# *MRP7/ABCC10* expression is a predictive biomarker for the resistance to paclitaxel in non-small cell lung cancer

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## Abstract

**We used the paclitaxel-resistant human small cell lung cancer subline PC-6/TAX1-1, selected from PC-6 cells by paclitaxel, to test whether MRP7/ABCC10 (ABCC10) confers paclitaxel resistance. We found that gene expression of both *ABCB1/MDR1* (*ABCB1*) and *ABCC10* was higher in PC-6/TAX1-1 cells than in PC-6 cells. The expression levels of *ABCC10* showed a significant inverse correlation with paclitaxel sensitivity ($r = 0.574$; $P < 0.05$) in 17 non-small cell lung cancer (NSCLC) cells unlike the expression levels of *ABCB1*. Pretreatment with the ABCC10 inhibitor sulfinpyrazone altered the sensitivity to paclitaxel in *ABCC10*-expressing NSCLC cells, concomitant with increased intracellular paclitaxel accumulation. These findings suggest that expression of the *ABCC10* gene is induced by paclitaxel and that ABCC10 confers paclitaxel resistance by enhancing the efflux for paclitaxel. To confirm this hypothesis, we tested the effect on paclitaxel cytotoxicity of decreasing the expression of *ABCC10* by small interfering RNA and found that this enhanced paclitaxel cytotoxicity in NCI-H23 cells concomitant with increased intracellular paclitaxel accumulation. These data indicate that ABCC10 may be one of the biomarkers for paclitaxel resistance in NSCLC. [Mol Cancer Ther 2008;7(5):1150–5]**

## Introduction

Paclitaxel is the first of a new class of anticancer agents with a novel mechanism of action. Paclitaxel exerts its antitumor activity by binding tubulin and stabilizing nonfunctional microtubule bundles, thereby blocking normal mitotic spindle development and subsequent cell division. Clinical utility of paclitaxel spans many tumor types, including treatment of ovarian, breast, lung, head and neck, and unknown primary cancers (1). As is the case with most chemotherapy drugs, paclitaxel is administered empirically with little individualization of dose other than adjustment for body surface area. Studies are under way to identify the optimal dose and schedule of administration for paclitaxel, and effective combination regimens, and to exploit the drug's radiosensitizing properties. Efforts to define pharmacodynamic relationships and clinical mechanisms of resistance and to assess potential mechanisms for the drug's anticancer activity are also under way (2).

The ATP-binding cassette transporter superfamily contains several family members that confer drug resistance to drug-sensitive cells by effluxing anticancer or antiviral agents or their metabolites from cells when expressed at high levels (3–6). Recently, the MRP/ABCC (ABCC) family member MRP7/ABCC10 (ABCC10) was shown to mediate the ATP-dependent transport of several anticancer agents, including paclitaxel (7, 8). However, the role of ABCC10 in the mechanism of resistance to anticancer agents has not been fully examined. Because paclitaxel is one of the most powerful novel antitumor agents and has become an integral part of several commonly used chemotherapy regimens in non-small cell lung cancer (NSCLC) management over the past few years (9), it is important to clarify the determinants of sensitivity and/or resistance to paclitaxel in NSCLC. Therefore, we examined the gene expression levels of *ABCC10* in relation to sensitivity and/or resistance to paclitaxel in NSCLC cells.

## Materials and Methods

### Cell Lines and Chemicals

The following human NSCLC cell lines were used in this study: 13 adenocarcinomas (A549, NCI-H23, PC-9, VMRC-LCD, VMRC-LCF, RERF-LC-AI, RERF-LC-MT, RERF-LC-OK, RERF-LC-MS, NCU-LC-201, ACC-LC-94, ACC-LC-176, and SK-LC-10), 2 squamous cell carcinomas (PC10 and QG56), and 2 large-cell carcinomas (NCI-H460 and SK-LC-6). Cells from a human small cell lung cancer cell line, PC-6, and those from the paclitaxel-resistant subline PC-6/TAX1-1, were kindly provided by Daiichi Pharmaceutical (10). The cisplatin-resistant subline PC-9/CDDP was provided as described previously (6). Cells from the gemcitabine-resistant subline H23/GEM-R and the 7-ethyl-10-hydroxycamptothecin-resistant subline were established as described previously (11, 12). Cells were cultured in RPMI 1640 (or, for A549, DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% (v/w) penicillin/streptomycin in a humidified chamber (37°C, 5% $CO_2$). Paclitaxel was provided by Bristol-Mayers Squibb. Sulfinpyrazone and verapamil were purchased from Wako Pure Chemical Industries.

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### Total RNA Extraction and Reverse Transcription-PCR

Total RNA was extracted with TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription-PCR was done in a volume of 20 μL by SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions. The sequences of the *ABCC10* and *ABCB1/MDR1* (*ABCB1*) primers and the PCR conditions were as described previously (13, 14). Amplified products were separated by 2% agarose gel electrophoresis, and bands were visualized by staining with ethidium bromide. We also did real-time PCR with the LightCycler FastStart DNA SYBR Green kit (Roche Diagnostics). We used melting curve analysis to control for specificity of the amplification products. The number of transcripts was calculated from a standard curve obtained by plotting the known input of six different concentrations versus the PCR cycle number at which the detected fluorescence intensity reached a fixed value. The PCR program was 45 cycles of 94°C for 15 s and 60°C for 1 min. For each sample, the data were normalized to that of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH).

### Concentration of Paclitaxel for 50% Cell Survival ($IC_{50}$)

Cells were cultured at 5,000 per well in 96-well tissue culture plates. To assess cell viability, stepwise 10-fold dilutions of the anticancer drug were added 2 h after plating, and the cultures were incubated at 37°C for 96 h. At the end of the culture period, 20 μL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt solution (CellTiter 96 AQueous One Solution Cell Proliferation Assay) was added, and cells were incubated for a further 4 h; the absorbance was measured at 490 nm using an ELISA plate reader. Mean values were calculated from three independent experiments carried out in quadruplicate. Chemosensitivity is expressed here as the drug concentration for $IC_{50}$, determined from the concentration-effect relationship using GraphPad Prism version 4 (GraphPad Software).

### Inhibition of ABCC10 Activity by Sulfinpyrazone

*ABCC10*-expressing NCI-H23 and RERF-LC-AI cells and *ABCB1*-expressing NCU-LC-201 cells ($1 \times 10^5$) were plated in six-well tissue culture plates. We used the general organic anion transporter inhibitor sulfinpyrazone as an ABCC10 inhibitor as reported previously (7). After 24 h, the cells were exposed to sulfinpyrazone or verapamil, an inhibitor of P-glycoprotein (Pgp) encoded by *ABCB1*, for 30 min, and we added paclitaxel in the presence of sulfinpyrazone or verapamil. After a further 48 h, we counted the viable cells with trypan blue staining to evaluate the cytotoxicity of paclitaxel.

### Intracellular Paclitaxel Concentration

We treated $2 \times 10^6$ NCI-H23 cells with paclitaxel (100 nmol/L) for 90 min. After washing three times with cold PBS, the cells were resuspended in RPMI 1640 (2 mL) and homogenized. After centrifugation, the supernatant was stored at -80°C until analysis. Paclitaxel concentrations were quantified by a high-performance liquid chromatography assay as described previously (15).

### Transfection and Small Interfering RNA Experiments

Cells ($2 \times 10^5$) were transfected with small interfering RNA (siRNA) oligonucleotide using Oligofectamine (Invitrogen) to produce a final RNA concentration of 50 nmol/L in serum-free Opti-MEM (Invitrogen) as described previously (5). At 24 h after transfection, we changed the medium to RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. After an additional 24 h, total RNA was extracted, or the cells were treated with paclitaxel for 48 h, and then we counted viable cells with trypan blue staining to evaluate the cytotoxicity. The siRNA oligonucleotides for *ABCC10* (predesigned siRNA, ID 117707) were purchased from Ambion. The negative control siRNA (Silencer Negative Control 1 siRNA), which does not induce nonspecific effects on gene expression, was also purchased from Ambion.

### Statistical Analysis

Spearman's test was used for correlation analysis between expression of *ABCC10* gene and $IC_{50}$ values for paclitaxel. The differences in the cell viability between samples were evaluated with Student's unpaired *t* test. The level of significance was set at 5% using two-sided analysis.

## Results

### Expression of ATP-Binding Cassette Transporters

We used real-time reverse transcription-PCR to compare the expression levels of *ABCB1* and *ABCC10* genes in cells of the paclitaxel-resistant small cell lung cancer cell line PC-6/TAX1-1 with those in parent PC-6 cells. Expression levels of both the *ABCB1* and the *ABCC10* gene were increased in PC-6/TAX1-1 cells compared with parental PC-6 cells (Fig. 1A). We confirmed the gene expression levels by real-time PCR, indicating that expression of the *ABCC10* gene was ~2.5-fold higher in PC-6/TAX1-1 cells than in PC-6 cells (Fig. 1A). We detected *ABCB1* gene expression in PC-6/TAX1-1 cells but not in PC-6 cells.

We compared the expression levels of the *ABCC10* gene in cisplatin-resistant, gemcitabine-resistant, and 7-ethyl-10-hydroxycamptothecin-resistant cell lines with those in the respective parent cell lines. In contrast to the above results, the expression levels did not differ in the pairs of resistant and parent cells (Fig. 1B). We confirmed the *ABCC10* gene expression levels by real-time reverse transcription-PCR (data not shown). We did not detect *ABCB1* in any of these cells.

### Relationship between Cytotoxicity of Paclitaxel and Expression of *ABCC10* Gene

Using quantitative real-time reverse transcription-PCR, the expression levels of *ABCC10* and *ABCB1* genes were determined in 17 NSCLC cell lines. We detected *ABCB1* expression in only 4 of the 17 (A549, VMRC-LCD, NCU-LC-201, and NCI-H460), whereas *ABCC10* was expressed in all NSCLC cell lines. $IC_{50}$ values (mean ± SD) for paclitaxel in each cell line were as follows: A549, 13.55 ± 0.98 nmol/L; NCI-H23, 0.39 ± 0.01 nmol/L; PC-9, 5.48 ± 0.46 nmol/L; VMRC-LCD, 0.06 ± 0.01 nmol/L; VMRC-LCF, 12.03 ± 0.73 nmol/L; RERF-LC-AI, 27.46 ± 3.43 nmol/L;

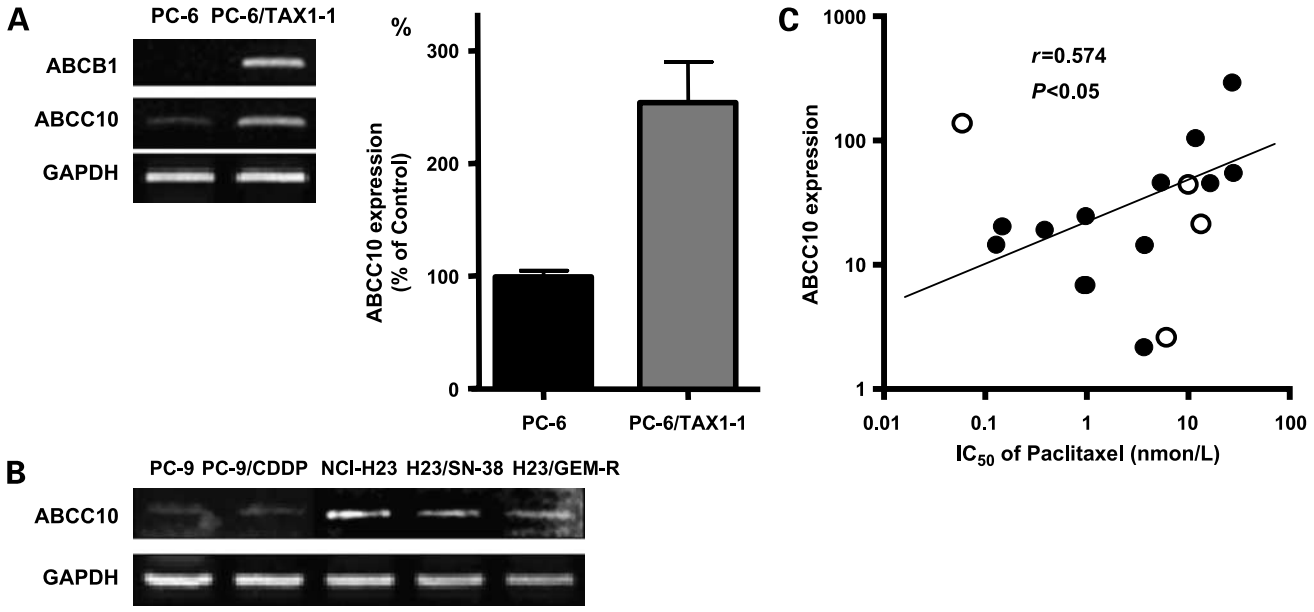


**Figure 1.** Expression of *ABCC10* and paclitaxel cytotoxicity. Comparison of *ABCC10* and *ABCB1* gene expression levels in the paclitaxel-resistant cell line PC-6/TAX1-1 and PC-6 cells. *ABCC10* gene expression was ~2.5-fold higher in PC-6/FU23-26 cells (**A**). The expression levels of the *ABCC10* gene in cisplatin-resistant, gemcitabine-resistant, and 7-ethyl-10-hydroxycamptothecin-resistant cell lines did not differ from those in the respective parent cell lines (**B**). There was a clear relation between *ABCC10* expression and paclitaxel sensitivity in NSCLC cell lines ($n = 17$). Each $IC_{50}$ value is the mean of three independent sensitivity tests done in quadruplicate. Expression levels are relative to expression by *glyceraldehyde-3-phosphate dehydrogenase*. Statistical significance of the correlations was determined by Spearman's correlation test. *Open circle,* NSCLC cells that express *ABCB1* gene; *closed circle,* NSCLC cells that did not express *ABCB1* gene (**C**).

RERF-LC-MT, 0.13 ± 0.05 nmol/L; RERF-LC-OK, 0.99 ± 0.07 nmol/L; RERF-LC-MS, 28.39 ± 5.59 nmol/L; NCU-LC-201, 6.18 ± 1.08 nmol/L; ACC-LC-94, 3.77 ± 0.57 nmol/L; ACC-LC-176, 3.72 ± 1.19 nmol/L; SK-LC-10, 0.15 ± 0.03 nmol/L; PC10, 16.72 ± 1.41 nmol/L; QG56, 0.95 ± 0.12 nmol/L; NCI-H460, 10.14 ± 2.19 nmol/L; and SK-LC-6, 1.01 ± 0.19 nmol/L. We found a clear inverse relationship between *ABCC10* gene expression and paclitaxel sensitivity ($IC_{50}$) in NSCLC cells ($r = 0.574$; $P < 0.05$; Fig. 1C). We also found a clear inverse relationship between *ABCC10* gene expression and paclitaxel sensitivity ($IC_{50}$) in 13 NSCLC cells that did not express *ABCB1* ($r = 0.720$; $P < 0.01$).

### Inhibition of Paclitaxel Cytotoxicity by Sulfinpyrazone

To elucidate whether ABCC10 actually affects paclitaxel sensitivity, we added the ABCC10 inhibitor sulfinpyrazone at a noncytotoxic concentration with paclitaxel to two *ABCC10*-expressing NSCLC cell lines (NCI-H23 and RERF-LC-AI) that do not express *ABCB1* and to a NSCLC cell line (NCU-LC-201) that expresses *ABCB1* and low levels of *ABCC10*. As shown in Fig. 2, the sensitivity of NCI-H23 and RERF-LC-AI cells to paclitaxel was significantly inhibited by sulfinpyrazone pretreatment (30 μmol/L, 30 min) but not by verapamil pretreatment (10 μmol/L, 30 min). In contrast, the sensitivity of NCU-LC-201 cells to paclitaxel was significantly inhibited by verapamil pretreatment (10 μmol/L, 30 min) but not by sulfinpyrazone pretreatment (30 μmol/L, 30 min).

ABCC10 has been shown to be an active transporter for paclitaxel (7, 8). To investigate mechanisms of paclitaxel, its intracellular accumulation was measured in NCI-H23 cells by high-performance liquid chromatography assay. Paclitaxel accumulation following exposure to paclitaxel (90 min, 100 nmol/L) was higher in NCI-H23 cells that had been pretreated with sulfinpyrazone than verapamil or vehicle NCI-H23 cells (Fig. 3).

### *ABCC10* Suppression and Enhanced Cytotoxicity to Paclitaxel by *ABCC10* siRNA

To confirm the alteration in paclitaxel cytotoxicity by ABCC10, NCI-H23 and RERF-LC-AI cells were transfected with siRNA directed against *ABCC10*; cells transfected with control siRNA or not transfected were used as controls, because sulfinpyrazone acts as an inhibitor of general organic anion transporters. Cells were harvested 48 h after transfection, and extracts were prepared and analyzed by quantitative real-time reverse transcription-PCR. Expression of the *ABCC10* gene was decreased at 48 h after siRNA transfection (Fig. 4A). Forty-eight hours after transfection with *ABCC10* siRNA or negative control siRNA, or with no transfection, NCI-H23 and RERF-LC-AI cells were treated with 1 or 2 or 4 nmol/L paclitaxel for 48 h. The cytotoxicity to paclitaxel was significantly higher for cells in the presence of *ABCC10* siRNA than for either control (Fig. 4B). Because negative control siRNA does not affect gene expression, these results indicate that decreasing the expression of *ABCC10* altered the paclitaxel cytotoxicity.

We also examined the intracellular concentration of paclitaxel in NCI-H23 cells after transfection of siRNA directed against *ABCC10*. The intracellular concentration of

paclitaxel was markedly higher for cells exposed to paclitaxel in the presence of *ABCC10* siRNA than for control cells (Fig. 4C).

## Discussion

We have found increased gene expression of *ABCC10* in paclitaxel-resistant small cell lung cancer cells. Further, we have analyzed the relationship between *ABCC10* expression levels and sensitivity to paclitaxel in NSCLC cell lines, finding a significant correlation. These results indicate that ABCC10 is a biological marker for paclitaxel treatment in NSCLC.

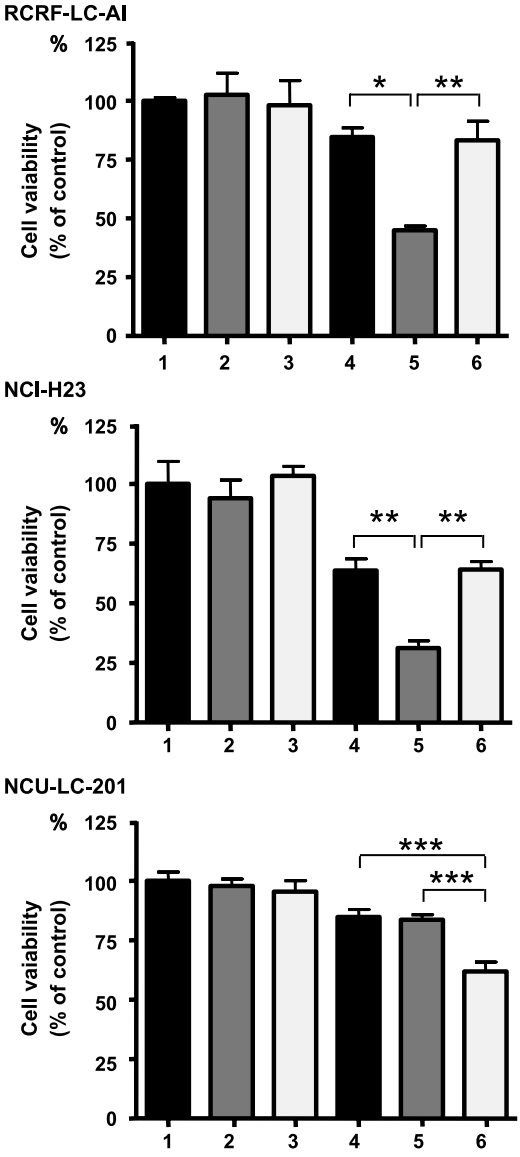


**Figure 2.** Modification of paclitaxel cytotoxicity by sulfinpyrazone or verapamil and intracellular paclitaxel concentration. NCU-LC-201, NCI-H23, and RERF-LC-AI cells were incubated with 30 μmol/L sulfinpyrazone or 10 μmol/L verapamil for 30 min before treatment with 2 nmol/L paclitaxel. After 48 h, we counted viable cells with trypan blue staining to evaluate the cytotoxicity of paclitaxel. *, $P < 0.001$; **, $P < 0.005$; ***, $P < 0.05$. *1,* vehicle; *2,* sulfinpyrazone; *3,* verapamil; *4,* paclitaxel; *5,* sulfinpyrazone + paclitaxel; *6,* verapamil + paclitaxel.

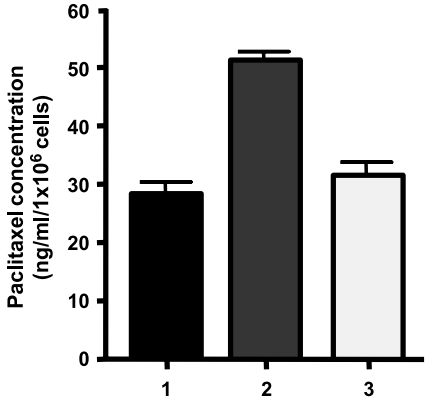


**Figure 3.** Intracellular paclitaxel concentration. The intracellular paclitaxel concentration was measured in NCI-H23 cells after exposure to paclitaxel for 90 min. *1,* paclitaxel; *2,* sulfinpyrazone + paclitaxel; *3,* verapamil + paclitaxel.

Previously conclusive evidences in paclitaxel-related drug resistance are the multidrug resistance mediated by overexpression of Pgp, encoded by *ABCB1* gene. Paclitaxel sensitivity has been shown previously, in 60 cancer cell lines, to have a strong negative correlation with *ABCB1* expression (16). Further, and similar to our findings, overexpression of Pgp/*ABCB1* has been detected in paclitaxel-resistant cells and inhibition of *ABCB1* activity has been shown to restore paclitaxel resistance in paclitaxel-resistant cancer cells (17–19). However, it was reported previously that the expression levels of *ABCB1* were low in clinical samples of NSCLC (20). We also found low expression levels of *ABCB1* in 17 NSCLC cell lines. These results allow us to hypothesize that Pgp/*ABCB1* does not mediate the major paclitaxel efflux and is not associated with paclitaxel sensitivity in NSCLC cells. Furthermore, acquisition of paclitaxel resistance via Pgp-mediated and non-Pgp-mediated mechanisms was shown in established paclitaxel-resistant ovarian carcinoma cells, in which the levels of α-tubulin and β-tubulin and binding of Taxol to microtubules were similar (18). These results support the existence of a non-Pgp-mediated paclitaxel efflux mechanism.

Multidrug resistance in cancer cells is often attributed to ABCC family members, which are involved in the efflux of anticancer drugs by cotransport with glutathione or glutathione-drug or glucuronide-drug conjugates. ABCC family members can be subdivided into two groups: (*a*) ABCC1-3, ABCC6, and ABCC10, which contain 17 transmembrane segments, and (*b*) ABCC4, ABCC5, ABCC11, and ABCC12, which lack one of the transmembrane regions and have only 12 transmembrane segments (3, 4). Recently, one of the ABCC family members, ABCC10, was shown to mediate the ATP-dependent transport of several anticancer agents, including paclitaxel (7, 8), although cancer cells transfected with *ABCC10* had only low level of resistance to paclitaxel. We therefore examined the relationship between

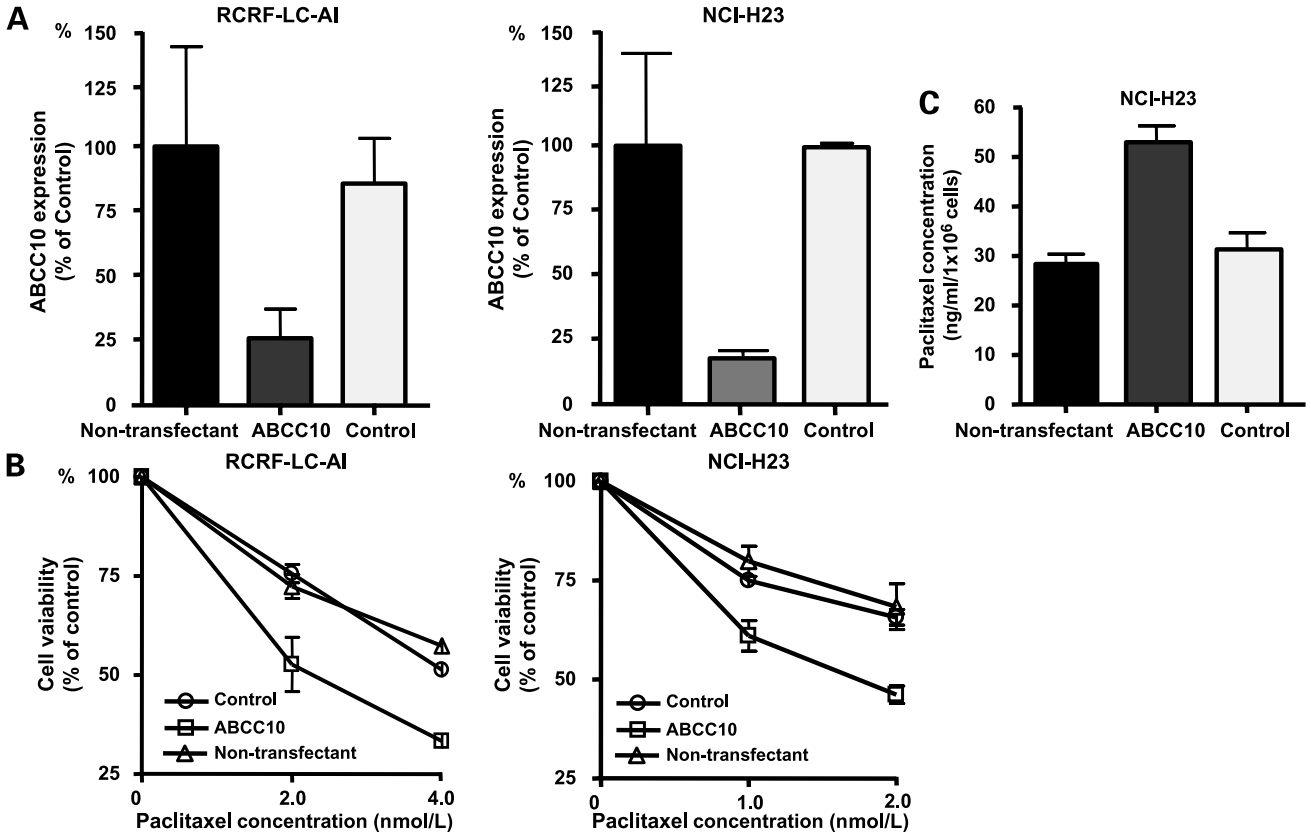


**Figure 4.** Modification of paclitaxel cytotoxicity by *ABCC10* siRNA. Gene expression levels of *ABCC10* in NCI-H23 and RERF-LC-AI cells transfected with siRNA directed against *ABCC10*, control siRNA-transfected, or nontransfected cells (**A**). Cytotoxicity to 1 or 2 or 4 nmol/L paclitaxel in cells transfected with siRNA directed against *ABCC10*, control siRNA-transfected, or nontransfected cells (**B**). The intracellular paclitaxel concentration was measured in NCI-H23 cells after exposure to paclitaxel for 90 min (**C**).

the expression levels of *ABCC10* and sensitivity to paclitaxel, finding a clear inverse relationship. In addition, inhibition of ABCC10 activity or expression altered the cytotoxicity of paclitaxel, concomitant with increasing intracellular paclitaxel concentration. Furthermore, we detected the gene expression of *ABCC10* in all NSCLC cell lines tested by real-time PCR in contrast to *ABCB1*. Although our results did not fully explore the role of ABCC10 in drug resistance, our results suggested that ABCC10 is associated with efflux of paclitaxel and that *ABCC10* expression may be one of the determinants of paclitaxel sensitivity in NSCLC.

We found increased expression of *ABCC10* in paclitaxel-resistant cells, which provides the first example of paclitaxel treatment inducing *ABCC10* expression. In paclitaxel-resistant cells, sulfinpyrazone pretreatment did not fully affect the paclitaxel cytotoxicity because of *ABCB1* up-regulation (data not shown). We cannot determine whether the paclitaxel efflux activity of ABCC10 is similar to that of Pgp; our results indicate that induction of *ABCB1* and *ABCC10* expression together by exposure to paclitaxel resulted in acquired resistance to paclitaxel.

We also investigated whether other anticancer agents affect *ABCC10* expression. Our comparison of the expression levels of the *ABCC10* gene in cisplatin-resistant, gemcitabine-resistant, and 7-ethyl-10-hydroxycamptothecin-resistant cell lines with those in the respective parent cell lines revealed no induction of *ABCC10* expression in any drug-resistant cells. These results suggest that the anticancer agents transported by ABCC10 are limited.

*ABCC10* transcripts are expressed in a wide range of normal tissues, including colon, skin, and testis (13). Clarifying the substrate for ABCC10 should help in determining the physiologic function of this pump; its expression in diverse cancer cell lines suggests that it has a ubiquitous role in cancer cells. Although the ability of paclitaxel to disrupt microtubule dynamics is well documented, paclitaxel resistance is a major obstacle to the successful treatment of lung cancer in the clinic. Understanding the molecular mechanisms underlying the antitumor effect of paclitaxel, and paclitaxel resistance, may lead to the design of biologically and pharmacologically targeted therapeutic strategies for paclitaxel-resistant tumors and to the improvement of chemotherapy effect and survival of NSCLC patients. Additional clinical research will be needed to investigate whether ABCC10 could be used as a predictive marker of sensitivity and/or resistance to paclitaxel in NSCLC.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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