Review Article

Breast cancer resistance protein: Molecular target for anticancer drug resistance and pharmacokinetics/pharmacodynamics

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Breast cancer resistance protein (BCRP) is a half-molecule ATP-binding cassette transporter that forms a functional homodimer and pumps out various anticancer agents, such as 7-ethyl-10-hydroxycamptothecin, topotecan, mitoxantrone and flavopiridol, from cells. Estrogens, such as estrone and 17β-estradiol, have been found to restore drug sensitivity levels in *BCRP*-transduced cells by increasing the cellular accumulation of such agents. Furthermore, synthetic estrogens, tamoxifen derivatives and phytoestrogens/flavonoids have now been identified that can effectively circumvent BCRP-mediated drug resistance. Transcellular transport experiments have shown that BCRP transports sulfated estrogens and various sulfated steroidal compounds, but not free estrogens. The kinase inhibitor gefitinib inhibited the transporter function of BCRP and reversed BCRP-mediated drug resistance both *in vitro* and *in vivo*. *BCRP*-transduced human epidermoid carcinoma A431 (A431/BCRP) and *BCRP*-transduced human non-small cell lung cancer PC-9 (PC-9/BCRP) cells showed gefitinib resistance. Physiological concentrations of estrogens (10–100 pM) reduced BCRP protein expression without affecting its mRNA levels. Two functional polymorphisms of the *BCRP* gene have been identified. The C376T (Q126Stop) polymorphism has a dramatic phenotype as active BCRP protein cannot be expressed from a C376T allele. The C421A (Q141K) polymorphism is also significant as Q141K-*BCRP*-transfected cells show markedly low protein expression levels and low-level drug resistance. Hence, individuals with C376T or C421A polymorphisms may express low levels of BCRP or none at all, resulting in hypersensitivity of normal cells to BCRP-substrate anticancer agents. In summary, both modulators of BCRP and functional single nucleotide polymorphisms within the *BCRP* gene affect the transporter function of the protein and thus can modulate drug sensitivity and substrate pharmacokinetics and pharmacodynamics in affected cells and individuals. (*Cancer Sci* 2005; 96: 457–465)

In cancer chemotherapy, there are two major problems to be overcome. One is the innate or acquired resistance of cancer cells to anticancer drugs. The other is the toxic effects of chemotherapeutic drugs on some normal tissues, such as bone marrow and the digestive organs. The study of the mechanisms of drug resistance in cancer cells has led to the identification of some of the genes and gene products that confer drug resistance. For example, a family of ATP-binding cassette (ABC) transporters, including the *MDR1* gene product P-glycoprotein (ABCB1)[1,2] and MRP1 (ABCC1),[3] have previously been shown to be responsible for multidrug resistance. Significantly, both P-glycoprotein and the MRP proteins have internally duplicated structures with two membrane-spanning domains and two ATP-binding domains.[1–3]

Breast cancer resistance protein (BCRP), also called ABCG2, ABCP and MXR, is a half-molecule ABC transporter with an N-terminal ATP-binding domain and a C-terminal transmembrane domain (TM) (Fig. 1).[4–9] We have shown that BCRP functions as a homodimer.[10] BCRP mediates resistance to several anticancer drugs, such as 7-ethyl-10-hydroxycamptothecin (SN-38, an active metabolite of irinotecan), mitoxantrone, topotecan and flavopiridol.[4–9] *BCRP*-transduced human myelogenous leukemia K562 (K562/BCRP) cells showed 25-fold higher resistance to SN-38, 10-fold higher resistance to mitoxantrone and 10-fold higher resistance to topotecan.[11] Hence, overexpression of BCRP in certain types of malignant cells would limit the effectiveness of some anticancer agents.

Breast cancer resistance protein is usually expressed in a variety of normal tissues, such as placenta, intestine, kidney, liver, mammalian gland, ovary, testis, endothelium and in hematopoietic stem cells.[12–14] BCRP is therefore assumed to play a role in the protective functions of the maternal–placental barrier, digestive tract and blood–testis barrier against toxic substances and metabolites. BCRP expression has also been reported in relapsed or refractory hematological malignancies.[15,16] It has been shown that BCRP expression may be associated with poor responses to chemotherapy.[16,17] It is thus possible that BCRP expression is responsible, at least in part, for many instances of clinical drug resistance. If this proves to be the case, overcoming BCRP-mediated drug resistance would contribute greatly to improving the efficacy of many cancer chemotherapy treatments.

Various organic compounds have been identified as BCRP inhibitors. Some of these inhibitors include estrogens,[18]

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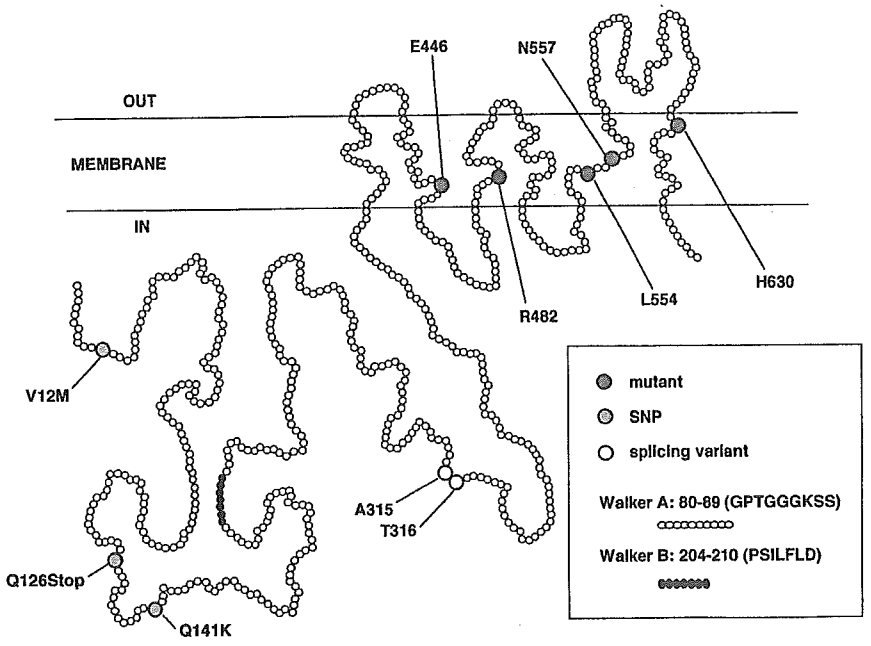

Fig. 1. Schematic structure of the breast cancer resistance protein, representing the positions of amino acid changes in mutants, single nucleotide polymorphisms and a splicing variant.

anti-estrogens,[19] flavonoids,[20] gefitinib[21–25] and imatinib.[26–28] Although a number of them are actively transported by BCRP, many act only as inhibitors of its function. In addition to anti-cancer agents, BCRP exports various dietary compounds, such as chlorophyll-derived dietary phototoxin and proto-porphyria, and dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.[29–31] As BCRP mediates the efflux of such agents from cells and tissues, suppression of its function by competitive inhibitors would be predicted to modulate the pharmacokinetics and pharmacodynamics of these drugs. This would potentially result in an increase in both the blood and tissue concentrations of the chemotherapeutics and cause more potent effects. However, there could also be increases in the undesirable side effects of these anti-cancer agents as a result of BCRP inhibition.

In this review we summarize the various factors that affect the expression and function of BCRP. These include substrates, inhibitors, regulators of protein expression and functional single nucleotide polymorphisms (SNP). We discuss the possible implications of the use of these factors in cancer chemotherapy.

Dimer formation of breast cancer resistance protein

We have demonstrated the homodimerization of BCRP using coexpression of Myc-tagged and hemagglutinin (HA)-tagged proteins (MycBCRP and HABCRP).[10] Exogenous BCRP migrated as a 70-kDa protein under reducing conditions in sodium dodecyl-sulfate–polyacrylamide gel electrophoresis, but migrated as a 140-kDa complex in the absence of reducing agents. The 140-kDa BCRP complex was found to be heat stable but to dissociate into 70-kDa species upon the addition of 2-mercaptoethanol. We immunoprecipitated the 140-kDa BCRP complex from lysates of PA317 cells doubly transfected with *MycBCRP* and *HABCRP*, using an anti-Myc antibody. The 140-kDa complex also reacted with anti-HA and anti-BCRP antibodies. In addition, following the addition of reducing agents, the 70-kDa species also reacted with these antibodies. These results clearly indicate that BCRP forms a homodimer, bridged by disulfide bonds.

To test for possible dominant-negative inhibition of the BCRP drug efflux pump, various mutant *BCRP* cDNAs were generated by polymerase chain reaction mutagenesis. These mutants were then expressed in parental PA317 cells and tested for drug-resistance properties. HA-tagged inactive *BCRP* cDNAs were subsequently transfected into MycBCRP-expressing cells and tested for their ability to lower drug resistance. Among the eight inactive mutant cDNAs, L554P-BCRP, with an amino acid change in TM5, was found to partially reverse drug resistance in *MycBCRP*-transfected cells (Fig. 1). This result suggests that homodimer formation may be essential for the transporter function of BCRP, and that dominant-negative inhibition of these complexes is a potential new strategy for circumventing drug resistance.[10]

Mutation analysis of breast cancer resistance protein

In earlier studies, *BCRP* cDNAs isolated from doxorubicin-selected and mitoxantrone-selected cells had mutations in R482.[5–7] To test for possible alterations in substrate specificity and in the drug resistance patterns of different mutant BCRP, we generated 32 such mutants with amino acid substitutions in the TM (seven E446 mutants in TM2, 15 R482 mutants in TM3, four N557 mutants in TM5 and six H630 mutants in TM6) and examined the resulting effects of these substitutions on cellular drug resistance (Fig. 1).[32] PA317 cells transfected with any one of the seven E446-mutant *BCRP* cDNA did not show drug resistance. In contrast, cells transfected with any of the 13 R482X-*BCRP* cDNA (X = N, C, M, S, T, V, A, G,

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E, W, D, Q and H, but not Y and K) showed a higher resistance to mitoxantrone and doxorubicin than wild-type *BCRP*-transfected cells. Cells transfected with N557D-*BCRP* cDNA showed a similar level of resistance to mitoxantrone, but lower resistance to SN-38 than wild-type *BCRP*-transfected cells. Cells transfected with N557E-*BCRP*, H630E-*BCRP* or H630L-*BCRP* cDNA showed similar degrees of resistance to mitoxantrone and SN-38. Cells transfected with R482G-*BCRP* or R482S-*BCRP* cDNA showed less intracellular accumulation of ^{3}H-mitoxantrone than wild-type *BCRP*-transfected cells. These results suggest that residues E446 in TM2, R482 in TM3, N557 in TM5 and H630 in TM6 play important roles in the drug recognition of BCRP.[32] R482-mutant BCRP was also shown to have a more effective pumping function for doxorubicin and mitoxantrone than the wild-type protein, but high levels of doxorubicin transport may not be associated with the physiological role of BCRP, as wild-type BCRP is less effective.[33,34] In fact, we and others have previously reported that wild-type BCRP effectively transports methotrexate and methotrexate polyglutamates. This suggests that BCRP may play a crucial role in the transport of folate derivatives.[33–35] In addition, the R482 mutation was found only in *in vitro* drug-selected cancer cells, and has not been found in any clinical specimens.

Estrogens and anti-estrogens inhibit breast cancer resistance protein

Breast cancer resistance protein is normally expressed in a variety of tissues, such as placenta, intestine, kidney, liver, mammalian gland, ovary, testis, endothelium and in hematopoietic stem cells.[12–14] Among these normal tissues, the highest level of BCRP expression has been seen in the placenta, and BCRP is therefore presumed to function in protecting the fetus against toxic compounds.[12] In addition, BCRP may mediate the transport of placenta-specific compounds across the blood–placenta barrier, as immunohistochemical analysis has shown that BCRP is highly expressed in the syncytiotrophoblast of the placenta, which produces female steroid hormones.[12] We therefore examined for possible interactions of female steroid hormones with BCRP.[18] The effects of steroid hormones and other related compounds on BCRP-mediated drug resistance were evaluated by a cell growth inhibition assay using K562/BCRP cells. Among the compounds tested, estrone and estradiol (17β-estradiol) were found to potentiate the cytotoxicity of SN-38, mitoxantrone and topotecan in K562/BCRP cells.[18] In contrast, these estrogens showed little effect on drug sensitivity in parental K562 cells.

The reversal activities (measured as the ratios of the IC_{50} values in the absence or presence of the steroid) of 10 μM estrone were 3.6-fold for SN-38, 7.5-fold for mitoxantrone and 4.1-fold for topotecan. Similarly, estradiol enhanced the cytotoxicity of these antitumor agents in K562/BCRP cells, but not in parental K562 cells. Drug resistance levels were also slightly abrogated by estriol in a dose-dependent manner but neither pregnenolone nor progesterone had any effect on the drug sensitivity of K562/BCRP cells. In order to determine whether this reversal might be associated with increased drug transport, effect of steroid hormones on the cellular accumulation of topotecan was evaluated by flow cytometric analysis. The intracellular accumulation of topotecan increased in the presence of estrone in a dose-dependent manner in K562/BCRP cells, whereas levels were not altered in parental cells. In addition, increased cellular accumulation of topotecan was also observed in the presence of both estradiol and estriol. In contrast, pregnenolone and progesterone showed only a marginal effect on topotecan uptake. These results suggest that estrogens reverse BCRP-mediated drug resistance by inhibiting its drug efflux function.[18]

We further examined the effects of other non-steroidal estrogens and anti-estrogens on BCRP-mediated drug resistance.[19] Initially, these compounds were tested for their effects on the cellular accumulation of topotecan in K562/BCRP cells. These compounds were then examined for their ability to reverse SN-38 and mitoxantrone resistance in K562/BCRP cells. Among the commercially available estrogen antagonists and agonists that were tested, synthetic estrogen diethylstilbestrol showed the strongest BCRP-reversing activity. Diethylstilbestrol was found to increase the cellular accumulation of topotecan and reverse BCRP-mediated drug resistance in K562/BCRP cells, but showed only marginal or no effect in parental K562 cells. The reversal activities of estrone and diethylstilbestrol were more prominent for mitoxantrone than for SN-38. Anti-estrogens, tamoxifen and toremifene were also found to enhance topotecan uptake in K562/BCRP cells. Various tamoxifen derivatives were subsequently screened for anti-BCRP activity and among the initial 14 compounds that were tested, TAG-11 showed the strongest effects. In a second screening of 25 TAG-11-related compounds, TAG-139 was found to show the strongest effect. Reversal of SN-38 and mitoxantrone resistance in K562/BCRP cells by TAG-139 was then found to be five-fold greater than the results with estrone. The dose-dependent characteristics of drug resistance reversal by estrone and TAG-139 treatment were very similar, suggesting that derivatives of these compounds interact with the same binding site of BCRP. Next, the possible effects of TAG-139 on P-glycoprotein-mediated and MRP1-mediated drug resistance were evaluated. TAG-139 strongly potentiated the cytotoxicity of doxorubicin and vincristine on K562/MDR cells. The reversal activity of TAG-139 was more prominent for doxorubicin than for vincristine. TAG-139 showed no effects on MRP1-mediated doxorubicin resistance or VP-16 resistance.[19]

To examine whether the BCRP-reversing activities of these compounds are associated with anti-estrogen activity, the effects of these agents on the binding of estradiol to estrogen receptors (ER) were evaluated.[19] Tamoxifen and 4-OH-tamoxifen strongly inhibited the binding of estradiol to ERα. TAG-11 showed weak inhibition of estradiol binding to ERα but TAG-72 and TAG-126, which both showed modest BCRP-reversing activity, strongly inhibited this binding. TAG-139, the most potent TAG-compound BCRP inhibitor, showed a weak interaction with ERα. Similar results were obtained with ERβ. These results show that BCRP-reversing activity and anti-estrogen activity can be disassociated. Therefore, it should be possible to develop BCRP-reversing agents that exhibit no other biological effects, including anti-estrogen activity. Such compounds would have great potential to be used clinically in overcoming BCRP-mediated drug resistance.[19]

Breast cancer resistance protein exports sulfated estrogens

Estrone and estradiol were both shown to reverse BCRP-mediated multidrug resistance. However, this did not necessarily indicate that BCRP exports these estrogens. To clarify this point, we generated *BCRP*-transduced porcine kidney LLC-PK1 (LLC/BCRP) cells, in which exogenous BCRP is expressed in the apical membrane of the cell monolayer, and investigated the transcellular transport of ^{3}H-labeled compounds using cells plated on microporous filter membranes.[36] The basal-to-apical transport (excretion) of ^{3}H-mitoxantrone, ^{3}H-estrone and ^{3}H-estradiol was greater in LLC/BCRP cells than in LLC-PK1 cells. However, thin layer chromatography of transported steroids revealed that the transport of estrone and estradiol was independent of BCRP expression. In contrast, increased excretion of estrone sulfate and estradiol sulfate was observed in LLC/BCRP cells, which was shown to be completely abrogated by BCRP inhibitors. In addition, the conversion of estrogens into their sulfated conjugates occurred at a similar rate between LLC/BCRP and LLC-PK1 cells, suggesting that the increased excretion of estrogen sulfates was attributable to BCRP-mediated transport.[36]

The uptake of ^{3}H-labeled compounds in membrane vesicles from K562/BCRP cells was also investigated.[36] ^{3}H-Labeled estrone sulfate, but not ^{3}H-labeled estrone or estradiol, was taken up by membrane vesicles from K562/BCRP cells, and this was ATP dependent. BCRP inhibitors suppressed the transport of estrone sulfate in membrane vesicles from K562/BCRP cells. Furthermore, sulfated steroidal compounds such as dehydroepiandrosterone sulfate, taurolithocholate and taurolithocholate sulfate, strongly inhibited the BCRP-mediated transport of estrone sulfate across K562/BCRP membrane vesicles, suggesting that BCRP has a high affinity for sulfated steroids. These results clearly demonstrate that BCRP does not transport either free estrone or estradiol but exports sulfate conjugates of these estrogens, which were the first identified physiological substrates of BCRP.[36,37]

Flavonoids inhibit breast cancer resistance protein

We carried out additional screens of estrogenic compounds for anti-BCRP activity and found that phytoestrogens and flavonoids, such as genistein, naringenin, acacetin and kaempferol, strongly potentiate the cytotoxicity of SN-38 and mitoxantrone in K562/BCRP cells.[20] Genistein and naringenin increased the cellular accumulation of topotecan in K562/BCRP cells. In addition, some glycosylated flavonoids, such as naringenin-7-glucoside, also effectively inhibited BCRP. These flavonoids showed marginal effects on drug sensitivity in K562 cells. Furthermore, neither genistein nor naringenin could reverse either P-glycoprotein-mediated vincristine resistance or MRP1-mediated VP-16 resistance. K562/BCRP cells accumulated less ^{3}H-genistein than parental K562 cells. Using a transcellular transport system we showed that ^{3}H-genistein transport in the basal-to-apical direction was greater in LLC/BCRP cells, which express exogenous BCRP in the apical membrane, than in parental LLC-PK1 cells. BCRP inhibitors abolished this increased transport of ^{3}H-genistein in LLC/BCRP cells.[38] Analysis by thin layer chromatography revealed that genistein was transported in its native but not in its metabolized form. These results suggest that genistein is in fact among the natural substrates of BCRP and competitively inhibits BCRP-mediated drug efflux. BCRP therefore seems to function as an efflux pump for genistein and other plant-derived flavonoids. These findings have two important clinical implications: (i) flavonoids and glycosylated flavonoids may be useful compounds for overcoming BCRP-mediated drug resistance in cancer cells; and (ii) the intake of flavonoids, mostly from food or drink, together with the administration of BCRP-substrate antitumor agents may alter the pharmacokinetics and consequently increase the toxicity of antitumor agents in cancer patients.[20]

Breast cancer resistance protein-transduced cells show gefitinib resistance

We evaluated the possible interaction of gefitinib, a selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, with BCRP.[21] *BCRP*-transduced human epidermoid carcinoma A431 (A431/BCRP) and *BCRP*-transduced human non-small cell lung cancer PC-9 (PC-9/BCRP) cells have acquired cellular resistance to gefitinib, suggesting that BCRP is one of the determinants of gefitinib sensitivity in specific cell types (Fig. 2). However, BCRP expression did not alter gefitinib sensitivity in cells that were intrinsically insensitive to this drug, such as K562/BCRP cells. Gefitinib reversed SN-38 resistance in both K562/BCRP cells and *BCRP*-transduced murine lymphocytic leukemia P388 (P388/BCRP) cells, but not in the corresponding parental cells. In addition, gefitinib sensitized human colon cancer HT-29 cells, which endogenously express BCRP, to SN-38. Gefitinib was also demonstrated to increase the intracellular accumulation of topotecan in K562/BCRP cells and suppressed ATP-dependent transport of estrone sulfate in membrane vesicles from K562/BCRP cells. These results suggest that gefitinib may overcome BCRP-mediated drug resistance by inhibiting the pump function of BCRP. Furthermore, P388/BCRP-transplanted mice that had been treated with a combination of irinotecan and gefitinib were observed to have significantly longer survival times than the P388/BCRP-transplanted mice treated with either irinotecan or gefitinib alone. In conclusion, gefitinib interacts with BCRP, the expression of which in gefitinib-responsive cells is likely to be one of the principal determinants of gefitinib sensitivity. Gefitinib also inhibits the transporter function of BCRP and reverses BCRP-mediated drug resistance both *in vitro* and *in vivo*.[21]

Other breast cancer resistance protein inhibitors

Imatinib, an inhibitor of BCR-ABL tyrosine kinase, has been reported to reverse BCRP-mediated drug resistance.[26,27] Imatinib was shown to significantly increase the accumulation of topotecan in the human osteosarcoma cell line Saos2, which expresses BCRP.[26] Another report showed that imatinib is a substrate of BCRP by demonstrating that its accumulation is low in a BCRP-overexpressing sub-line, MCF-7/MR.[27] Moreover, Ko-143, a specific inhibitor of BCRP, increased

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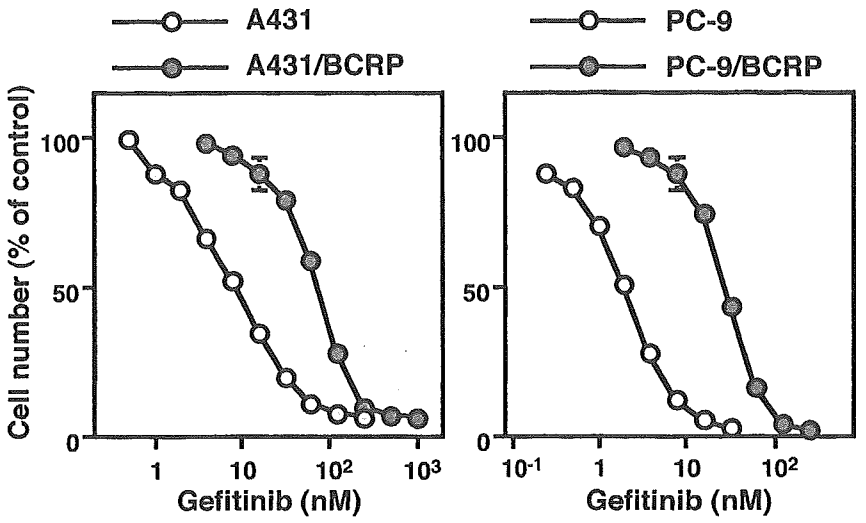

Fig. 2. Resistance to gefitinib of breast cancer resistance protein (*BCRP*)-transduced human epidermoid carcinoma A431 (A431/BCRP) and *BCRP*-transduced human non-small cell lung cancer PC-9 (PC-9/BCRP) cells. Cells were cultured for 5 days with increasing concentrations of gefitinib. Cell numbers were counted with a Coulter counter. Data are represented as mean ± SD from triplicate determinations.

the accumulation of imatinib in MCF-7/MR cells.[27] In a separate study, imatinib was efficiently transported by mouse bcrp1 in *bcrp1*-transfected Madin–Darby canine kidney strain II (MDCKII) monolayers.[28] Furthermore, the clearance of intravenously injected imatinib was significantly decreased by 1.6-fold in *bcrp1*-knockout mice, compared with wild-type mice.[28] Taken together, BCRP seems to be a determinant for imatinib sensitivity. Another potent tyrosine kinase inhibitor, CI1033, has also been shown to enhance the uptake and cytotoxicity of SN-38 and topotecan in *BCRP*-transfected cells.[39] CI1033 accumulation was diminished in BCRP-expressing cells, suggesting that it may be transported by BCRP.[39] It has been reported that BCRP exports dietary toxins and carcinogens such as chlorophyll-derived dietary phototoxin and protoporphyria, and dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.[29] This study suggests that humans or animals with low or absent BCRP activity may be at an increased risk of developing diet-dependent phototoxicity. BCRP thus seems to play a protective role against such toxins from normal food constituents.

Suppression of breast cancer resistance protein expression by estrogens

Because estrogens induce the expression of various genes via ER-mediated pathways, we examined the possible effect of estrogens on BCRP expression.[40] We found that estrogens, such as estradiol at physiological concentrations (10–100 pM), markedly decrease endogenous BCRP expression in estrogen-responsive and ERα-positive human breast cancer MCF-7 cells. These effects did not occur, however, in estrogen-non-responsive human lung cancer A549 cells. To examine the effects of estrogens on BCRP expression in various ERα-positive and ERα-negative cells, the human breast cancer cell lines MCF-7, T-47D and MDA-MB-231, the ovarian cancer cell line SKOV-3 and the lung cancer cell line A549 were transduced with *BCRP* retrovirus. Among these cell lines, MCF-7 and T-47D were both estrogen responsive and ERα positive. Estradiol significantly reduced exogenous BCRP expression, driven by a retroviral constitutive promoter, in estrogen-responsive MCF-7/BCRP and T-47D/BCRP cells, but not in estrogen-non-responsive MDA-MB-231/BCRP and SKOV-3/BCRP cells. Estradiol also significantly potentiated the cytotoxicity of SN-38, but not vincristine, in MCF-7/BCRP cells, and increased cellular topotecan uptake in MCF-7/BCRP cells. The anti-estrogen compound tamoxifen was shown to reverse estradiol-mediated BCRP downregulation in MCF-7 and MCF-7/BCRP cells. Treatment of MCF-7/BCRP cells with an ERα small interfering RNA abolished estradiol-mediated BCRP downregulation, suggesting that interaction of estradiol and ERα is necessary for this suppression. Estradiol did not alter endogenous *BCRP* mRNA levels in MCF-7 cells or exogenous *BCRP* mRNA levels in MCF-7/BCRP cells. Pulse-chase labeling experiments using MCF-7/BCRP cells suggested that decreased protein biosynthesis and maturation, but not alterations in protein turnover, might underlie estradiol-mediated BCRP downregulation. These data indicate that estrogens downregulate BCRP expression by novel post-transcriptional mechanisms (Fig. 3). Significantly, this was the first demonstration that small molecules can control cellular BCRP protein expression.[40] Analysis of the regulation of BCRP expression by estrogens would assist in the development of a more rational anticancer treatment protocol, particularly against malignancies in women.[40,41]

Functional single nucleotide polymorphisms in the *BCRP* gene

Single nucleotide polymorphisms in the *BCRP* gene and *BCRP* cDNA variants were screened in genomic DNA samples from healthy Japanese volunteers and from 11 *BCRP*-expressing human cancer cell lines, respectively. From these analyses, we identified three *BCRP* coding SNP, G34A (V12M), C376T (Q126Stop) and C421A (Q141K), and a splicing variant, Δ315-6, that lacked nucleotides 944–949 (deletion of A315 and T316) (Fig. 1).[42] It was expected that the C376T polymorphism in exon 4, which substituted Gln126 with a stop codon, would have the highest impact of the *BCRP* SNP, as an active BCRP protein cannot be expressed from the C376T allele. Of the 124 healthy Japanese volunteers that we sampled in our study, three were heterozygous for the C376T

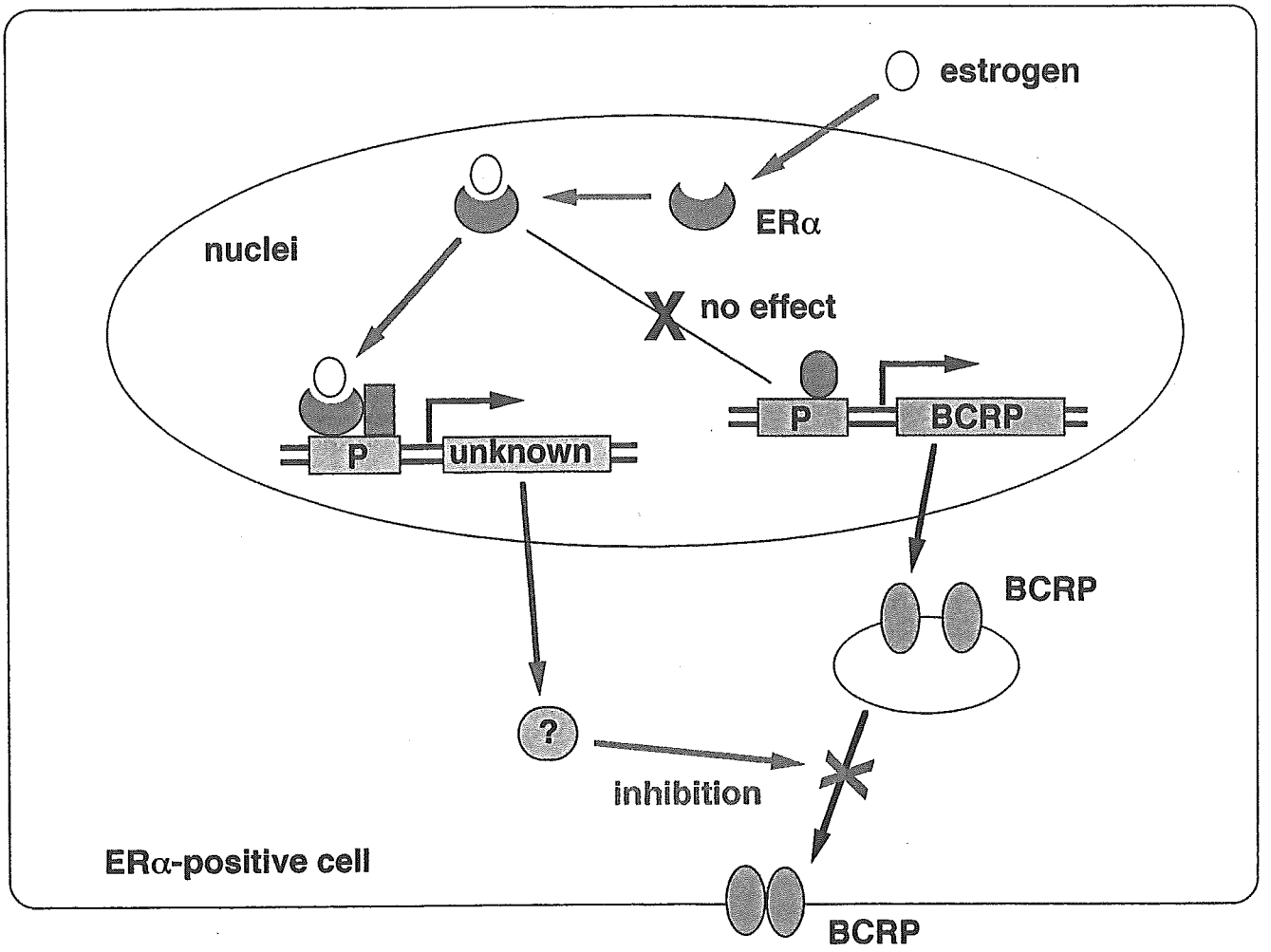

Fig. 3. Putative mechanism of the suppression of breast cancer resistance protein (BCRP) expression by estrogens. Estrogens, such as estradiol at physiological concentrations (10–100 pM), markedly decrease BCRP expression in estrogen-responsive and estrogen receptor (ER)α-positive cells. Estrogen does not affect the *BCRP* mRNA expression in ERα-positive cells. The BCRP downregulation seems to be associated with alterations in protein maturation, but not protein turnover.

allele. In addition, Q141K-*BCRP*-transfected PA317 cells showed markedly lower levels of both BCRP protein expression and drug resistance than wild-type *BCRP*-transfected cells (Fig. 4a). It was noteworthy in this case that Q141K-*BCRP*-transfectants and wild-type *BCRP*-transfectants expressed similar levels of *BCRP* transcripts (Fig. 4a). V12M-*BCRP*-transfected and Δ315-6-*BCRP*-transfected PA317 cells showed similar and somewhat lower BCRP protein expression and drug resistance levels compared with wild-type *BCRP*-transfected cells. Among the normal subjects in our analysis, 67 were wild type, 48 were heterozygous and nine were homozygous for the C421A allele. It has been reported that the C421A SNP is prevalent in 50–60% of Asians and 20–30% of Caucasians.[42] Low expression levels of the Q141K BCRP protein have been confirmed using various experimental systems.[43–45] These results suggest that some individuals harbor a C421A polymorphic *BCRP* gene and express low amounts of Q141K BCRP (Fig. 4b).

The possible significance of the C421A *BCRP* SNP was evaluated recently in a phase I study of diflomotecan, a new camptothecin derivative anticancer agent.[46] In this study, five patients who were heterozygous for the C421A allele showed three-fold higher plasma levels of diflomotecan than 15 patients who were wild type. Following intravenous administration of this drug, the area-under-curve (AUC) analysis of patients with the C421A allele and in patients who were homozygous wild type was 138 ng·h/mL·mg and 46.1 ng·h/mL·mg, respectively ($P = 0.015$).[46] A similar trend was noted after oral administration of this agent, although the differences were found not to be statistically significant. This study suggests that the *BCRP* genotype of a patient may impact strongly on the resulting pharmacokinetics of diflomotecan administration. Although the C376T allele is rare, a combination of the C376T and C421A SNP or a homozygous C421A genotype would be expected to occur frequently in Japanese individuals, considering the high incidence of this allele in Japan. In summary, individuals with C376T or C421A polymorphisms may express low amounts of BCRP, resulting in hypersensitivity of normal cells to anticancer drugs such as irinotecan, topotecan and diflomotecan.[42–45,47]

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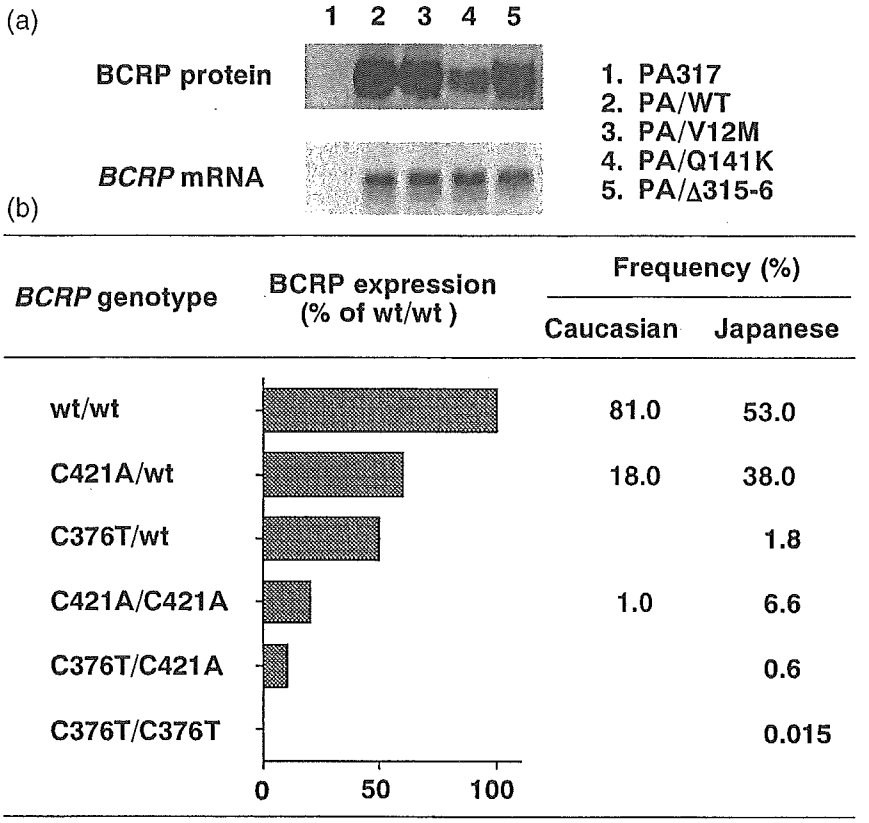

Fig. 4. Effect of C376T (Q126Stop) and C421A (Q141K) single nucleotide polymorphisms in the breast cancer resistance protein (*BCRP*) gene on protein expression. (a) Upper panel: western blotting of BCRP in PA317 cells and *BCRP* transfectants. PA317 cells transfected with wild-type, G34A, C421A and Δ944-949 *BCRP* cDNAs were termed PA/WT, PA/V12M, PA/Q141K and PA/Δ315-6, respectively. Western blot analysis processed under non-reducing conditions. The BCRP dimer was detected as a band at approximately 140 kDa (ref. 42). Lower panel: northern blot analysis of PA317 cells and *BCRP* transfectants. The blot was hybridized with an internal *BCRP* cDNA probe (ref. 42). (b) Putative BCRP expression levels in the C376T and/or C421A allele carriers. Left, putative BCRP expression levels relative to that of homozygous wild-type allele carriers. Right, putative frequencies of each genotype with respect to nucleotides 376 and 421 of the *BCRP* gene.

Conclusions

Breast cancer resistance protein, an ATP-binding cassette transporter, confers resistance to a series of anticancer agents, such as mitoxantrone, SN-38 (an active metabolite of irinotecan), topotecan and flavopiridol. BCRP expression is one of the key determinants of the sensitivity of cells to these drugs. We found that estrone and estradiol reverse drug resistance in K562/BCRP cells. Estrone and estradiol also increase the cellular accumulation of topotecan in K562/BCRP cells, but not in the parental K562 cells. BCRP-dependent and ATP-dependent uptake of estrone sulfate, but not estrone or estradiol themselves, was also observed in K562/BCRP vesicles. BCRP-dependent excretion of estrone sulfate was observed in porcine kidney LLC/BCRP cells. Taken together, BCRP exports estrone sulfate, and sulfated estrogens seem to be physiological substrates of BCRP. Based on these findings, we have identified various BCRP inhibitors among the different estrogens, anti-estrogens, phytoestrogens, flavonoids and kinase inhibitors. Some compounds, such as the flavonoid derivatives and the EGFR kinase inhibitor gefitinib, were effective against P388/BCRP *in vivo*.

Many clinical studies of P-glycoprotein inhibitors have shown that the inhibition of drug efflux pumps not only increases the sensitivity of malignant cells to anticancer agents but also modulates the pharmacokinetics and pharmacodynamics of these drugs, and increases their concentrations in blood and tissues.[48] In the case of BCRP inhibitors, it is possible that these factors could also alter the bioavailability and pharmacokinetics of the drugs that are targeted by BCRP.[49,50] An example of this is seen with GF120918, a dual inhibitor of P-glycoprotein and BCRP that increases the oral bioavailability of topotecan through the inhibition of BCRP function.[50] The camptothecins are good BCRP substrates and are being used increasingly in cancer chemotherapy. Modulation of BCRP activity by inhibitors should alter the pharmocokinetics of such chemotherapeutic drugs in a number of clinical contexts. These effects might be used advantageously in improving several aspects of chemotherapy, such as a reduction of the variability in exposure to orally administered topotecan and potentiation of the cytotoxic activity of irinotecan. In addition, however, unintentional side effects may be caused by modulations to drug bioavailability by the inhibition of transporters. These must be considered in any new strategies that combine chemotherapeutic treatments with BCRP inhibitors.

We have identified two important functioning *BCRP* SNP, C376T (Q126Stop) and C421A (Q141K), that greatly diminish the expression of this protein. No active BCRP can be expressed from the C376T allele. Furthermore, cells transfected with Q141K-*BCRP* cDNA express low amounts of BCRP protein and show only low levels of drug resistance. Polymorphisms within the *BCRP* genes of individuals that cause low transporter expression are likely to be associated with the hypersensitivity of their normal cells to substrate anticancer agents. Because BCRP may play crucial roles in the absorption and excretion of anticancer drugs such as DNA topoisomerase I inhibitors, the use of *BCRP* SNP should be considered carefully during the clinical development of novel anticancer agents and BCRP-reversing drugs.

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Role of Cys-603 in dimer/oligomer formation of the breast cancer resistance protein BCRP/ABCG2

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Breast cancer resistance protein (BCRP/ABCG2) is a half-molecule ATP-binding cassette transporter that we have previously suggested might function as a homodimer, bridged by disulfide bonds. In the present study, we carried out cysteine-scanning mutagenesis, substituting Ser for Cys, and established 12 PA317 transfectants expressing BCRP mutants with possible disruptions to their S–S bonds. Western blot analysis of BCRP from the wild-type transfectants (PA/WT) confirmed that the wild-type protein migrates as a 140-kDa dimer under non-reducing conditions, but as a 70-kDa monomer under reducing conditions. However, under non-reducing conditions the BCRP-C603S mutant migrated both as a 70-kDa monomer and a 140-kDa dimer, whereas all other mutant BCRP migrated only as dimers. PA317 cells transfected with C603S-*BCRP* (PA/C603S) showed either similar or only marginally lower SN-38 resistance than PA/WT cells, despite the reduced levels of BCRP dimer in these cells. Moreover, the degree of SN-38 resistance in the mutant BCRP transfectants was found to be associated with the monomer expression levels under reducing conditions. Reverse transcription–polymerase chain reaction analysis showed that the *BCRP* mRNA levels were similar in the transfectants. We subsequently generated six C603X mutants of BCRP (X = D, H, R, Y, A and W) and carried out western blot analysis and drug sensitivity assays. The results were equivalent to those from the PA/C603S cells, with some variations that again corresponded to the monomer levels. Our findings suggest that Cys-603 is an important residue in the covalent bridge between BCRP monomers but that a functioning unit of BCRP may not necessarily require covalent linkages. (*Cancer Sci* 2005; 96: 866–872)

ATP-binding cassette (ABC) transporters, such as the *MDR1* gene products P-glycoprotein[1–4] and MRP1,[5] are known to be involved in multidrug resistance. Breast cancer resistance protein (BCRP/ABCG2/MXR) is a new member of the ABC transporter family,[6–8] with a C-terminal transmembrane domain and an N-terminal ATP-binding domain,[9] consisting of 655 amino acids. BCRP has been studied as a molecular target for anticancer drug resistance because of its ability to confer resistance to mitoxantrone, 7-ethyl-10-hydroxycamptothecin (SN-38) and topotecan in cells by pumping out these structurally unrelated drugs.[10–16]

We previously showed that BCRP might form a homodimer, bridged by disulfide bonds, and that BCRP function was impaired by dominant-negative mutants through S–S-dependent homodimerization.[17] In our present study we removed each of the possible disulfide bonds that may be involved in BCRP dimerization by site-directed mutagenesis of the cysteine residues in this protein. As shown in Fig. 1, BCRP has six cysteines in the cytoplasm, three of which are intramembranous and three that are extracellular. We constructed 12 BCRP mutants for these analyses, each with a serine substitution in place of a cysteine, using site-directed mutagenesis of *BCRP* cDNA, and examined the resulting effects on both protein structure and function. We show from our data that Cys-603 is likely to be an important residue for the stable oligomerization of BCRP.

Materials and Methods

Cell culture and drug sensitivity assay

PA317 amphotropic retrovirus packaging cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, at 37°C in a humidified incubator with 5% CO_2. The sensitivity of the mutant *BCRP*-transfected cells to SN-38 was evaluated by the inhibition of cell growth after incubation at 37°C in the presence of various concentrations of SN-38. After a 5-day incubation of cells with these agents, cell numbers were determined using a Coulter cell counter and drug concentrations that inhibited cell growth by 50% (IC_{50}) were determined from growth inhibition curves. Statistical analysis was carried out using the Student's *t*-test.

Generation of wild-type and mutant BCRP vectors, and transfections

Mutant *BCRP* cDNAs containing cysteine-to-serine substitutions were synthesized using a site-directed mutagenesis kit (Takara, Kyoto, Japan), according to the manufacturer's instructions. Our previously described *BCRP* cDNA[17] was used as a template for these reactions. The nucleotide sequences of the mutant BCRP cDNA clones were confirmed using an automated DNA sequencer. The wild-type and mutant *BCRP* cDNAs were then cloned into pHaL-IRES-DHFR bicistronic retrovirus vectors. PA317 cells were transfected with the vectors according to the method of Chen and Okayama.[18] In

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Abbreviations: DHFR, dihydrofolate reductase; IRES, internal ribosome entry site.

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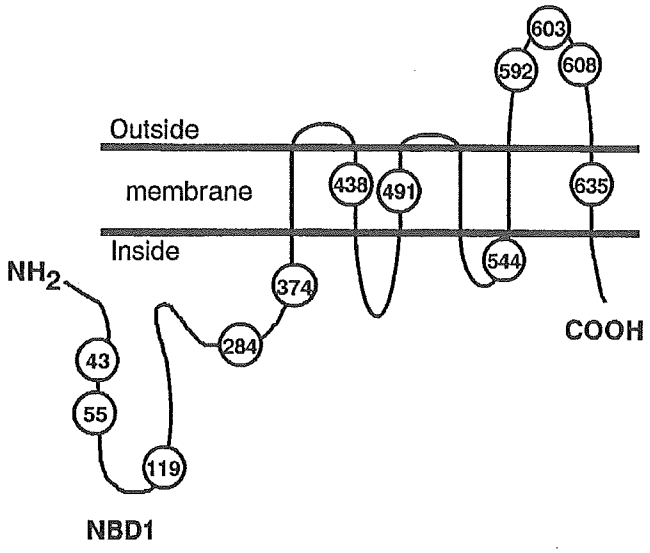

Fig. 1. Schematic diagram of a breast cancer resistance protein (BCRP) molecule indicating the position of its 12 cysteine residues. We constructed 12 mutants of BCRP by replacing each of these cysteines with serine by site-directed mutagenesis of the cDNA template.

cells transfected with pHa-BCRP-IRES-DHFR, a single mRNA is transcribed under the control of a retroviral long terminal repeat of Harvey murine sarcoma virus (LTR) promoter, and two gene products are translated independently from a bicistronic mRNA. It has been shown previously that cells expressing DHFR are resistant to methotrexate.[19] The transfectants were therefore selected by exposure to 120 ng/mL methotrexate for 5 days and by subsequent exposure to 1 ng/mL mitoxantrone for an additional 5 days to eliminate residual BCRP-non-expressing cells. The resulting *BCRP* transfectants were pooled, and were named using the prefix 'PA' (parental).

Western blot analysis under both reducing and non-reducing conditions

Exponentially growing cells were harvested, washed and solubilized in T buffer (10 mM Tris-HCl, pH 8.0, 0.1% Triton-X 100, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride) with or without 1 mM dithiothreitol. Cellular debris was removed by centrifugation and the supernatant was subjected to immunoblotting. The protein concentrations of the cell lysates were measured by the Bradford method using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA). Protein samples were then solubilized with 2% sodium dodecylsulfate (SDS), 50 mM Tris-HCl, pH 7.5, in the presence or absence of 5% 2-mercaptoethanol. Before loading, samples treated under reducing conditions were heated at 70°C for 10 min and samples subjected to non-reducing conditions were heated at 20°C for 5 min. Samples (20 μg or the appropriate amount of protein per lane) were resolved by 5–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes. The blots were then incubated with mouse anti-BCRP monoclonal antibody (BXP-21, Chemicon International, Temecula, CA, USA) for 1 h. BXP-21 recognizes an internal epitope of a 126-amino acid region of BCRP (amino acids 271–396). After washing, the blots were incubated with peroxidase-conjugated antimouse immunoglobulins (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membrane-bound peroxidase was visualized on Hyperfilm ECL Plus after enhancement with a chemiluminescence detection kit (Amersham Pharmacia Biotech).

Fluorescence-activated cell sorter analysis

After selection by methotrexate and mitoxantrone, the cell-surface expression levels of the BCRP protein were analyzed by fluorescence-activated cell sorter (FACS). Briefly, trypsinized cells (5×10^5) were incubated with 20 μg/mL of biotinylated antihuman BCRP (5D3) (eBioscience, San Diego, CA, USA), washed and incubated with R-phycoerythrin-conjugated streptavidin (BD Biosciences, San Jose, CA, USA). The epitope for this antibody is localized in one of the extracellular domains of BCRP, which corresponds to amino acids 417–428, 500–504 or 567–628. Fluorescence staining was then analyzed using FACSCalibur (BD Biosciences).

Semi-quantitative reverse transcription–polymerase chain reaction analysis

BCRP mRNA expression in wild-type and mutant *BCRP* transfectants was examined by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was extracted from 8×10^6 cells using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) and subsequent RT-PCR was carried out using an LA-RT-PCR kit (Takara), according to the manufacturer's instructions. The primers for PCR described below were previously reported.[20] First strand cDNA was synthesized with 0.3 μg of total RNA and a 315-bp *BCRP* cDNA fragment, and then amplified with the primers 5′-CAG GTG GAG GCA AAT CTT CGT-3′ (forward) and 5′-A CAC ACC ACG GAT AAA CTG A-3′ (reverse). As an internal control, amplification of *GAPDH* mRNA (551-bp fragment) was carried out with the primers 5′-ATC ACC ATC TTC CAG GAG CGA-3′ (forward) and 5′-GCT TCA CCA CCT TCT TGA TGT-3′ (reverse). The PCR conditions were as follows: 95°C for 9 min, then increasing cycle numbers of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s.

Results

Expression of BCRP in mutant BCRP transfectants

The expression of BCRP in the PA317 transfectants was evaluated by both western blotting and FACS analyses. BCRP protein in PA/WT (BCRP-WT) cells migrated as a 70-kDa monomer under reducing conditions and as a 140-kDa dimer protein under non-reducing conditions. Under reducing conditions each of the mutants migrated as a 70-kDa species, as observed for the wild-type protein (Fig. 2a). Under non-reducing conditions, however, the BCRP-C603S mutant was found to migrate as both a 70-kDa monomer and a 140-kDa dimer, whereas the BCRP-WT and each of the other mutant proteins migrated only as 140-kDa dimers (Fig. 2b). Also under reducing conditions, the BCRP protein levels in PA/C43S, PA/C55S, PA/C284S and PA/C603S cells were slightly lower than the levels in the wild-type transfectants.

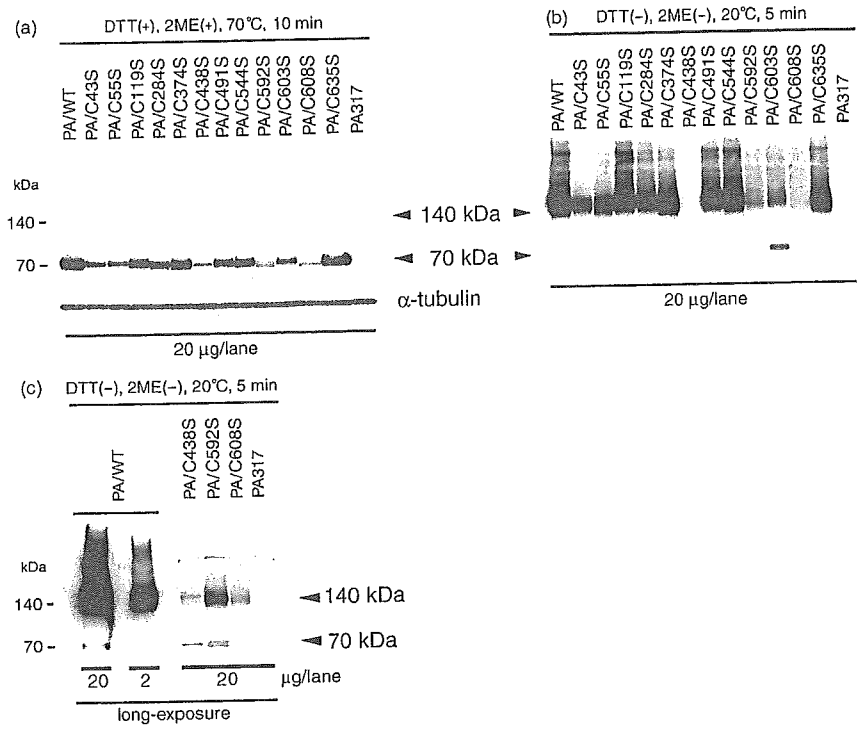

Fig. 2. Detection of mutant breast cancer resistance proteins (BCRP), containing serine substitutions, by western blot analysis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out (a) under reducing conditions with heating at 70°C for 10 min, or (b) under non-reducing conditions with heating at 20°C for 5 min. α-Tubulin expression was analyzed as a loading control. (c) Western blot analysis of three BCRP mutants with a long exposure showing weak band intensities under non-reducing conditions. Cellular protein (20 μg/lane) was separated by 5–20% SDS-PAGE and then transferred onto nitrocellulose membranes. BCRP was detected using mouse anti-BCRP monoclonal antibody (BXP-21).

Because BCRP protein expression levels in PA/C438S, PA/C592S and PA/C608S cells were found to be remarkably decreased under non-reducing conditions (Fig. 2b), they were further examined by overexposing the immunoblot (Fig. 2c). Following this increased exposure time, these mutants could also be detected as both a 70-kDa monomer and a 140-kDa dimer, as is the case for BCRP-C603S, although their expression levels were less than 10% of wild-type. The ratio of monomer to dimer in these mutants was also far higher than PA/WT, as observed for PA/C603S.

FACS analysis of our mutant BCRP transfectants was undertaken to examine the levels of exogenous protein expressed on the cell surfaces of these cells (Fig. 3). For the PA/C119S, PA/C374S, PA/C491S, PA/C544S and PA/C635S species, these exogenous mutant protein levels were equivalent to PA/WT. In contrast, the PA/C43S, PA/C55S, PA/C284S and PA/C603S mutants expressed intermediate amounts of BCRP and, in PA/C438S cells, little surface expression of BCRP was observed. In addition, the results of our FACS analyses of these 10 BCRP mutants were in accordance with the western blot analyses carried out under reducing conditions (Fig. 2a). For the PA/C592S and PA/C608S species, which may have mutations in the epitope for the 5D3 antibody located on a extracellular domain of BCRP, BCRP expression on the cell surface was undetectable by FACS, but protein could be detected at low levels by western blotting.

Semi-quantitative reverse transcription–polymerase chain reaction

Reverse transcription–polymerase chain reaction revealed that each of the transfectants expressed similar levels of *BCRP* mRNA (Fig. 4). Hence, the observed differences in BCRP expression, particularly in the PA/C438S, PA/C592S, PA/C603S and PA/C608S cells, are not attributable to low transcript levels.

SN-38 sensitivity of the mutant BCRP transfectants

The results of our SN-38 sensitivity measurements in the mutant *BCRP* transfectants, in which the exogenous BCRP expression levels were almost equivalent to PA/WT cells, are shown in Fig. 5a. Transfectants expressing a high level of exogenous BCRP mutant, including PA/C544S and PA/C635S, showed almost the same degree of resistance as PA/WT cells. The SN-38 sensitivity levels of the mutant *BCRP* transfectants that expressed either intermediate or small amounts of exogenous protein were also determined and are shown in Fig. 5b,c. Significantly, the degree of drug resistance (IC_{50} of mutant *BCRP* transfectants/IC_{50} of PA/WT) was found to be coincident with the levels of BCRP monomer detected under reducing conditions (Fig. 2a), with a correlation coefficient of 0.88.

Protein expression and SN-38 sensitivity levels of PA317 cells transfected with mutant BCRP containing Cys-603 substitutions

Because the monomeric form of BCRP was detectable in PA/C603S cells under non-reducing conditions (Fig. 2b), we chose to further analyze this specific cysteine residue by generating additional amino acid substitutions at this position. The resulting PA/C603X mutants (X = D, H, R, S, Y, A and W) were also investigated by western blotting under the same conditions used for PA/C603S. Even without the use of reducing agents, both a monomeric band and decreased amounts of the BCRP dimer could be observed for each of these mutants, whereas BCRP-WT was again present only as a dimer (Fig. 6b). Under reducing conditions, the

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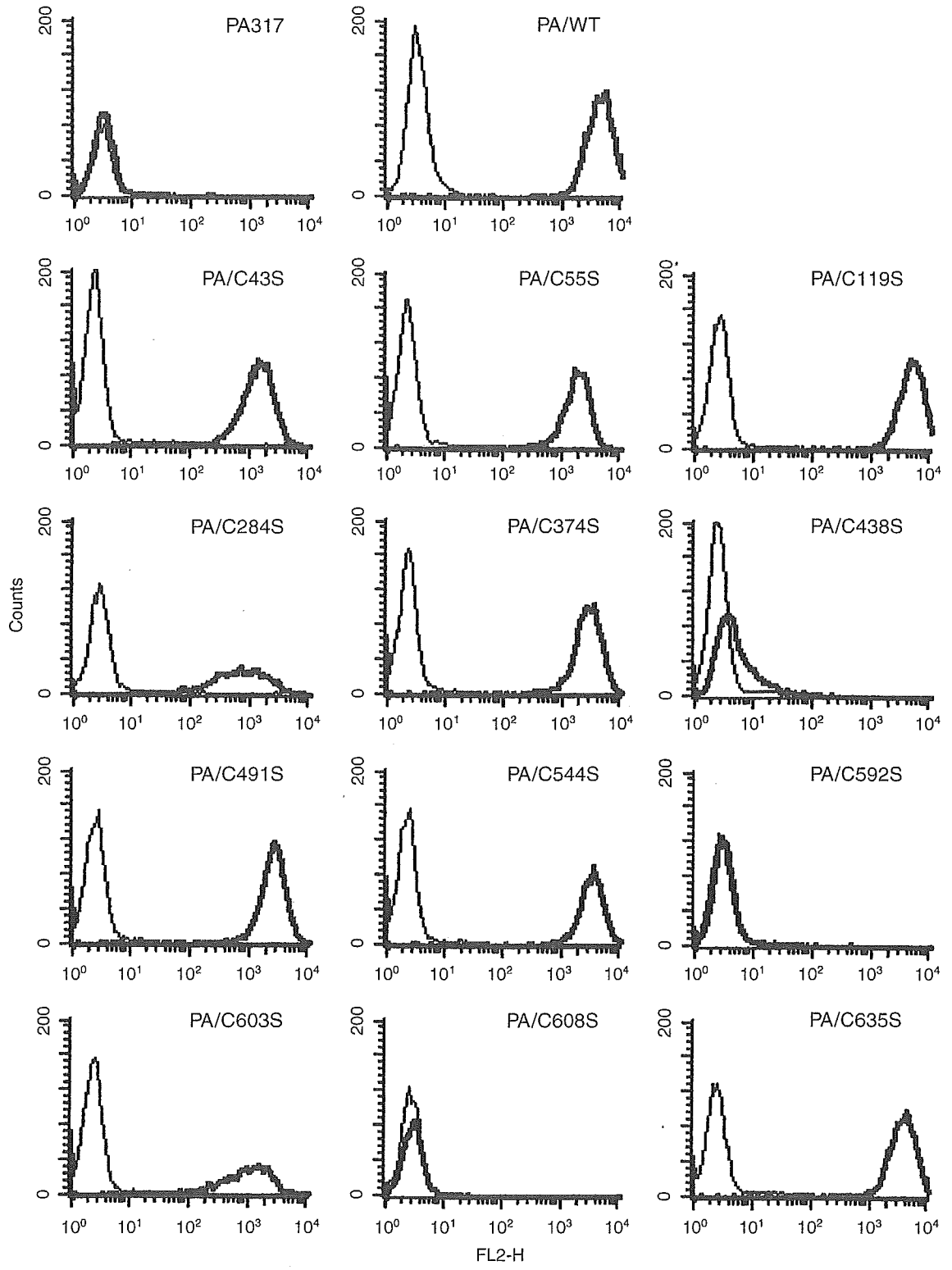

Fig. 3. Expression analysis of mutant breast cancer resistance proteins (BCRP) on the cell surfaces of PA317 transfectants by FACS. Trypsinized cells were incubated with (bold line) or without (fine line) the biotinylated antihuman BCRP monoclonal antibody 5D3, followed by incubation with R-phycoerythrin-conjugated streptavidin.

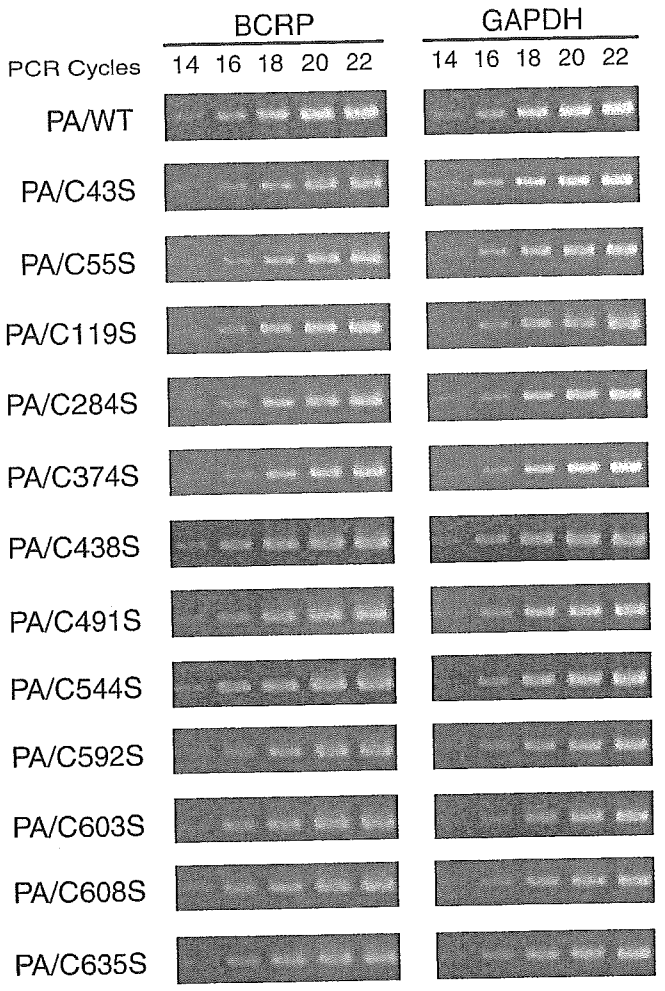

Fig. 4. Semi-quantitative reverse transcription–polymerase chain reaction of mRNA in wild-type and mutant breast cancer resistance protein (*BCRP*) transfectants. First-strand cDNA was synthesized with 0.3 μg of total RNA and a *BCRP* cDNA fragment (315 bp) was amplified by PCR using the indicated cycle numbers. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (551 bp fragment) was carried out as an internal control.

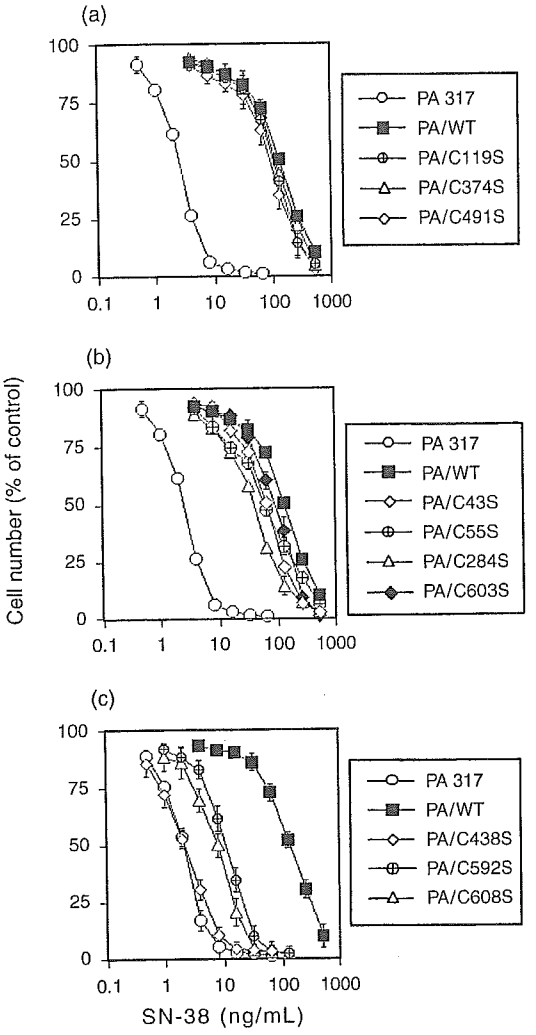

Fig. 5. SN-38 sensitivity assay of mutant breast cancer resistance protein (*BCRP*)-transfected PA317 cells. Drug sensitivity to SN-38 was examined by cell growth inhibition assays as described in Materials and Methods. All data are representative of the mean values ± SD from triplicate determinations. (a) SN-38 sensitivity of the indicated mutant *BCRP* transfectants, which expressed similar levels of BCRP protein to PA/WT. (b) SN-38 sensitivity of four mutant *BCRP* transfectants, which expressed lower levels of BCRP protein than PA/WT. PA/C43S, PA/C55S, PA/C284S and PA/C603S transfectants acquired similar or somewhat lower degrees of SN-38 resistance than PA/WT cells. (c) SN-38 sensitivity of mutants that expressed very small amounts of monomer BCRP. PA/C592S and PA/C608S showed decreased drug resistance and the sensitivity of PA/C438S cells was similar to that of the parental PA317 cells.

intensities of the 70-kDa BCRP monomeric mutant bands were similar to wild-type with the exception of PA/C603S and PA/C603Y, which had somewhat lower expression levels (Fig. 6a). The SN-38 resistance measurements in the PA/C603X cells were also found to be slightly lower than PA/WT, and correlated with the levels of the BCRP monomer in each case (Fig. 6b,c). Similar results were found for PA/C603H, PA/C603R, PA/C603G and PA/C603W cells (data not shown). Taken together, these findings suggest that Cys-603 is directly involved in the covalent attachment of BCRP monomers.

Discussion

In the present study, we undertook cysteine-scanning mutagenesis of BCRP to investigate the involvement of specific cysteine residues in the conformation of the functioning BCRP molecule. In a previous study we used a bicistronic vector, pHa-BCRP-IRES-DHFR, to examine the function of *BCRP* single nucleotide polymorphism (SNP).[21] It has been shown that cells expressing DHFR are resistant to methotrexate.[19] Cells were therefore transfected with wild-type, G34A, C421A or 944–949-deleted *BCRP* cDNAs and were then selected with methotrexate. The methotrexate-resistant colonies were mixed and then analyzed. In cells transfected with pHa-BCRP-IRES-DHFR, a single mRNA is transcribed under control of a retroviral LTR promoter, and two gene products are translated independently from a bicistronic mRNA. The upstream *BCRP* cDNA is translated cap-dependently, and the downstream *DHFR* cDNA is translated under control

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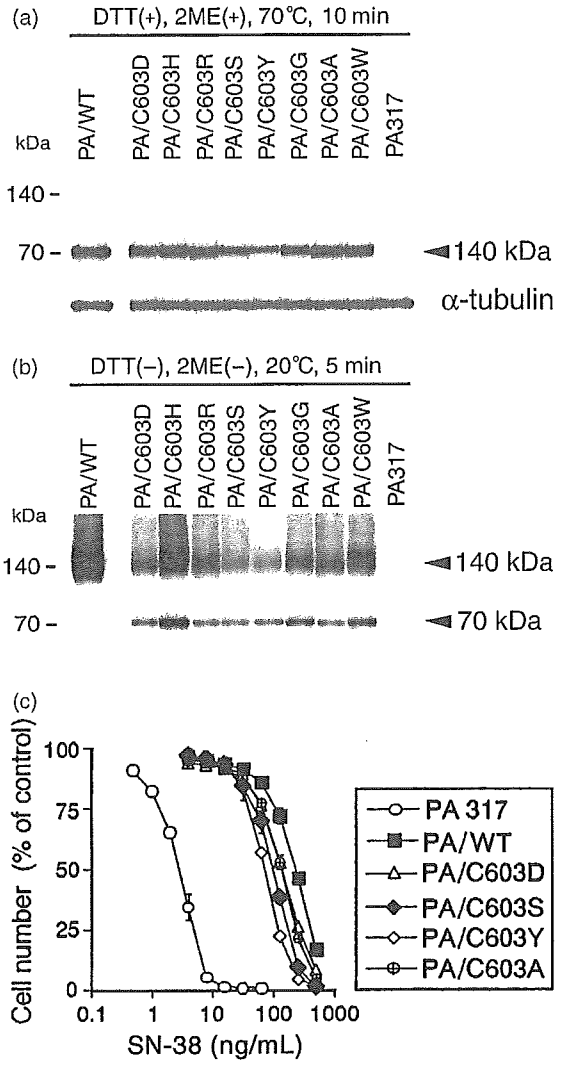

Fig. 6. Western blot analysis of breast cancer resistance protein (BCRP) expression and SN-38 drug sensitivity in PA/C603X mutants. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out (a) under reducing conditions with heating at 70°C for 10 min and (b) under non-reducing conditions with heating at 20°C for 5 min. α-Tubulin expression was analyzed as a loading control. BCRP was detected using the mouse anti-BCRP monoclonal antibody BXP-21. Cellular protein (20 μg) was loaded in each lane. (c) SN-38 sensitivity of parental PA317, PA/WT, PA/C603D, PA/C603Y, PA/C603S and PA/C603A. Drug sensitivity was examined by cell growth inhibition assays as described in Materials and Methods. All data are representative of the mean values ± SD from triplicate determinations.

of the IRES. Because only one mRNA is transcribed, cells expressing *DHFR* theoretically always coexpress the *BCRP* cDNA. Consequently, when the transfected cells are selected with methotrexate, most methotrexate-resistant cells coexpress BCRP at similar levels and the mRNA levels of the mixed population will closely reflect the levels in individual clones. We subsequently showed from mixed populations of resistant clones that these four BCRP transfectants expressed similar levels of exogenous *BCRP* mRNA. Moreover, subsequent FACS analysis showed that almost all of the methotrexate-selected cells expressed BCRP on the cell surface. We then showed that BCRP protein expression from C421A *BCRP* cDNA was markedly lower than wild-type. In our current study we used the same strategy to examine amino acid substitution of Cys residues in the BCRP protein. Cells transfected with pHa-BCRP-IRES-DHFR that carried wild-type or mutant BCRP cDNAs were used for further study after drug selection.

Under non-reducing conditions, BCRP-WT and 8/12 Cys–Ser mutant BCRP species were found to migrate as a 140-kDa dimer. In contrast, considerable levels of the monomeric form of BCRP were observed for the PA/C603S mutant, which correspondingly showed decreased dimer levels (Fig. 2b). These findings suggest that Cys-603 is significantly involved in covalent bond formation between BCRP monomers. Under non-reducing conditions, small quantities of both monomeric and dimeric forms of BCRP could be observed for the PA/C438S, PA/C592S and PA/C608S transfectants as well as in PA/C603S cell extracts following overexposure of the blot (Fig. 2c). The Cys-592 and Cys-608 residues, located on the same extracellular domain, possibly participate in crosslinking with Cys-603. In order to elucidate the possible involvement of Cys-592 and Cys-608 in BCRP dimerization, and to confirm the protein structure, we are now preparing mutant BCRP with double or triple mutations in cysteines 592, 603 and 608, which may completely inhibit dimer formation. The intensity of the BCRP-C603S monomer band under non-reducing conditions was far stronger than the other mutants (Fig. 2b). This suggests that the loss of possible disulfide bridges involving Cys-603 does not alter the stability of the corresponding mutant BCRP. In the case of BCRP-C438S, which has a mutation in the second transmembrane domain, little expression of BCRP was observed by either western blotting or FACS. In addition, the SN-38 sensitivity of PA/C438S cells was equivalent to the parental PA317 cells (Fig. 5c). Hence, the mutation in Cys-438 is likely to result in a remarkable loss of BCRP activity. In contrast, C43S, C55S and C284S mutations did not interfere with the overall function of BCRP, as these mutants conferred high levels of resistance to SN-38 (Fig. 5b).

The drug resistance levels induced by exogenous PA/C603S were almost equivalent to PA/WT (Fig. 5b), although the intensity of the 140-kDa BCRP-C603S band under non-reducing conditions was far less than BCRP-WT (Fig. 2b). Significantly, the degree of SN-38 resistance was found to be associated with the expression levels of the BCRP monomer, detected under reducing conditions by western blotting, as shown in Fig. 2a and Fig. 5. This finding suggests that a functioning unit of BCRP may not necessarily require a covalent bond. This hypothesis is supported by the recent study of Mitomo *et al.*, which reported that the methotrexate transport ability of BCRP was little affected by disruption of interpeptide disulfide bonds following treatment with 2-mercaptoethanol.[22] It is known that typical ABC transporter proteins have two transmembrane segments and two ATP-binding sites,[23–25] and that some half-molecule ABC transporters form homodimers or heterodimers.[26–29] Some studies have also reported that BCRP-WT forms either a homodimer or a homo-oligomer,[30,31] and Xu and coworkers have recently suggested that BCRP exists and functions as a homotetramer.[32] In a previous study,[17] we showed that a 140-kDa BCRP complex that forms in cells coexpressing

Myc-tagged BCRP and hemagglutinin (HA)-tagged BCRP could be immunoprecipitated with anti-Myc antibodies. The precipitate also reacted with anti-HA, and a 70-kDa HA-BCRP monomer was detectable under reducing conditions. These results suggest that BCRP forms a homodimer bridged by disulfide bonds and that it may form other homo-oligomers. Dominant-negative inhibition of BCRP function was also demonstrated, suggesting that homodimerization is essential for BCRP function. So far, however, no studies have directly shown the existence of covalent bonds between monomers and counterpart molecules in a BCRP functioning unit. Hence, further studies are needed to confirm the structure of BCRP.

In conclusion, the BCRP cysteine residue at position 603 is significantly involved in a covalent bridge between BCRP monomers, but a functioning unit of BCRP may not necessarily require these covalent bonds.

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SURGERY TODAY

Review Article

Microsatellite Instability in Gastrointestinal Tract Cancers: A Brief Update

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Abstract
Microsatellite instability (MSI) was initially reported in colorectal cancer and, particularly, in hereditary nonpolyposis colorectal cancer (HNPCC). Since mutations in the genes functioning in DNA mismatch repair (MMR) were found in HNPCC kindred, this phenotype has been connected to a deficiency in MMR. The MSI$^+$ phenotype is associated with various human malignancies. As MSI$^+$ tumors appear to form a unique clinicopathological and molecular entity that is clearly distinct from that of classical colorectal tumors, which are accompanied by chromosomal instability (CIN), an exclusive pathway of tumorigenesis has been proposed in colorectal cancer. However, this scheme, comprising two mutually exclusive pathways, is now being reexamined, in light of a series of evidence accumulating in the literature, which relates to (a) distinction between high-level MSI (MSI-H) and low-level MSI (MSI-L), (b) heterogeneity in MSI-H, particularly in the sporadic and hereditary settings, (c) molecular mechanisms underlying the MSI$^+$ phenotypes, and (d) relationships between the MSI$^+$ and CIN phenotypes. Several molecular mechanisms may underlie repeat instability in eukaryotic cells. The relationship between MSI and defective MMR may be more complicated than has been suspected. The role of MMR deficiency in tumorigenesis in the digestive tract appears to be diverse and is not simple, even in the colorectum.

Key words Replication error (RER) · DNA mismatch repair · hMSH2 · hMLH1

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Introduction

Microsatellites are one of the most abundant classes of intergenic repeat sequences that contain short repetitive motifs such as one to five base pairs. Numerous microsatellites have been mapped throughout the human genome. Although sequences in this category are highly polymorphic in human populations, microsatellites appear stable within a relatively short time such as the life span of the individual. In 1993, instability of microsatellites at the somatic level was reported in colorectal cancer[1,2] and, particularly, in the familial cancer-prone syndrome, hereditary nonpolyposis colorectal cancer (HNPCC).[3] Since mutations in one of the genes functioning in DNA mismatch repair (MMR) were found in HNPCC kindred,[4,5] this phenotype, 'microsatellite instability (MSI),' is regarded as an important phenotype of cells deficient in MMR and, consequently, as a marker of high risk for familial predisposition or second malignancies. MMR is an important DNA repair system, which counteracts base mismatches and strand misalignments that occur during DNA replication and recombination.[6] Repetitive sequences such as microsatellites are particularly prone to polymerase slippage and, consequently, strand misalignment. If uncorrected, these errors are fixed as a mutation, i.e., addition or deletion of one or more repeat units, after a next round of replication. Thus, the phenomenon of unstable microsatellites, i.e., MSI, in which cells accumulate this type of repeat length alteration in microsatellites, is considered to reflect cellular MMR deficiency. MSI analysis is indeed an efficient approach for detecting defective MMR, since MMR genes have no marked hot spots for mutation. As the MSI$^+$ phenotype is frequently associated with various human malignancies, analyses of MSI have been prevalent, particularly in the field of oncology, and numerous data have accumulated in the literature. However, the reported frequency for MSI$^+$

tumor in each malignancy differs widely in the literature.[7]

Analysis of MSI is now commonplace. However, a precise designation of MSI+ is sometimes attended with difficulty. The 1997 National Cancer Institute (NCI) workshop, "Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition,"[8] suggested that the variety of microsatellites used was a major cause of the discrepancies among data from various laboratories and recommended a panel of five microsatellites as "working reference panel." In addition to selection of targets for analysis, methodological problems may also account for the variability in results. In fact, in some of the conventional and popular techniques used for MSI analysis, various methodological problems remain unsolved.[9] Recently, more caution has been taken in the methodological aspects of assay techniques and their effects on the results of MSI analyses, and improved sensitivity and accuracy in analyses has elucidated previously unrecognized aspects of MSI in cancer. In this article, recent advancements in our knowledge on MSI in gastrointestinal tract cancers are summarized, and problems that have newly emerged in this field are also discussed.

Realities of MSI in Gastrointestinal Tract Cancers

Colorectal Cancer

MSI has initially been reported in colorectal cancer.[1,2] It is now widely accepted that MSI is most frequently observed in colorectal cancer and cancers occurring in the endometrium, both of which feature HNPCC, as defined in the Amsterdam Criteria II.[10] However, frequencies for MSI+ tumors reported in the literature are not uniform even in these malignancies.[7] These discrepancies may derive not only from methodological problems left in assay techniques, but also from a variety of MSI phenomena in cancer. In 1997, the National Cancer Institute (NCI) sponsored a workshop entitled "The International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition," in order to review and unify the confused field.[8] In this workshop, it has been concluded that the diversity in data derives mainly from the variety of microsatellite markers used and, therefore, a panel of five microsatellites was recommended as "working reference panel." In addition, MSI was recommended to be classified into two different grades: MSI-H and MSI-L. The first has been defined as ones showing microsatellite alterations in "the majority of markers" (e.g., ≥30%–40%) and the second as exhibiting changes only in "a minority of markers" (e.g., <30%–40%). This distinction of MSI, i.e., MSI-H and MSI-L, has been widely accepted since then. In this workshop, MSI-H has been defined as bona fide MSI derived from defective mismatch repair (MMR). However, MSI-L has not been well characterized. Recently, controversy on the existence of this category of MSI has been raised.[11] Laiho et al. reported that MSI-L was observed in 80% of colorectal tumors without alterations in the BAT26 mononucleotide microsatellite, which are typical in MSI-H tumors, when 377 makers were analyzed.[11–14] Moreover, no significant difference in clinicopathological and molecular variables was observed between MSI-L tumors and ones without MSI. Halford et al. similarly concluded that MSI-L occurs in most colorectal tumors, and that the difference between MSI-L and the phenotype with stable microsatellites is not qualitative but quantitative.[14] On the other hand, some reports have shown significant correlations between MSI-L and mutation in *K-ras* or *p53*, which implies that MSI-L tumors form a unique entity.[12,15,16] Each microsatellite is located in a different chromosome context and is exposed to a different risk for accidents in the processes of DNA metabolism, including polymerase slippage. It appears difficult to distinguish MSI+ tumors with different molecular backgrounds, merely from the frequency of observed changes in a given set of markers.

What is notable in MSI data is not only differences in the frequency of alterations, but also differences in the form of changes. Oda and colleagues[9,17,18] reported that MSI+ tumors are classifiable into two distinct subgroups, Type A and Type B, according to the length change observed in dinucleotide microsatellites (Fig. 1). Based upon findings in mouse or human cell lines with a known defect in MMR genes (Ref. 19 and unpublished data), Type A MSI was defined as length changes of ≤6 base pairs. Type B changes are more drastic and involve modifications of ≥8 base pairs. In fact, Thibodeau et al., one of those who first reported MSI, noticed these qualitative differences.[2] They divided microsatellite changes into two categories, Type I and II mutations. The former was defined a "significant increase (expansion) or decrease (deletion) in the apparent fragment size," and the latter as a "single 2-bp change." This classification may be close to the former distinction. Intriguingly, inspection of published data reveals that MSI thus far reported in tumors occurring in HNPCC individuals is predominantly Type B/Type I mutation. In addition, in colorectal cancer, Type B MSI tends to occur in the majority of markers analyzed, whereas Type A/Type II mutations have a tendency to be noted in a limited number of markers. Therefore, in colorectal cancer, Type A/B MSI, or Type II/I mutations, may correspond to MSI-L/H, respectively. The Bethesda classification, i.e., MSI-H and MSI-L, is based on these observations. Type A MSI/Type II mutations are ob-

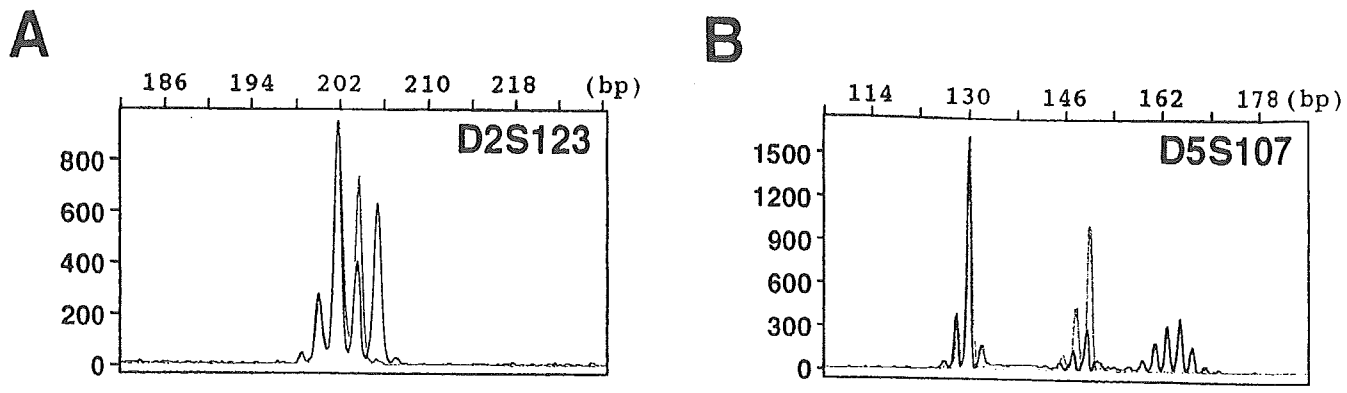

Fig. 1A,B. Type A and Type B microsatellite instability observed in human cancers. Dinucleotide microsatellite sequences, shown on the *top right* of each panel, were amplified by polymerase chain reaction with primers differentially labeled with fluorescence, then mixed and run on a same lane in an automated sequencer. The *x*-axis indicates the quantitatively detected amount of each DNA fragment; and the *y*-axis corresponds to its length standardized using size markers run in the same lane. Results typical for each subtype of microsatellite instability are shown: *red lines*, cancer; *green lines*, normal mucosa. **A** Type A alterations are defined as length changes of ≤6 base pairs. **B** Type B changes involve more drastic modifications of ≥8 base pairs. The most important characteristic of Type B is the "jump"-like discontinuous change. Type B alterations can sometimes appear as if a "third" allele is present in addition to the parental alleles

served in approximately 25% of sporadic colorectal tumors.[17,18] Intriguingly, MSI observed in mouse and human cell lines with a known defect in MMR genes is uniformly Type A (Ref. 19 and Oda et al., in preparation). In addition, a series of findings suggests that tumors exhibiting Type A MSI and Type B tumors arise from different molecular backgrounds. It is well known that MSI-H/Type B tumors occur more frequently in the proximal colon. On the other hand, it has been recently reported that all the microsatellite changes observed in tumors occurring in the rectum are Type A.[17] Moreover, while it is widely accepted that mutations in oncogenes or tumor suppressor genes, such as *K-ras*, *p53*, etc., are infrequent in MSI-H/Type B tumors, Type A MSI appears to be strongly correlated with mutations in these genes (Oda et al., in preparation). This observation may be consistent with several recent reports that have shown correlations between MSI-L and mutations in *K-ras* or *p53*.[12,15,16] Thus, MSI$^+$ tumors appear to include at least two distinct entities with different molecular backgrounds, i.e., MSI-H/Type B tumors and those with Type A MSI, at least in colorectal cancer. This may be rather possible since more than two distinct molecular mechanisms may contribute to microsatellite changes occurring in eukaryotic cells, as discussed later.

Is the molecular background of MSI-H/Type B MSI well understood? In more than 90% of tumors occurring in HNPCC individuals, this type of MSI is observed.[20] As mutations in major MMR genes have been found in HNPCC kindred,[4,5] this phenotype of MSI has been directly connected to defective MMR. However, microsatellite changes observed in MMR gene-knockout mice, including those in tumors occurring in the mouse bodies, were uniformly Type A (Oda et al., in preparation), which strongly suggests that Type A MSI is a direct consequence of defective MMR, and that deficiency in MMR itself is not sufficient for Type B MSI. Previously unrecognized molecular abnormalities, in addition to defective MMR, may underlie the development of Type B MSI. This hypothesis may be consistent with the fact that a generally reported figure of mutation frequency in major MMR genes in HNPCC kindred is sometimes lower than 50%.[21–26] Needless to say, the relationship between molecular abnormalities causing these genomic changes and pathogenesis in HNPCC remains unclear. However, such drastic and multicentric changes in the genome may influence the structure of chromatin domains and, consequently, expression of the genes within. In tumors exhibiting typical MSI, i.e., MSI-H or Type B, point mutations in representative tumorigenic genes, such as *APC*, *K-ras*, or *p53*, are rare and, instead, mutations are found in mononucleotide runs within genes of a different variety, including *TGFβRII*,[27] *IGF2R*,[28] *BAX*,[29] *Caspase 5*,[30] and others.[31,32] Alterations in these genes functioning in growth control and apoptosis have been highlighted as a cause of tumorigenesis. In MSI$^+$ tumors, insertion/deletion mutations are found in short reiterative motifs comprised of a single nucleotide in the coding region of genes such as ones mentioned above, which may cause a shift of the reading frame, leading to a change in protein structure and, possibly, in gene function. Proteins with altered structures appear to be expressed in MSI$^+$ tumor cells because T-cell clonality reactive to these altered structures is indeed found in patients with MSI$^+$ tumors.[33] However, the pathogenic significance of these gene alterations remains unclear.

Clinical and pathological features of MSI-H tumors appear to be more established. It is now widely accepted that MSI-H tumors occur more frequently in the proximal colon and possess several distinct clinicopathological features.[2,34–37] Histopathologically, they frequently exhibit poor differentiation, mucinous component, and lymphocyte infiltration.[35,37–40] It also appears to be a consensus that colorectal cancer patients with MSI$^+$ tumors have a significant survival advantage, compared with those without MSI,[39,41–44] although some reports in the literature differ in conclusion. Survival advantage in patients with MSI$^+$ colorectal tumors is now explained by their unique biological potential rather than by an altered sensitivity to chemotherapy in DNA mismatch repair-defective tumor cells.[44] In fact, MSI$^+$ colorectal tumors appear to arise via two distinct pathways in tumorigenesis, i.e., MSI-H tumors occurring in HNPCC individuals in which germ line mutations are found in major MMR genes and sporadic MSI-H colorectal cancer. It was originally expected that all MSI-H tumors harbor an inherited defect, i.e., mutation in MMR genes. However, most MSI-H tumors are sporadic and germ line mutations are rare. Population-based studies suggested that germ line mutation in major MMR genes accounts for 2%–5% of colorectal cancers.[45,46] At present, generally accepted frequencies of mutation in the two major MMR genes, *hMSH2* and *hMLH1*, are lower than 30% in all sporadic MSI-H colorectal tumors. Instead, in sporadic MSI-H tumors, a role of epigenetic silencing of *hMLH1* is assumed to be more important. It has been reported that colorectal tumors that do not express *hMLH1* comprise approximately 70% of all MSI$^+$ tumors.[47–51] In HNPCC, germ line mutations in major MMR genes are sometimes found in combination with loss of heterozygosity (LOH) in the paired allele, fulfilling Knudson's model.[52] Recently, LOH plus inactivation of an intact allele of *hMLH1* by promoter methylation has been reported in an HNPCC individual.[53] Epigenetic events may play a critical role in the development of MSI-H tumors, particularly in the sporadic setting. In this context, distinction of MSI-H tumors comprised of two distinct categories, i.e., familial MSI-H and nonfamilial MSI-H colorectal cancer, has been emphasized by Jass and colleagues,[40,50,54] who found differences in clinicopathologic features between these tow categories. Indeed, familial MSI-H and nonfamilial MSI-H colorectal cancer show a line of significant histopathological differences, including mucinous component, lymphocyte infiltration, and concomitant serrated adenoma, which was recently suggested to be a precursor lesion for sporadic MSI-H colorectal cancer.[55,56] Intriguingly, in their analyses, HNPCC tumors were more similar to traditional colorectal carcinoma, with the exception of lymphocyte infiltration.[40] MSI-H tumors occurring in HNPCC individuals and sporadic MSI-H tumors may be more different than have been suspected.

Emergence of cancer with a definite genetic background has raised a discussion about methods to detect populations at high risk. MSI-H, or Type B MSI, appears to be a hallmark for HNPCC. However, fragment analysis using an automated sequencer or radiolabeled polymerase chain reaction (PCR) is not common or readily available in many medical facilities. As mentioned above, loss of expression of hMLH1 is highly coincident with MSI-H. Therefore, immunohistochemistry (IHC) of MMR proteins has been regarded as a candidate to substitute MSI analysis, since this approach is technically less complicated and more prevalent, and is inexpensive. Indeed, the results of IHC well correlate with those of MSI analysis. Ninety percent of sporadic MSI-H tumors exhibit loss of expression of either hMLH1 or hMSH2.[46,57–60] Several reports conclude that IHC is sensitive, specific, and cost-effective for detecting potential HNPCC carriers.[58] On the other hand, discrepancy derived from the existence of missense mutations has been pointed out.[59–61] In addition, HNPCC comprises a limited part of MSI-H colorectal tumors. Needless to say, genetic testing is more direct and specific. However, this approach is extremely expensive and not cost-effective. From this point of view, MSI analysis is less expensive and specific. The revised Bethesda guideline for MSI analysis[62] will also facilitate detection of patients at risk. At present, combination of family history according to the Amsterdam Criteria,[10] IHC, MSI analysis, and sequencing has been suggested as a practical approach, and some testing algorithms have been proposed.[57,63]

In colorectal cancer genetic instability, or genomic instability, in tumors has been regarded as deriving from two mutually exclusive pathways: chromosomal instability (CIN), frequently associated with mutations in various oncogenes or tumor suppressor genes such as *APC*, *K-ras*, and *p53*, and "microsatellite mutator phenotype (MMP),"[64,65] which is characterized by MSI.[66,67] However, recent studies on genetic instability in colorectal cancer suggest that this distinction may be an oversimplification, and that these two pathways are not always independent and overlap in some tumors (Fig. 2). Heterogeneity in MSI-H tumors, i.e., familial MSI-H and non-familial MSI-H colorectal cancer, and the entity of MSI-L or Type A MSI have been discussed above. In addition, tumors in which MSI and LOH are coincident[68] and biologically distinct diploid tumors without MSI[69] have been reported recently. In the former report, more than 20% of MSI-H tumors exhibited LOH events at acknowledged tumor suppressor loci. It is widely accepted that tumors with MSI-H are largely diploid whereas aneuploidy is frequently observed in tumors without MSI. Diploid tumors with