

## MRP8/ABCC11 directly confers resistance to 5-fluorouracil

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### Abstract

Multidrug-resistance-associated protein, MRP8/ABCC11 (ABCC11), is an efflux pump for nucleotide analogues and 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP). To test whether ABCC11 directly confers 5-fluorouracil (5-FU) resistance, we used the 5-FU-resistant subline PC-6/FU23-26 selected from PC-6 human small-cell lung cancer cells by 5-FU and found that it increases the resistance by ~25-fold. The intracellular FdUMP accumulation was reduced in PC-6/FU23-26 cells concomitant with the overexpression of the *ABCC11* gene. These findings suggest that ABCC11 confers 5-FU resistance in the sublines by enhancing the efflux for the active metabolite FdUMP. Previously, methotrexate also increased the efflux by *ABCC11*, and we found cross-resistance to methotrexate in PC-6/FU23-26 cells. To confirm our hypothesis, we examined whether decreasing the expression of ABCC11 in PC-6/FU23-26 cells by small interfering RNA altered the cytotoxicity to 5-FU and methotrexate and found that this enhanced 5-FU and methotrexate cytotoxicity in PC-6/FU23-26 cells. These data indicate that expression of the *ABCC11* gene is induced by 5-FU, and that ABCC11 is directly involved in 5-FU resistance by the efflux transport of the active metabolite FdUMP. [Mol Cancer Ther 2007;6(1):122–7]

### Introduction

5-Fluorouracil (5-FU) and its derivatives are antimetabolite drugs that are widely used in cancer chemotherapy. The effects of 5-FU have been attributed to the inhibition of thymidylate synthase (TS) and the incorporation of its metabolites into RNA and DNA. 5-FU enters cells rapidly

and is converted intracellularly by metabolic enzymes. Dihydropyrimidine dehydrogenase is the first and rate-limiting enzyme for converting 5-FU to an inactive metabolite, dihydrofluorouracil. In contrast, the active metabolite of 5-FU is converted to 5'-fluoro-2'-deoxyuridine by thymidine phosphorylase, with the subsequent phosphorylation by thymidine kinase resulting in the active metabolite 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP). The primary mode of action of FdUMP is believed to be TS inhibition, which reduces the thymidine pool and increases the uracil pool, leading to the inhibition of DNA synthesis (1). It has thereby been shown that TS and dihydropyrimidine dehydrogenase gene expression and/or activity are associated with the efficacy of 5-FU (1–4).

Members of the multidrug-resistance-associated protein (MRP/ABCC) superfamily are primary active transporters that confer drug resistance by effluxing anticancer agents or their metabolites from cells (5). Nine structurally and functionally related MRP/ABCC family members have been identified. MRP4/ABCC4 (ABCC4) and MRP5/ABCC5 (ABCC5), which lack a transmembrane domain present in other family members, and MRP1/ABCC1 (ABCC1), MRP2/ABCC2 (ABCC2), MRP3/ABCC3 (ABCC3), MRP6/ABCC6 (ABCC6), and MRP7/ABCC10 (ABCC10) have been shown to mediate the ATP-dependent transport of several anticancer agents and antiviral nucleosides (5–7). More recently, the new family members MRP8/ABCC11 (ABCC11) and MRP9/ABCC12 (ABCC12) have been identified (8, 9), which share the greatest structural similarity with ABCC4 and ABCC5. ABCC11, like ABCC4 and ABCC5, also enhances the cellular extrusion of cyclic nucleosides and confers resistance to nucleoside analogues (10, 11).

The metabolism of 5-FU is very complicated, and ABCC5 and ABCC11 have been shown to be active transporters for FdUMP and are suggested as conferring resistance to 5-FU and certain fluoropyrimidines (10, 12). Usually, a decrease in the intracellular concentration of drugs due to drug-efflux pumps is considered as a determinant for drug resistance; however, the role of MRP/ABCC transporters in 5-FU resistance remains undetermined. In the present study, the 5-FU-resistant small-cell lung cancer subline PC-6/FU23-26, derived by continuously exposing PC-6 human small-cell lung cancer cells to 5-FU, was used to investigate whether MRP/ABCC transporters directly confer the 5-FU resistance.

### Materials and Methods

#### Cell Lines and Chemicals

Cells from a human small-cell lung cancer cell line, PC-6, those from their 5-FU-resistant subline PC-6/FU23-26, 7-ethyl-10-hydroxycamptothecin (SN-38)-resistant subline PC-6/SN2-5, and paclitaxel-resistant subline PC-6/TAX1-1

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were kindly provided by Daiichi Pharmaceutical (Tokyo, Japan; refs. 4, 13). The human lung adenocarcinoma cell line PC-9 and cisplatin-resistant human lung adenocarcinoma cell line PC-9/CDDP were provided as described previously (4). NCI-H23 cells and those from their gemcitabine-resistant human lung adenocarcinoma cell line H23/GEM-R were established as described previously (14). PC-6/FU23-26 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% (v/w) penicillin/streptomycin and 10  $\mu\text{mol/L}$  of 5-FU, and were then cultured in 5-FU-free medium for 7 days before each experiment. 5-FU was obtained from Kyowa Hakko Kogyo (Tokyo, Japan); gemcitabine was obtained from Eli Lilly Pharmaceuticals (Indianapolis, IN); 1- $\beta$ -D-arabinofuranosylcytosine was purchased from Calbiochem/EMD Biosciences (San Diego, CA); and methotrexate was purchased from Wako Chemicals (Osaka, Japan).

#### Total RNA Extraction and Real-time PCR

Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription (RT)-PCR was done in a volume of 20  $\mu\text{L}$  using the SuperScript<sup>™</sup> III Platinum<sup>®</sup> One-Step qRT-PCR kit (Invitrogen) according to the manufacturer's instructions. The sequences of the primer sets of ABC transporters were determined as described previously (8, 15–17). We did real-time PCR with the LightCycler FastStart DNA SYBR Green kit (Roche Diagnostics, Indianapolis, IN). We used the melting-curve analysis to control for the 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. For each sample, the data were normalized to that for the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*.

#### Intracellular FdUMP Concentration

We treated PC-6 and PC-6/FU23-26 cells with 100  $\mu\text{mol/L}$  of 5-FU for 2 h. After thrice washing with cold PBS, the pellet was stored at  $-80^{\circ}\text{C}$  until analysis.

The intracellular FdUMP concentration was determined using the FdUMP binding assay for TS as described previously (18). Phosphate buffer (50 mmol/L, pH 7.4) containing 20 mmol/L 2-mercaptoethanol, 100 mmol/L NaF, and 15 mmol/L cytidine-5'-monophosphate was added to the pellet,  $\sim 5 \times 10^7$  cells. Three cycles of freeze-thawing of the suspensions were conducted, and the cells were homogenized. Acetic acid of 1 mol/L was added to the homogenate, and the supernatant was lyophilized. The lyophilized resultant was reconstituted in 10 mmol/L Tris-HCl buffer (pH 8.0) and centrifuged. The supernatant was adjusted to pH 7 to 8 by aqueous ammonia and applied to DEAE-cellulose column.  $\text{NH}_4\text{HCO}_3$  solution was applied to the column, and the obtained fraction of FdUMP was lyophilized. The lyophilized resultant was reconstituted in 10 mmol/L Tris-HCl buffer (pH 7.4). The aliquot was mixed with [<sup>3</sup>H]-FdUMP,  $\text{CH}_2\text{FH}_4$ , and TS enzyme and

incubated at  $25^{\circ}\text{C}$  to obtain a covalent ternary complex ([<sup>3</sup>H]-FdUMP-TS- $\text{CH}_2\text{FH}_4$ ). Charcoal suspension was added to the solution to remove the excess of [<sup>3</sup>H]-FdUMP, and radioactivity of the complex in the solution was determined. The solution including a known amount of FdUMP was treated in the same manner, and the standard curve was constructed to determine the concentration of FdUMP in the treated cell. To correct the recovery of FdUMP from the sample during the treatment, a tracer of [<sup>3</sup>H]-FdUMP was added to the homogenate obtained from the cell pellet, and the recovery was determined by the same treatment.

#### Chemosensitivity Assay

Cells were cultured at 5000 cells/well in 96-well tissue culture plates. To assess cell viability, stepwise 10-fold dilutions of the antimetabolic agents were added 2 h after plating, and the cultures were incubated at  $37^{\circ}\text{C}$  for 96 h. At the end of the culture period, 20  $\mu\text{L}$  of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution (CellTiter 96<sup>®</sup> Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI) was added, and cells were incubated for a further 4 h, and the absorbance was measured at 490 nm using an ELISA plate reader. Mean values were calculated from three independent experiments done in quadruplicate. Chemosensitivity was quantified as the 50% cell survival ( $\text{IC}_{50}$ ), as determined from the concentration-effect relationship using GraphPad Prism (version 4, GraphPad Software, San Diego, CA).

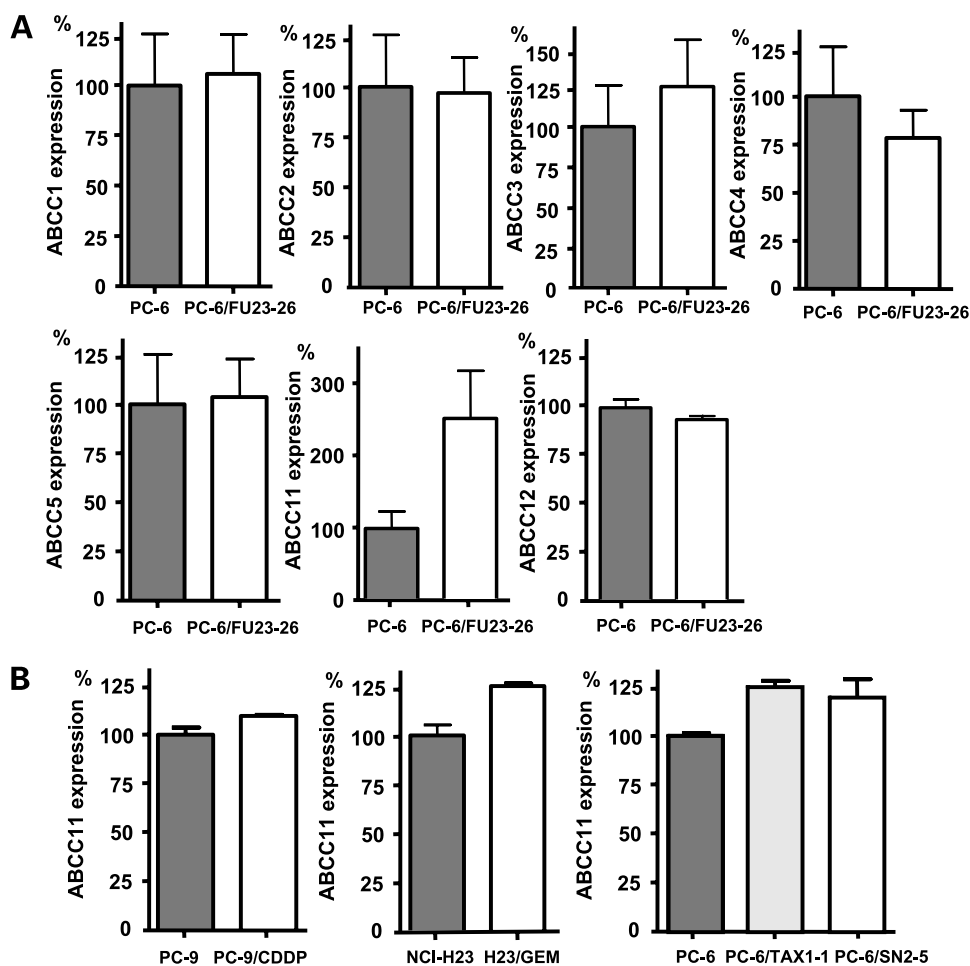
#### Modification of 5-FU Cytotoxicity by RNA Interference

Approximately  $2 \times 10^5$  cells were transfected with small interfering RNA (siRNA) oligonucleotide using Lipofectamine<sup>™</sup> 2000 (Invitrogen) to produce a final RNA concentration of 50 nmol/L in serum-free Opti-MEM medium (Invitrogen) as described previously (19). At 24 h after transfection, we changed the medium to RPMI 1640 supplemented with 10% heat-inactivated FBS. After an additional 24 h, total RNA was extracted, or the cells were treated with 5-FU or methotrexate for 48 h, and then the viable cells were counted using trypan blue staining to evaluate the cytotoxicity of 5-FU or methotrexate. The siRNA oligonucleotides for ABCC11 (predesigned siRNA, ID 118187) were purchased from Ambion (Austin, TX). The negative-control siRNA (Silencer negative control siRNA), which does not exert nonspecific effects on gene expression, was also purchased from Ambion.

## Results

#### Expression of ABC Transporters

We used real-time RT-PCR to compare the expression levels of *ABCC1-5*, *ABCC11*, and *ABCC12* genes in cells of the 5-FU-resistant small-cell lung cancer cell line PC-6/FU23-26 with those in parent PC-6 cells. The *ABCC11* gene expression was  $\sim 2.5$ -fold higher in PC-6/FU23-26 cells than in PC-6 cells, whereas the expressions of *ABCC1-3*, *ABCC4*, *ABCC5*, and *ABCC12* were the same in both cell types (Fig. 1A).



**Figure 1.** Expression of MRP/ABCC transporters in drug-resistant cells. Comparison of gene expression levels of the MRP/ABCC transporters between cells of the 5-FU-resistant cell line PC-6/FU23-26 and PC-6 cells. The *ABCC11* gene expression was ~2.5-fold higher in PC-6/FU23-26 cells, whereas the expressions of the *ABCC1-3*, *ABCC4*, *ABCC5*, and *ABCC12* were not altered (A). The expression levels of the *ABCC11* gene in cisplatin-, paclitaxel-, gemcitabine-, and SN-38-resistant cell lines did not differ from those in the respective parent cell lines (B).

We compared the expression levels of the *ABCC11* gene in cisplatin-, paclitaxel-, gemcitabine-, and SN-38-resistant cell lines with those in the respective parent cell lines. In contrast to the above results, the expression levels of *ABCC11* genes did not differ in the pairs of resistant and parent cells (Fig. 1B).

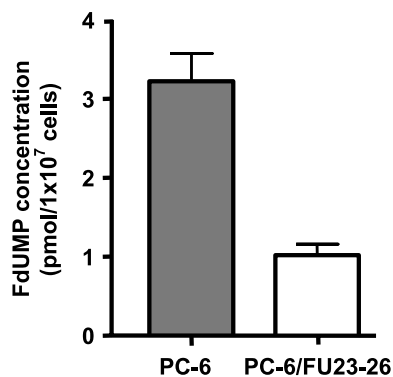
#### FdUMP Concentration

ABCC11 has been shown to be an active transporter for FdUMP, which is an active metabolite for TS inhibition (10). To investigate mechanisms of 5-FU resistance, the intracellular accumulation of FdUMP was measured in PC-6 and PC-6/FU23-26 cells. After treatment with 100  $\mu\text{mol/L}$  of 5-FU for 2 h, a FdUMP binding assay was used to measure the FdUMP concentration. As shown in Fig. 2, the FdUMP accumulation following 2 h of exposure to 5-FU was lower in PC-6/FU23-26 cells than in the parent PC-6 cells.

#### Cross-Resistance to Antimetabolic Agents in PC-6/FU23-26 Cells

It has previously been shown that ABCC11 is also able to transport methotrexate (11), and hence, we examined the drug cytotoxicity against antimetabolic agents of PC-6 and PC-6/FU23-26 cells by MTS assay. The resistance to 5-FU

was ~25-fold higher in PC-6/FU23-26 cells than in the parent cells (Table 1). PC-6/FU23-26 cells also exhibited cross-resistance to methotrexate (~56-fold), but not to gemcitabine and 1- $\beta$ -D-arabinofuranosylcytosine.



**Figure 2.** Intracellular FdUMP concentration. A FdUMP binding assay showed that the intracellular FdUMP concentration was markedly lower in PC-6/FU23-26 cells than in PC-6 cells after exposure to 5-FU for 2 h.

**Table 1.** IC<sub>50</sub> values of various antimetabolic drugs in PC-6 and PC-6/FU23-26 cells

	PC-6	PC-6/FU23-36	RR
	IC <sub>50</sub> (95% CI)	IC <sub>50</sub> (95% CI)	
5-FU (μmol/L)	0.78 (0.58–1.07)	19.65 (4.20–91.99)	25.2
Methotrexate (μmol/L)	0.12 (0.09–1.71)	6.28 (1.90–20.70)	52.3
Gemcitabine (nmol/L)	6.13 (2.31–16.20)	3.75 (2.950–4.760)	0.6
1-β-D-Arabinofuranosylcytosine (nmol/L)	40.70 (29.30–56.40)	36.20 (28.80–45.50)	0.8

Abbreviation: RR, resistance ratio: (IC<sub>50</sub> in resistant subline)/(IC<sub>50</sub> in the parental cells).

### Enhanced Cytotoxicity to 5-FU in PC-6/23-26 Cells by ABCC11 siRNA

To examine the alteration of 5-FU or methotrexate cytotoxicity by ABCC11, PC-6/FU23-26 cells were transfected with siRNAs directed against ABCC11, and control siRNA-transfected or nontransfected cells were used as controls.

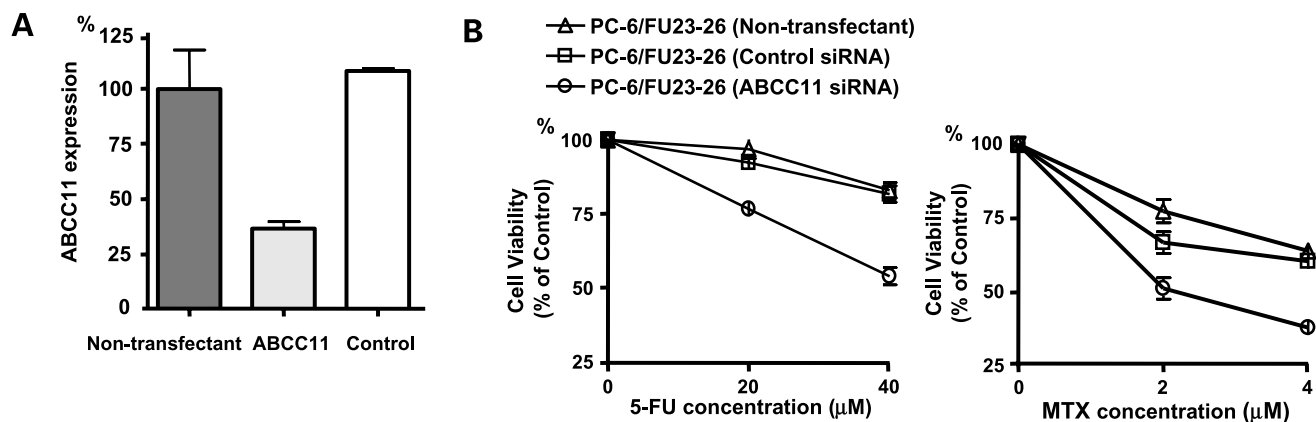
Cells were harvested at 48 h after transfection, and extracts were prepared and analyzed by quantitative real-time RT-PCR. The expression level of the *ABCC11* gene was decreased at 48 h after siRNA transfection (Fig. 3A). Forty-eight hours after transfection with ABCC11 siRNA or negative-control siRNA, or with no transfection, cells were treated with 20 and 40 μmol/L 5-FU or 2 and 4 μmol/L methotrexate for 48 h, and then the viable cells were counted using trypan blue staining to evaluate the cytotoxicity of 5-FU or methotrexate. The cytotoxicity to 5-FU was higher for cells exposed to 5-FU in the presence of ABCC11 siRNA than for cells transfected with negative-control siRNA and for nontransfected cells (Fig. 3B). The cytotoxicity to methotrexate was also altered by the transfection of ABCC11 siRNA (Fig. 3B).

Because negative-control siRNA does not affect gene expression, these results indicate that decreasing the expression of ABCC11 by siRNA altered the cytotoxicity to these agents.

### Discussion

In this study, we found that the expression of *ABCC11* was induced by 5-FU exposure and conferred to acquired resistance to 5-FU. We believe that this is the first description of a direct relationship between 5-FU resistance and MRP/ABCC family members in 5-FU-resistant cancer cells.

MRP/ABCC family members are often attributed with multidrug resistance in cancer cells by effluxing anticancer agents by cotransporting drugs with glutathione or glutathione- or glucuronide-drug conjugates (5, 16, 17). MRP/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 (5). Some studies have shown that later group members



**Figure 3.** Modification of 5-FU cytotoxicity by ABCC11 siRNA. Gene expression levels of *ABCC11* in PC-6/FU23-26 cells transfected with siRNAs directed against ABCC11, and in control siRNA-transfected or nontransfected PC-6/FU23-26 cells (A). Cytotoxicity to 20 and 40 μmol/L 5-FU or 2 and 4 μmol/L methotrexate (MTX) was measured in PC-6/FU23-26 cells transfected with siRNAs directed against ABCC11, and in control siRNA-transfected or nontransfected PC-6/FU23-26 cells. The viable cells were counted using trypan blue staining to evaluate the cytotoxicity of 5-FU or methotrexate. The cell viability following 5-FU or methotrexate treatment for 48 h differed between PC-6/FU23-26 cells transfected with ABCC11 siRNA and control siRNA-transfected or nontransfected PC-6/FU23-26 cells (B).

(although ABCC12 is still under investigation) are transporters of cyclic nucleosides and nucleoside analogues due to the active efflux of the monophosphorylated metabolites of these drugs (6, 7, 10). Furthermore, recent transfection studies have showed that ABCC5 and ABCC11 are able to efflux FdUMP, which is the monophosphorylated and active metabolite of 5-FU (10, 12). We therefore compared the gene expression levels of ABC transporters in 5-FU-resistant cells with those in the respective parent cells. We found that FdUMP accumulation was apparently reduced in PC-6/FU23-26 cells, concomitant with the increased expression of *ABCC11*. Furthermore, suppression of *ABCC11* expression by siRNA resulted in the restoration of 5-FU cytotoxicity. Our data indicate that the induction of *ABCC11* expression by exposure to 5-FU results in the acquisition of resistance to 5-FU by the efflux transport of the active metabolite FdUMP.

5-FU derivatives are clinically used as combined cancer chemotherapies, and hence, it is important to identify potential drug interactions when using 5-FU. We therefore also investigated whether other anticancer agents affect *ABCC11* expression. Our comparison of the expression levels of the *ABCC11* gene in cisplatin-, paclitaxel-, gemcitabine-, and SN-38-resistant cell lines with those in the respective parent cell lines revealed no induction of *ABCC11* expression in any drug-resistant cells. We have previously shown that the IC<sub>50</sub> of 5-FU did not differ markedly between paclitaxel-, gemcitabine-, and SN-38-resistant cells and their respective parent cells, whereas the IC<sub>50</sub> of 5-FU in cisplatin-resistant cells was decreased compared with that in the parent cells (4). Because these anticancer agents do not induce *ABCC11* expression, these results together suggest that anticancer agents transported by ABCC11 are limiting.

ABCC11 has a splicing variant in which exon 28 is deleted, although its functionality remains undetermined (8). Furthermore, Yoshiura et al. (20) recently reported that ABCC11 has a functional single-nucleotide polymorphism: that of G538 to A538, which caused a lower excretory activity for cyclic guanosine 3',5'-monophosphate and thereby determined the earwax type. We did not examine this single-nucleotide polymorphism or expression of splicing variants in this study, and hence, additional research is needed to elucidate whether this single-nucleotide polymorphism or splicing variant changes the activity for FdUMP efflux in cancer cells.

ABCC/MRP transporters play a role in the cellular efflux and resistance to methotrexate (21), and recently, it was reported that ABCC11 also mediates the transport of methotrexate (11). We found that ABCC11-overexpressed PC-6/FU23-26 cells exhibited cross-resistance to methotrexate, suggesting that ABCC11 also confers resistance to methotrexate. Another ABC transporter, BCRP/ABCG2 (ABCG2), has also been shown to be capable of mediating antifolates including methotrexate; however, in contrast to ABCC/MRP transporters, ABCG2 is also able to transport methotrexate polyglutamates (22). Therefore, ABCG2 may be a major contributor to methotrexate resistance. We did

not find the increased expression of the *ABCG2* gene in PC-6/FU23-26 cells (data not shown). Furthermore, FdUMP is not a substrate for ABCG2, indicating that ABCG2 was not major determinant for the methotrexate resistance observed in this study. Nonetheless, whether ABCC11 is induced by methotrexate or whether ABCC11 mediates transport of another antifolate remains to be elucidated.

ABCC11 transcripts are expressed in a wide range of normal tissues, including liver, breast, prostate, and testis (8, 9). Clarifying the substrate for ABCC11 should help in determining the physiologic function of this pump, and the expression of ABCC11 in diverse cancer cell lines suggests that it has a ubiquitous role in cancer cells. Because 5-FU is widely used in many cancer treatments, investigating ABCC11 expression in clinical cancers is needed to determine whether ABCC11 could be used as a predictive marker for 5-FU treatment.

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# Molecular Cancer Therapeutics

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