Expression and Functional Analyses of Breast Cancer Resistance Protein in Lung Cancer

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ABSTRACT

Purpose: **Breast cancer resistance protein (BCRP/ABCG2), an ATP binding cassette half-transporter, confers resistance to mitoxantrone, doxorubicin, and topoisomerase I inhibitors of irinotecan and topotecan. Recently, we reported that BCRP efficiently transported SN-38 (the active metabolite of irinotecan) with a high affinity in lung cancer cells *in vitro* (K. Nakatomi *et al.*, Biochem. Biophys. Res. Commun., *288:* 827–832, 2001). The aim of this study is to explore the role of BCRP in the drug resistance of lung cancer.**

Experimental Design: **The *BCRP* mRNA expression in lung cancer cells and 23 untreated non-small cell lung cancer (NSCLC) tissues was quantitated by real-time reverse transcription-PCR. To evaluate the drug-efflux function of BCRP, the intracellular topotecan accumulation and drug sensitivity were measured in lung cancer cells with various levels of the *BCRP* mRNA expression by flow cytometric and tetrazolium dye assay, respectively.**

Results: **The levels of *BCRP* mRNA expression in the cell lines were significantly correlated with the BCRP function and the sensitivity to SN-38 and topotecan. In NSCLC tissues, the *BCRP* mRNA expression levels were widely dispersed. Five (22%) of 23 tissues expressed higher levels of the *BCRP* mRNA than that in NCI-H441 cells with active BCRP function conferring high resistance to topotecan *in vitro*.**

Conclusions: **Some NSCLC tissues expressed sufficient levels of the *BCRP* mRNA to confer drug resistance *in vitro*.**

INTRODUCTION

Chemotherapy is the mainstay in the treatment of advanced cancers, however, treatment failure is frequently attributable to MDR[2]. The cellular mechanisms of MDR include the decrease of intracellular drug accumulation, alterations of targets, the increase of DNA repair and detoxification activity, and so on (1). Among these, ABC transporters, especially Pgp and MRPs, are well-established mechanisms (2). BCRP/ABCG2 of an ABC half-transporter was first isolated from human breast cancer MCF7/AdrVp-resistant cells (3). BCRP confers resistance against mitoxantrone, doxorubicin, and topoisomerase I inhibitors of topotecan and irinotecan through the efflux of these drugs (3–5). Recently, we reported that BCRP ATP dependently transported SN-38 (the active metabolite of irinotecan) with a high affinity, using plasma membrane vesicles overexpressing BCRP (6). Thus, BCRP is probably an important mechanism of resistance against topoisomerase I inhibitors, which have become key drugs for chemotherapy of lung cancer (7, 8).

BCRP is expressed in the normal small intestine, colon, liver, and mammary gland of the breast but is at quite a low level in the lung (3, 9, 10). BCRP-overexpressing MDR cells have been isolated from breast, colon, gastric, lung, or ovarian carcinomas, including our SN-38-resistant SCLC cells (5, 11–13). A few clinical studies reported that some acute myeloid leukemias and breast cancers expressed BCRP at the mRNA or protein level (14–17), however, the association between the expression and clinical resistance remains undetermined. Here, to explore the role of BCRP in lung cancer, we examined the *BCRP* mRNA expression and the BCRP function in culture cells and tissue specimens by real-time quantitative RT-PCR and flow cytometry, respectively.

MATERIALS AND METHODS

Cell Lines and Reagents. SN-38-resistant PC-6/SN2-5 cells were selected from parental PC-6 human SCLC cells by continuous exposure to SN-38 of 4.8 nM, as reported previously (18). PC-6/SN2-5 cells overexpressed the *BCRP* mRNA and showed cross-resistance to topotecan and mitoxantrone, whereas PC-6 cells expressed quite a low level of the *BCRP* mRNA (13). All other unselected human lung cancer cell lines were obtained from American Type Culture Collection (Manassas, VA), Japanese Cancer Research Resources Bank (Tokyo, Japan), and

Received 9/12/02; revised 2/14/03; accepted 3/21/03.
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[2] The abbreviations used are: MDR, multidrug resistance; ABC, ATP binding cassette; Pgp, P-glycoprotein; MRP, MDR protein; BCRP, breast cancer resistance protein; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

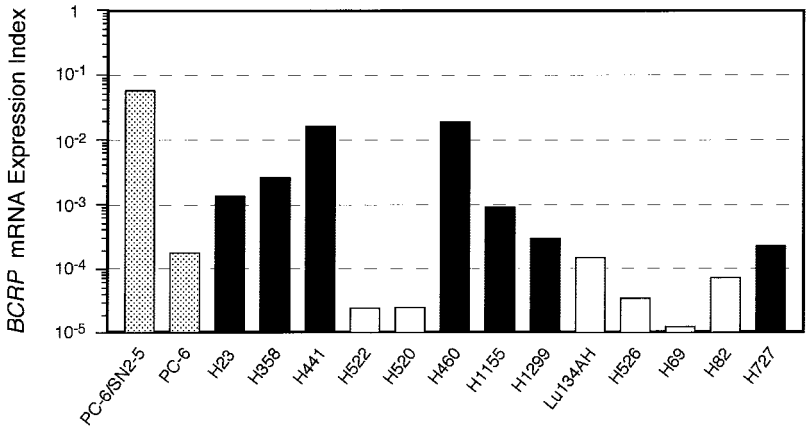

Fig. 1 The *BCRP* mRNA expression levels in lung cancer cell lines. The *BCRP* mRNA expression index was calculated from the following formula: the values of the *BCRP* gene copies divided by the values of the *GAPDH* gene copies. ■ represents cell lines with higher expression indices than that in PC-6 cells. (NSCLC cells, NCI-H23 to NCI-H1299; SCLC cells, Lu134AH to NCI-H82; carcinoid cells, NCI-H727).

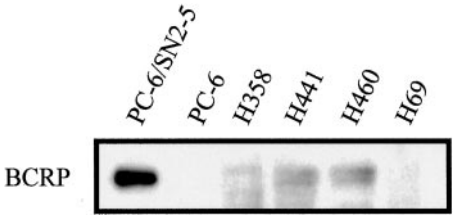

Fig. 2 Immunoblot of BCRP in lung cancer cell lines. BCRP protein migrated at M_r ~70,000. Relative BCRP protein expression was 0.285 in NCI-H358, 0.554 in NCI-H441 and 0.701 in NCI-H460, and BCRP was not observed in PC-6 and NCI-H69 cells. Relative BCRP protein expression in cells was calculated using the following formula: density of band in cells divided by the density of band in PC-6/SN2-5 cells.

RIKEN Cell Bank (Ibaraki, Japan). These cells have no mutations at the codon 482 of BCRP (19), which determines specificity of BCRP substrates (2, 20). These cells were cultured at 37°C under a humidified atmosphere of 5% CO_2 in complete RPMI 1640 cell culture medium (Life Technologies, Inc., Grand Island, NY).

FCS was purchased from Life Technologies, Inc. SN-38 was a gift from Yakult Honsha Co. (Tokyo, Japan), and topotecan was from SmithKline Beecham Co. (Tokyo, Japan). All other reagents were purchased from Sigma Chemicals (St. Louis, MO).

Tissues Samples. Lung tumor tissues were obtained from 23 patients with NSCLC who underwent surgery in Nagasaki University Hospital between September 1998 and February 2001. No patients had received chemotherapy or radiotherapy before surgery. The tumor tissues were immediately frozen in liquid nitrogen and stored at −80°C until analyses.

Real-Time Quantitative RT-PCR Analysis. Total RNA was obtained from each cell line and tumor tissue sample according to the manufacturer's protocol (Isogen; Nippongene, Tokyo, Japan). After the removal of DNA contamination with a MessageClean Kit (GenHunter Co., Nashville, TN), 1 μg of total RNA was reverse transcribed with oligo$(dT)_{20}$ according to the manufacturer's protocol (Thermoscript RT-PCR System; Life Technologies, Inc.). The primer for the *BCRP* gene was reported previously (13). To determine the *BCRP* gene standard curve, RT-PCR products amplified by the primer were subcloned into the pGEM-T Easy Vector according to the manufacturer's protocol (pGEM-T Easy Vector System; Promega, Tokyo, Japan). The sequence of the clone obtained was confirmed by direct sequencing. The vector was digested with *Pst*I to produce a single band and was performed with template dilutions ranging from 10.4 to 1.04×10^6 copies. The fluorescence signal was obtained at 72°C, just below the melting temperature (*T*m) of the product and above the *T*m of the primer dimers. Real-time RT-PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics Ltd., Lewes, United Kingdom) according to the manufacturer's instructions. Complementary DNA obtained from 40 ng of total RNA and 0.3 μM primers were included in the LightCycler-FastStart DNA Master Sybr Green I mix (Roche Diagnostics). The reaction condition was 40 cycles with denaturation for 15 s at 95°C, annealing for 5 s at 63°C, and extension for 15 s at 72°C. To confirm the amplification specificity, the PCR products from each primer were subjected to agarose gel electrophoresis. To represent the *BCRP* mRNA expression level, we used the *BCRP* mRNA expression index calculated from the following formula: the values of the *BCRP* gene copies divided by the values of the *GAPDH* gene copies.

Immunoblotting of BCRP. Whole cell proteins from each cell line were obtained, and aliquots of 15 μg of protein were separated on a 7.5% SDS-PAGE gel, then electrotransferred to nitrocellulose membranes, as described previously (13). Using BXP-21 (Kamiya Co., Seattle, WA) of antihuman BCRP antibody (1:500) and the enhanced chemiluminescence detection system (Amersham Co., Bucks, United Kingdom), immunoblotting was performed, as described previously (13). The density of each band was measured on a densitometer and compared with that of PC-6/SN2-5 cells overexpressing BCRP protein.

Flow Cytometric Analysis with Topotecan. For the detection of functional activity of BCRP as a transporter, topotecan was used as a fluorescent probe because topotecan fluorescence is easily detectable by a conventional FACScan (21), and topotecan is a good substrate for BCRP regardless of any mutations. In the preliminary experiments, we measured the intracellular topotecan fluorescence in the PC-6 and PC-6/

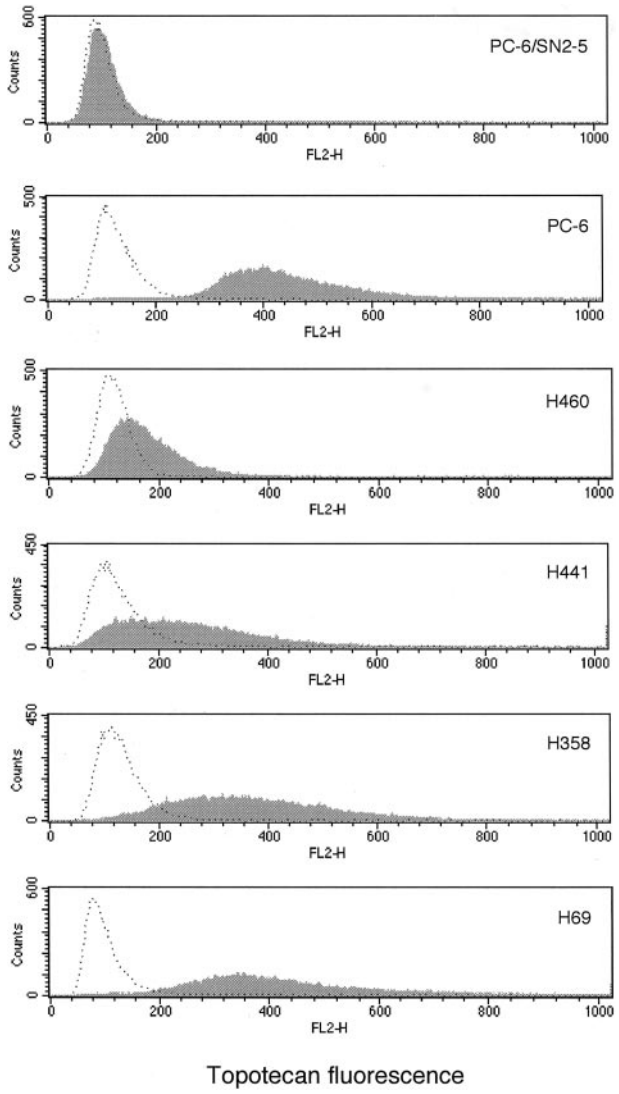

Fig. 3 Intracellular topotecan fluorescence in cells with various levels of *BCRP* mRNA expression. Flow cytometric assays were performed in the presence (*shaded line*) or absence (*dotted line*) of topotecan. PC-6/SN2-5 and PC-6 cells were used as positive and negative controls for BCRP functional activity, respectively. The topotecan fluorescence value in each cell line was calculated from the following formula: (the mean fluorescence with topotecan) − (the mean fluorescence without topotecan). (Topotecan fluorescence value: 12 in PC-6/SN2-5, 306 in PC-6, 54.2 in NCI-H460, 123 in NCI-H441, 239 in NCI-H358, and 317 in NCI-H69).

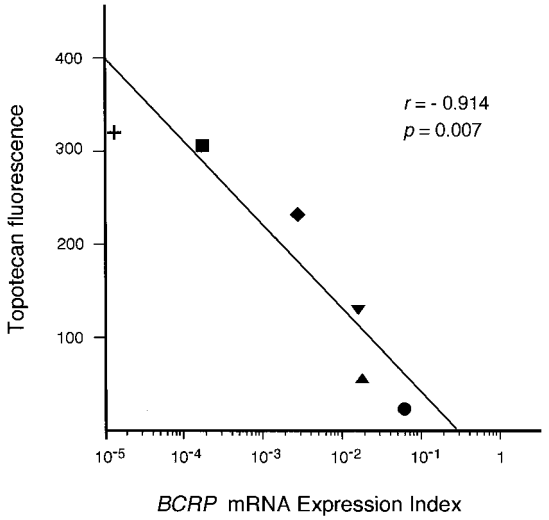

Fig. 4 Relationship between the *BCRP* mRNA expression levels and topotecan fluorescence values in the cell lines. The topotecan fluorescence value in each cell line was calculated from the following formula: (the mean fluorescence with topotecan) − (the mean fluorescence without topotecan). The mean values in three independent experiments are plotted, and SD values were very small (data not shown). (●, PC-6/SN2-5; ▲, NCI-H460; ▼, NCI-H441; ♦, NCI-H358; ■, PC-6; +, NCI-H69 cells).

SN2-5 cells by flow cytometry. There was little topotecan-derived fluorescence in PC-6/SN2-5 cells at 10, 20, or 30 μM topotecan exposure, whereas the fluorescence was dose dependently detectable in PC-6 cells (data not shown). Accordingly, the flow cytometric assay was performed just after a 15-min exposure with 30 μM topotecan. Briefly, 2×10^6 cells were loaded for 15 min at 37°C with 30 μM topotecan in RPMI 1640 and were then washed twice in ice-cold saline. The fluorescence of topotecan was analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with an argon laser. Topotecan-derived fluorescence of 3×10^4 events was measured through a 488-nm bandpass filter at an excitation wavelength of 585 nm. Topotecan accumulation after the 15-min exposure was expressed in fluorescence units. In all flow cytometric assays, parallel samples were stored on ice to control for nonspecific drug binding to plasma membranes, and cells without exposure to topotecan were included as control for autofluorescence.

Anticancer Drug Sensitivity. Sensitivity to SN-38 and topotecan in human lung cancer cell lines was determined by a tetrazolium dye assay, as described previously (22). Briefly, cells (7.5×10^3 cells/well) were seeded in 96-well plates with different concentrations of each test drug. The drug concentration producing IC_{50} was determined graphically for each drug using the relative survival curves. Each study was performed in triplicate and repeated three times.

Statistical Analysis. Data were tested for statistical significance by the Pearson's correlation method, and a $P < 0.05$ denoted the presence of a significant difference.

RESULTS

BCRP mRNA and BCRP Protein Expression in Lung Cancer Cell Lines. The *BCRP* mRNA and BCRP protein expression were examined in 13 (8 NSCLCs, 4 SCLCs, and 1 carcinoid) and 6 drug-unselected human lung cancer cell lines, respectively (Figs. 1 and 2). The *BCRP* mRNA expression indices in PC-6 and PC-6/SN2-5 cells as controls were 1.9×10^{-4} and 5.9×10^{-2}, respectively, and the other cells expressed various levels of the *BCRP* mRNA. Seven cell lines had higher expression indices than that in PC-6 cells, and NCI-H460 cells expressed ~30% of the *BCRP* mRNA expression level observed

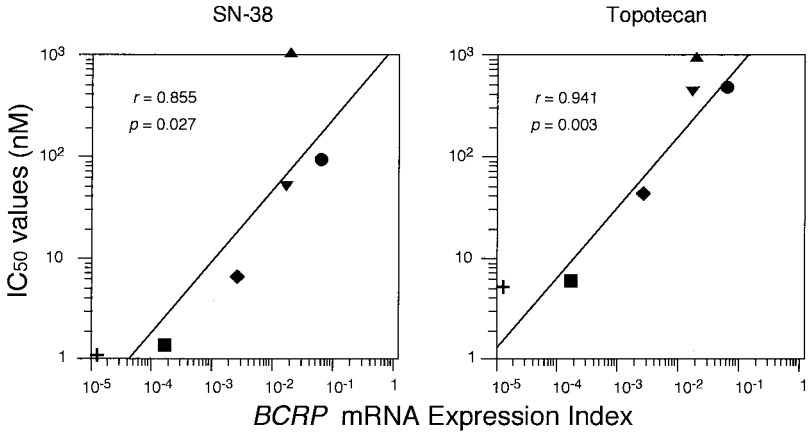

Fig. 5 Relationship between the *BCRP* mRNA expression index and drug sensitivity to SN-38 and topotecan in each cell line. The mean values of IC_{50} in three independent experiments are plotted, and SD values were very small (data not shown). A correlation was evaluated by the Pearson's correlation method. (●, PC-6/SN2-5; ▲, NCI-H460; ▼, NCI-H441; ◆, NCI-H358; ■, PC-6; +, NCI-H69 cells).

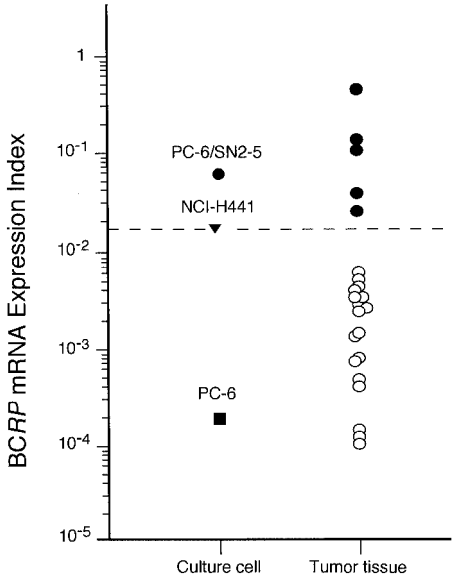

Fig. 6 The *BCRP* mRNA expression levels in NSCLC tumor tissues. The *BCRP* mRNA expression index was calculated from the following formula: the values of the *BCRP* gene copies divided by the values of the *GAPDH* gene copies. *Horizontal dotted line* represents the level of the *BCRP* mRNA expression in NCI-H441 cells, and ● in tumor tissues represent tumors with higher expression indices than that in NCI-H441 cells.

in PC-6/SN2-5 cells. Moreover, the levels of the BCRP protein expression were almost correlated with those of the mRNA expression in 6 cell lines analyzed here.

Functional Activity of BCRP in Cells. To examine the drug-efflux function of BCRP, a flow cytometric assay was performed using topotecan as a probe. In this experiment, 6 cell lines with various levels of the *BCRP* mRNA expression were used (Fig. 3) because these cell lines did not express the *MDR1* gene in semiquantitative RT-PCR (data not shown) and expressed higher levels of the *BCRP* mRNA than that in drug-sensitive PC-6 cells. Because topotecan is also a substrate for Pgp, preliminary experiments were performed to exclude the effects of Pgp/*MDR1* on topotecan transport. These cells were loaded with 30 μM topotecan in the presence of 3 mM cyclosporin A, a Pgp inhibitor, and no increase in the intracellular topotecan-derived fluorescence was observed (data not shown). As shown in Fig. 3, intracellular topotecan fluorescence was detected in PC-6 cells, but there was little in PC-6/SN2-5 cells overexpressing BCRP. In the cell lines tested here, the topotecan fluorescence values were correlated inversely with the *BCRP* mRNA expression levels (Fig. 4).

BCRP mRNA Expression and Drug Sensitivity in Cell Lines. We examined the relationship between the *BCRP* mRNA expression and the sensitivity (IC_{50}) to SN-38 or topotecan in the above 6 cell lines (Fig. 5). There was a significant correlation between the *BCRP* mRNA expression levels and the sensitivity to SN-38 or topotecan.

BCRP mRNA Expression in Tumor Tissues. The *BCRP* mRNA expression in 23 untreated NSCLC tumor tissues (11 adenocarcinomas, 10 squamous cell carcinomas, and 2 adenosquamous carcinomas) was quantitated by real-time RT-PCR (Fig. 6). The *BCRP* mRNA expression indices in the tumor tissues were widely dispersed. The maximum plasma concentration of topotecan in humans is reported to be ~130 nM (23). Three cell lines (PC-6/SN2-5, NCI-H460, and NCI-H441 cells) tested here had IC_{50}s of >130 nM for topotecan (Fig. 5), indicating that these cells were quite resistant to topotecan. Therefore, the *BCRP* mRNA expression level in NCI-H441 cells was thought to be critical in functioning as an efflux pump conferring topotecan-resistance *in vitro*. We then fixed the level in NCI-H441 cells as a border *BCRP* expression level conferring resistance in tumor tissues. As a result, 5 (22%) of 23 NSCLC tissues expressed higher levels of the *BCRP* mRNA than that in NCI-H441 cells.

DISCUSSION

We examined the *BCRP* mRNA expression and measured BCRP-mediated topotecan efflux in lung cancer cell lines. The intracellular topotecan fluorescences correlated inversely with the *BCRP* mRNA expression levels, indicating that the *BCRP* mRNA levels probably reflected the functional activity of BCRP. The *BCRP* mRNA levels also correlated inversely with the sensitivity to SN-38 and topotecan *in vitro*. Then, we quantitated the *BCRP* mRNA expression and predicted the BCRP

activity in NSCLC tumor tissues. Consequently, we showed that some NSCLC tissues expressed sufficient levels of the *BCRP* mRNA to function as a drug-efflux pump conferring resistance *in vitro*. The present findings indicate that BCRP may confer intrinsic drug resistance in NSCLC *in vivo*.

To evaluate the contribution of resistance-related molecules, mRNA and protein expression and functional activity are usually analyzed in culture cells and tissue specimens. In ABC transporters, the mRNA expression levels are reported to correlate with the protein levels (9, 24, 25). A very recent study on BCRP also showed a good correlation between the mRNA and protein levels in breast cancer (15), and we showed a similar result in this study. Compared with analyses of mRNA/protein expression, it is difficult to measure the functional activity of molecules such as detoxification and DNA-repair proteins, except for ABC transporters. Especially in solid tumor tissues, the functional activity has generally been predicted by the gene/protein levels. The present *in vitro* study is the first to show a good correlation between the mRNA levels and the drug-efflux activity in BCRP.

ABC transporters MRP1, MRP2, and MRP3 are understood to function as glutathione- or glucuronide-drug conjugate transporters or to cotransport drugs with glutathione. Thus, the MRP-mediated drug resistance probably requires intracellular drug conjugation activity. In contrast, we reported that BCRP preferentially transported SN-38 with a high affinity rather than SN-38-glucuronide *in vitro* (6). This strongly indicates that BCRP as well as Pgp interacts directly with drugs and immediately effluxes them, reflecting good correlations among the BCRP expression, the efflux function and the drug sensitivity in this study. Likewise, as shown in this study, the functional analysis using topotecan of a relatively specific substrate for BCRP would be useful in non-Pgp cells.

We showed that some NSCLC cell lines and tumor tissues expressed sufficient levels of the *BCRP* mRNA to confer drug resistance *in vitro*. However, there is a limitation in evaluating total *BCRP* mRNA expression in clinical samples because they include tumor cells but also various normal cells. In the normal lung, the *BCRP* mRNA is undetectable in Northern blots (3), but the endothelial layer of veins, seromucinous glands, and capillary endothelium weakly expressed BCRP at the protein level (10). Likewise, the venous and capillary endothelial cells of most human tissues express BCRP in immunostaining (9). Thus, the *BCRP* mRNA expression in tissues is partly influenced by the density of blood vessels. We believe our RT-PCR to be the most sensitive and specific method in the detection of BCRP, but the BCRP localization in tumor tissues remains undetermined. Considering the high *BCRP* expression in some cell lines, we think that the *BCRP* mRNA in our NSCLC tissues partly originated from tumor cells and that BCRP possibly confers clinical resistance. To explore the clinical contribution of BCRP in drug resistance of lung cancer, additionally detailed immunohistochemical analyses are needed.

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