317
PA/WT
PA/F431L
PA/F431L cl.16
PA/F431L cl.15
Counts
FL2-H
d
Cell number (% of control)
SN-38 (nM)
PA317
PA/WT
PA/F431L
PA/F431L-cl.6
PA/F431L-cl.15

Frequencies of Germ-line Mutations Within the *BCRP* Gene

Due to the possible significance of the T623C and T1291C *BCRP* mutations, we examined the frequencies of five germ-line mutations, G151T, C496G, T623C, T742C and T1291C *BCRP*, among Japanese populations. We analyzed 200–350 samples in this study, depending on the frequency of each mutation. As shown in Table I, allele frequencies for the T623C *BCRP* and T1291C *BCRP* allele were 0.3% and 0.6%, respectively. A healthy volunteer was heterozygous for the T623C *BCRP* allele. Two healthy volunteer and a cancer patient were heterozygous for the T1291C *BCRP* allele. We previously reported that allele frequency for the C376T *BCRP*, that encodes a truncated protein, was 1.2% in the Japanese population (17). From both our previous and present results, we conclude that there are in fact two non-functional germ-line mutations/SNPs in the *BCRP* gene, C376T and T623C. It should be noted however that we have not thus far identified any individuals who are homozygous for either the C376T or T623C alleles, nor have we observed individuals who are heterozygous for a combination of these two alleles.

DISCUSSION

In our current study, we have examined the effect of the nine germ-line mutations/SNPs, G151T, C458T, C496G, A616C, T623C, T742C, T1291C, A1768T, and G1858A *BCRP*, resulting in the amino acid changes G51C, T153M, Q166E, I206L, F208S, S248P, F431L, N590Y, D620N, respectively, on BCRP protein expression and function. We first obtained both the wild-type and mutant *BCRP* cDNAs and expressed each in PA317 cells. The resulting mixed populations of cells were designated a PA/WT, PA/V12M, PA/G51C, PA/Q141K, PA/T153M, PA/I206L, PA/F208S, PA/S248P, PA/F431L, PA/N590Y and PA/D620N. PA/F208S cells were found to express marginal levels of BCRP (65-kDa) (Figs. 2a and 3a), which were slightly lower than wild-type BCRP, but did not appear on the cell surface (Figs. 2d and 3c). Moreover, PA/F208S cells did not show any drug resistance (Fig. 3c and Table II). PA/F431L cells expressed a 65-kDa and 70-kDa species of BCRP (Figs. 2a and 4a). In addition, although PA/F431L cells expressed BCRP at cell surface levels that were similar to PA/WT cells (Figs. 2d and 4c), they showed only marginal resistance to SN-38 (Fig. 4d and Table II). PA/T153M and PA/D620N cells expressed lower levels of BCRP and also showed lower resistance to SN-38, compared with PA/WT cells (Fig. 2a and Table II).

Our previously described bicistronic pHa-BCRP-IRES-DHFR construct (20–23) was used for the establishment of the mutant *BCRP* transfectants in the present study. In the resulting transfectants, *BCRP* and *DHFR* products are translated independently from single bicistronic mRNAs that are transcribed under the control of a retroviral long terminal repeat promoter. The upstream *BCRP* cDNA is translated in a cap-dependent manner, and the downstream *DHFR* is translated under the control of the IRES. Hence, cells expressing *DHFR*, resulting in methotrexate resistance, theoretically always coexpress the *BCRP* cDNA. It is noteworthy that methotrexate itself is reported to be a substrate of BCRP (24,25), but wild-type BCRP-transfected cells show only marginal resistance to this drug. In addition, BCRP expression does not affect the survival of cells transfected with the bicistronic DHFR vector, and mixed population of the methotrexate-resistant colonies (>100) were used in this study.

The amino acid positions of the germ-line mutations/SNPs examined in this study are represented by the black circles in Fig. 1. G51C, T153M, Q166E, I206L, F208S, and S248P are located in the intracellular domain, and F431L, N590Y, and D620N reside in the transmembrane domain. Walker A (80–89), Walker B (204–210), and Signature C (186–190) in the ATP binding site of BCRP are indicated by the gray circles in Fig. 1 and are conserved in other members of the ATP transporter family (26). The I206L BCRP and F208S BCRP mutants harbor amino acid substitutions within the Walker B region, which is likely to have a significant impact upon the functioning of the ATP binding site. PA/F208S cells express a marginal amount of a smaller BCRP protein species(65 kDa), which is not expressed on the cell surface (Figs. 2a, c, d, 3a and c). Moreover, PA/F208S cells do not show any drug resistant properties. Considering no expression of F208S BCRP mutant on the cell surface of PA/F208S, the lack of drug resistance property in the transfectant is probably due to the absence of cell surface transporter. On the other hand, PA/I206L cells show similar levels of BCRP expression and the resistance to SN-38 as PA/WT cells. Further studies are ongoing to evaluate the ATP-binding and -hydrolyzing activity of I206L BCRP and F208S BCRP mutants.

We recently examined the effects of a T3587G germ-line mutation in the human *MDR1* gene and found that the resulting I1196Y P-glycoprotein (P-gp), that also contains an amino acid substitution within the Walker B domain, did not have ATP-binding activity (27). In our T3587G *MDR1* transfectants, I1196S P-gp also did not appear on the cell surface, and the transfected cells were drug sensitive. These results are very similar to our current data for the T623C (F208S) *BCRP* germ-line mutation. Surprisingly, both the Ile residue of I1196Y P-gp and the Phe of F208S BCRP occupy the amino acid positions in the Walker B motifs of P-gp and BCRP, respectively. A number of ongoing studies in our laboratory are therefore currently focused on the mechanisms underlying the maturation and stability of mutant ABC transporters as this may have a significant impact upon the effectiveness of cancer chemotherapy regimens.

The loss of function of particular mutant ABC transporters has been extensively studied for multidrug resistance associated protein 2 (MRP2) in relation to Dubin-Johnson syndrome, an inherited disorder defined by chronic hyperbilirubinemia (28–30). R768W MRP2, which has the amino acid substitution in Signature C of the first ATP binding site of the protein, confers high serum bilirubin concentrations in the affected patients (28), and the mutant protein is not completely glycosylated (29). Q1382R MRP2 is a mutation

◀ **Fig. 4.** BCRP protein and mRNA expression in PA/F431L clones as described in Figs. 2 and 3. **a**, Western blot analysis of BCRP in PA/F431L clones **b**, Semi-quantitative RT-PCR of *BCRP* mRNA in PA/F431L clones. **c**, BCRP cell surface expression analysis of PA/F431L clones by FACS. **d**, Drug resistance levels in the PA/F431L clones. PA317 (*open circle*), PA/WT (*closed circle*), PA/F431L (*closed triangle*), PA/F431L clone 6 (*closed lozenge*), and PA/F431L clone 15 (*closed square*) cells were cultured for 5 days with various concentrations of SN-38 and assayed as described in Fig. 3d.

between the Walker A and the Signature C regions of the second ATP-binding site, and causes loss of ATP hydrolysis activity (29). Furthermore, the deletion of Arg-1392 and Met-1393 in MRP2, located between the Walker A and the Signature C regions of the second ATP-binding site, leads to both impaired maturation and trafficking of the protein (30). Based upon these earlier reports, it is evident that various amino acid substitutions in the ATP binding domain can result in a dysfunctional ABC transporter.

The F431L residue is located in the second transmembrane domain (Fig. 1) and PA/F431L cells express two species of BCRP of 70-kDa and 65-kDa in size. The 65-kDa F431L BCRP product has the same molecular weight as F208S BCRP by SDS-PAGE (Fig. 2a and c). From the results of our analysis of PA/F431L clones, these two products seemed to be generated from a single cDNA species (Fig. 4b). The 70-kDa BCRP expression levels in PA/F431L cells were also much higher than the 65-kDa BCRP protein in the same cells (Figs. 2c and 4c). Although PA/F431L cells express higher quantities of 70-kDa BCRP compared with PA/Q141K, PA/T153M, and PA/D620N cells (Fig. 2a) these cells in fact show a lower resistance to SN-38 than these other three transfectants (Table II). From these results, we speculate that this residue might in fact be important in the recognition of SN-38, and that the F431L substitution may result in lower transporter function than the wild-type protein. We previously reported that seven mutants of BCRP at residue E446, located in the second transmembrane domain, did not show any drug resistance and that 13 mutants of BCRP at R482, residing in the third transmembrane domain, showed higher resistance to mitoxantron and doxorubicin than wild-type BCRP (31).

PA/T153M and PA/D620N cells showed low-levels of BCRP expression and drug resistance to SN-38 compared with PA/WT cells (Fig. 2a and Table II). These results were similar to the data obtained for PA/Q141K cells and based upon these data, we hypothesize that the decrease in the resistance levels to SN-38 may not be due to functional alterations but to decreased protein expression. Similar results were obtained using NIIH3T3/T153M and NIH3T3/D620N cells (date not shown).

The possible significance of the C421A *BCRP* was recently evaluated in a phase I study of diflomotecan, a new camptothecin derivative anticancer drug (18). In this study, five patients who were heterozygous for the C421A allele showed three-fold higher blood drug concentrations of diflomotecan than 15 patients who had wild-type allele (18). Following intravenous administration of this drug, the area-under-curve (AUC) of the individuals with the C421A allele and patients who were homozygous wild-type were 138 ng·h/mL and 46.1 ng·h/mL, respectively ($P = 0.015$). This study has therefore clearly shown that germ-line mutations/SNPs in the *BCRP* gene that cause a reduction in expression or loss of functions of the protein will alter the pharmacokinetics of BCRP substrate anticancer agents.

CONCLUSION

We have characterized two important *BCRP* germ-line mutations, T623C (F208S) and T1291C (F431L). T623C *BCRP* cDNA encodes a non-functional BCRP, and T1291C *BCRP* cDNA encodes a low-functional protein product. Polymorphisms within the *BCRP* genes of individuals that severely disrupt transporter activity are thus likely to be associated with hypersensitivity to substrate anticancer agents. Because BCRP may play crucial roles in the absorption and excretion of anticancer drugs, the monitoring of *BCRP* germ-line mutations/SNPs should be considered carefully during the clinical development of novel anticancer agents and BCRP-reversing drugs.

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