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ORIGINAL ARTICLE

ABCC11 gene polymorphism as a potential predictive biomarker for an oral 5-fluorouracil derivative drug S-1 treatment in non-small cell lung cancer

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Abstract

Purpose ABCC11/MRP8 (ABCC11) is an ATP-binding cassette transporter that is involved in regulating cellular sensitivity and resistance for many anti-cancer drugs. Since 5-fluorouracil (5-FU) is one of the substrates for ABCC11, we examined whether ABCC11 is a predictive marker for an oral 5-FU derivative drug S-1 treatment in non-small cell lung cancer (NSCLC).

Methods Real-time PCR and MTS assay were carried on 21 human NSCLC cell lines. The drug resistance capabilities of ABCC11 are evaluated by analyzing the resistance profiles of a clone of HeLa cell in which the pump was ectopically expressed. Blood samples of 106 NSCLC patients were collected.

Results There was a significant correlation between dihydropyrimidine dehydrogenase (*DPD*) gene expression and the IC_{50} for 5-FU. We then classified NSCLC cell lines into two groups based on the phenotype of the SNP538 (G > A) in ABCC11: a combined G/G and G/A group, and an A/A group. The distribution of the IC_{50} for 5-FU in combination with a potent inhibitor of DPD 5-chloro-2, 4-dihydropyrimidine (CDHP), which is contained in S-1, showed a significant reduction in the A/A group compared with the combined G/G and G/A group. Next, the clinical usefulness of the *ABCC11* SNP in treatment containing S-1 was examined in 106 NSCLC patients, and the disease control rate was found to be significantly better in the A/A group than in the combined G/G and G/A group.

Conclusions These results indicate that the SNP538(G > A) in the *ABCC11* gene is a potential determinant for S-1 treatment.

Keywords ABCC11/MRP8 · 5-fluorouracil (5-FU) · S-1 · Single-nucleotide polymorphism (SNP) · Thymidylate synthase (TS)

Introduction

5-Fluorouracil (5-FU) was synthesized in 1957 as an anti-metabolite agent and is still widely used in cancer chemotherapy [1]. 5-FU is converted to an active metabolite called 5′-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP)

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intracellularly. This FdUMP inhibits thymidylate synthase (TS), which reduces the thymidine pool and increases the uracil pool, leading to the inhibition of DNA synthesis. 5-FU is also converted to an inactive metabolite, dihydrofluorouracil, by dihydropyrimidine dehydrogenase (DPD). Thus, TS and DPD expressions and/or activities in tumor cells are associated with the efficacy of 5-FU [2–4].

Because DPD is the key enzyme in 5-FU degradation, 5-FU is not active in primary lung cancers with high DPD activity, which causes rapid degradation of 5-FU [5]. Thus, a DPD-inhibitory fluoropyrimidine (DIF) is now used for lung cancer chemotherapy [6]. Among such chemotherapies, S-1 is one of the most useful oral fluoropyrimidine formulations, and it is a combination of tegafur (which is a prodrug metabolized to 5-FU), a potent DPD inhibitor, 5-chloro-2, 4-dihydropyrimidine (CDHP), and potassium oxonate (which reduces gastrointestinal toxicity) [7]. S-1 has demonstrated marked activity against non-small-cell lung cancer (NSCLC), as well as a broad array of other solid tumors, including gastric, colorectal, breast, cervical, and pancreatic cancers [8].

Although DPD and TS are sensitivity determinant factors for 5-FU, there are no known biomarkers of DIF. The ATP-binding cassette (ABC) transporter superfamily contains several family members that confer anti-cancer drug resistance by effluxing anti-cancer agents or their metabolites from cells when expressed at high levels. 5-FU, methotrexate (MTX), and pemetrexed (PEM) are substrates for the multidrug-resistance associated protein, MRP8/ABCC11 (ABCC11, NCBI Reference Sequence: NG_011522.1), one of the ABC transporters [9–11]. We previously showed that ABCC11 is directly involved in 5-FU resistance by the efflux transport of its active metabolite FdUMP [12]. Therefore, in this study, we focused on ABCC11 to examine the usefulness of analysis of ABCC11 for S-1 treatment of NSCLC.

Materials and methods

Cell lines and chemicals

The following 21 human NSCLC cell lines were used in this study: NCI-H23, SK-LC-10, VMRC-LCF, SK-LC-1, SK-LC-6, NCI-H460, Calu-1, A549, UMRC-LCD, ACC-LC-314, ACC-LC–MS, ACC-LC-176, NCU-LC-201, ACC-LC-94, PC-14, RERF-MC-MT, PC-9, ACC-LC-174, RERF-LC-AI, PC-10, and QG56. All cell lines were provided by Aichi Cancer Center (Nagoya, Japan). These cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and 1% (v/w) penicillin/streptomycin in a humidified chamber (37 °C, 5% CO_2). 5-FU was provided by Kyowa Hakko Kogyo (Tokyo, Japan), and the CDHP was provided by Taiho Pharmaceutical Co. Ltd. (Tokyo, Japan). MTX was obtained from Wako Chemicals (Osaka, Japan).

Total RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Alameda, CA, USA) according to the manufacturer's instructions. Real-time PCR was performed using the StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) and *ABCC11* gene primers and probe set of Taqman gene expression assays (Hs00261567, Applied Biosystems). Melting curve analysis was used to control for specificity of the amplification products. The number of transcripts was calculated from a standard curve obtained by plotting the input of four different known transcript concentrations versus the PCR cycle number at which the detected fluorescence intensity reached a fixed value. The PCR program consisted of 45 cycles of 94 °C for 15 s and 60 °C for 1 min. For each sample, the data were normalized to those of the Taqman RNase P control reagents kit (Applied Biosystems).

Concentration of 5-FU resulting in 50% cell survival (IC_{50})

Cells were cultured at 5000 cells per well in 96-well tissue culture plates. To assess cell viability, stepwise tenfold dilutions of 5-FU or 5-FU plus CDHP (at a 1:1 ratio) 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 of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt solution (MTS) (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) were added, and the cells were incubated for a further 4 h. The absorbance was then measured at 490 nm using an ELISA plate reader. Cellular chemosensitivity is expressed as the relationship between 5-FU or 5-FU plus CDHP concentration and its effect using GraphPad Prism (version 4, GraphPad Software, La Jolla, CA, USA).

Protein extraction and western blotting

Equal amounts of total cell lysates were solubilized in the sample buffer (50 mM Tris–HCl [pH 6.8], 2% SDS, 1 mM EDTA, 10% glycerol), which contained Complete, Mini, Protease Inhibitor Cocktail Tablets and the PhosSTOP Phosphatase Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN, USA). Subsequently, these lysates were electrophoresed on a 10% Ready Gel Tris–HCl Gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to Immobilon-P filters (Millipore, Billerica, MA, USA). The filters were first incubated with primary antibodies for 3 h (ABCC11) or 1 h (α-tubulin) at room temperature and then with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare Bioscience, Amersham Place, UK). The anti-ABCC11 antibody was purchased from MyBiosource (MBS7045487 Rabbit anti-Human ABCC11 polyclonal antibody, San Diego, CA, USA), and the anti-α-tubulin antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of ABCC11-transfected HeLa cells

ABCC11 538G was amplified using human prostate cDNA as a template by six forward and reverse primers (Fw1 including Bam HI recognition sequence and Kozak sequence; tataggatcccaccATGACTAGGAAGAGGACATACTG, Fw2; AAGCCACTTCGGACATGTTGCAGGACACAG, Fw3; AAAGTCATGGCTGTCAACATCGTGCTGCAG, Rv1; AACATGTCCGAAGTGGCTTCCTTGTGCATC, Rv2 including NotI recognition sequence; tatagcggccgcTCTCAGTGAAGAAGTGGCTGTGGCCATG, Rv3; ATGTTGACAGCCATGACTTTAAAGGAGTAG), and these segments were finally combined with PCR. ABCC11 538A was also amplified using two additional primers (Fw4; CAGTGTACTCAGGCCAATATTGATTATAC, Rv4; AATATTGGCCTGAGTACACTGGCAATGCAG) to introduce specific mutation. These DNA fragments were separated with 0.8% agarose gel electrophoresis. These samples were purified using the PreLink gel Extraction Kit (Invitrogen, Carlsbad, CA, USA).

This ABCC11 coding sequence was inserted into the pcDNA3.1 expression vector (Invitrogen). Each ABCC11 expression vector and parental plasmid was introduced into HeLa cells by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For stable ABCC11 expression, the cells with 400 µg/ml geneticin 418 (G418) (Invitrogen) in the culture medium for 21 days were selected, followed by isolation of G418-resistant colonies. Cells with suitable ABCC11 expression were clonally expanded. The selected stable cell lines were maintained in culture medium containing 400 µg/ml G418. The ABCC11-538G, ABCC11-538A, and mock-introduced HeLa cells were named HeLa/ABCC11-538G, HeLa/ABCC11-538A, and HeLa/Mock, respectively.

Intracellular MTX concentration

HeLa, HeLa/Mock, HeLa/ABCC11-538G, and HeLa/ABCC11-538A cells (1×10^7) were treated with 80 M MTX for 2 h. After washing five times with cold PBS, the cells were resuspended in RPMI (1.5 mL) and homogenized. After centrifugation, the supernatant was stored at 80 °C until analysis. The intracellular MTX concentration was determined using an FPIA [13].

Genomic DNA extraction and detection of the 538(G > A) SNP in the *ABCC11* gene

Genomic DNA was extracted from 21 NSCLC cell lines using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. The single-nucleotide polymorphism (SNP) (538G > A) in the *ABCC11* gene was detected using StepOnePlus Real-Time PCR Systems (Applied Biosystems) and Taqman Drug Metabolism Genotyping Assays (Assay ID; C_25999969, JSNP ID; rs17822931, Applied Biosystems) according to the manufacturer's instructions.

Patient samples

The study group included 106 Japanese patients with pathologically confirmed primary NSCLC, who were treated with S-1 monotherapy or combination therapy of S-1 and platinum-containing drug (cisplatin or carboplatin) at the Nagoya City University Hospital from 2007 to 2017. The creatinine clearance (Cockcroft-Gault Equation) of all patients who had been treated with S-1 was higher than 50 ml/min, which was enough to tolerate S-1. After written, informed consent had been obtained, a blood sample was collected. Clinical staging and recurrence or progression information was determined based on results from available chest radiography, computerized tomography, bone scans, positron emission tomography scans, and magnetic response imaging. Approval for the study was obtained from our institutional ethics committee.

Statistical analysis

Differences in gene expression levels, intracellular MTX accumulation, and IC_{50} values for 5-FU plus CDHP between samples were evaluated using Student's unpaired *t* test. Correlations between expressions of *ABCC11, TS,* and *DPD* genes and IC_{50} values for 5-FU were evaluated using Spearman's test. Comparisons between groups were performed using Student's unpaired *t* test and Chi-squared test. The level of significance was set at 5% using two-sided analysis.

Results

Relationship between 5-FU cytotoxicity and expression of TS and DPD in 21 NSCLC cell lines

We previously found a relationship between *DPD* gene expression and 5-FU sensitivity in NSCLC cells [4]. In this study, we again determined the expression levels of the *TS* and *DPD* genes in 21 NSCLC cell lines using real-time PCR and compared the IC_{50} values for 5-FU in these cell lines with the relative expression levels of the *TS* and *DPD* genes.

The sensitivities of 21 NSCLC cell lines to 5-FU and 5-FU plus CDHP are summarized in Table 1. There was a significant correlation between *DPD* gene expression and 5-FU sensitivity in all 21 NSCLC cell lines (Fig. 1a), whereas the expression levels of the *TS* genes were not significantly correlated with 5-FU sensitivity (Fig. 1b).

Relationship between the 5-FU sensitivity of 21 NSCLC cell lines and the status of SNP538 in ABCC11

We also determined the expression levels of the *ABCC11* genes in these 21 NSCLC cell lines using real-time PCR and compared the IC_{50} values for 5-FU in these cell lines with the relative expression levels of the *ABCC11* gene. However, there was no correlation between *ABCC11* gene expression and 5-FU sensitivity of the 21 NSCLC cell lines (Fig. 1c). Since we considered that DPD inhibition was necessary when using 5-FU derivative drugs in clinical treatment of NSCLC, we further used the potent DPD inhibitor CDHP together with 5-FU to exclude the influence of DPD. However, there was also no significant correlation between *TS* or *ABCC11* gene expression and sensitivity for 5-FU plus CDHP (Supplementary Fig. 1a, b).

The *ABCC11* gene has a nonsynonymous SNP 538G > A (rs17822931; Gly180Arg), which is important for determination of the type of human earwax that is expressed [14]. We previously reported that the adenocarcinoma cell lines that have the SNP538 A/A in ABCC11 showed a significant reduction in the IC_{50} of PEM compared with that of the combined G/G and G/A *ABCC11* SNP groups [11]. To determine if expression of this SNP might modulate 5-FU or 5-FU plus CDHP sensitivity of the cell lines, we, therefore, classified the NSCLC cell lines into two groups based on the phenotype of SNP538 (G > A in ABCC11: a combined G/G and G/A group and an A/A group). Eight of the cell lines had a G/G genotype, two cell lines had a G/A genotype, and 11 cell lines had an A/A genotype. We compared the distribution of the IC_{50} for 5-FU between the G/G and G/A group and the A/A group, but no significant difference was found (Fig. 1d). We also compared the distribution of the IC_{50} for 5-FU in combination with CDHP in the NSCLC cell lines and found that the A/A group showed a significant reduction in the IC_{50} compared with that of the combined G/G and G/A group (Fig. 1e).

Cytotoxicity for MTX and 5-FU plus CDHP in ABCC11-538G and ABCC11-538A-transfected cells

Next, ABCC11 showing 538G or 538A expression vector and parental plasmid were introduced into HeLa cells, and HeLa/ABCC11-538G, HeLa/ABCC11-538A, and HeLa/mock cells, respectively, were established. Parental

Table 1 Sensitivities of 21 NSCLC cell lines to 5-FU and 5-FU plus CDHP

			IC_{50} for 5-FU (M)	95% CI (M)	IC_{50} for 5-FU plus CDHP (M)	95% CI (M)
NCI-H23	ad	G/G	2.622×10^{-6}	2.176×10^{-6} to 3.160×10^{-6}	1.294×10^{-7}	8.770×10^{-8} to 1.909×10^{-7}
SK-LC-10	ad	G/G	4.980×10^{-6}	4.417×10^{-6} to 5.614×10^{-6}	1.083×10^{-7}	7.486×10^{-8} to 1.567×10^{-7}
VMRC-LCF	ad	G/G	1.697×10^{-5}	8.899×10^{-6} to 3.184×10^{-5}	1.599×10^{-5}	9.055×10^{-6} to 2.737×10^{-5}
SK-LC-1	ad	G/G	1.597×10^{-5}	1.362×10^{-5} to 1.880×10^{-5}	2.721×10^{-6}	2.080×10^{-6} to 3.559×10^{-6}
SK-LC-6	la	G/G	5.944×10^{-6}	4.396×10^{-6} to 8.048×10^{-6}	3.231×10^{-7}	2.397×10^{-7} to 4.353×10^{-7}
NCI-H460	la	G/G	1.584×10^{-4}	1.339×10^{-4} to 1.824×10^{-4}	5.292×10^{-5}	3.464×10^{-5} to 8.087×10^{-5}
Calu-1	sq	G/G	1.612×10^{-5}	1.166×10^{-5} to 2.230×10^{-5}	1.323×10^{-5}	9.105×10^{-6} to 1.922×10^{-5}
A549	ad	G/G	4.618×10^{-5}	1.965×10^{-5} to 1.085×10^{-4}	3.653×10^{-5}	$2.421\times10^{-5}$to 5.519×10^{-5}
UMRC-LCD	ad	G/A	2.295×10^{-6}	1.645×10^{-6} to 3.203×10^{-6}	2.179×10^{-6}	1.478×10^{-6} to 3.096×10^{-6}
ACC-LC-314	ad	G/A	1.037×10^{-5}	4.627×10^{-6} to 2.507×10^{-5}	6.348×10^{-6}	4.155×10^{-6} to 9.700×10^{-6}
ACC-LC–MS	ad	A/A	6.881×10^{-6}	4.087×10^{-6} to 1.159×10^{-5}	7.562×10^{-8}	4.461×10^{-8} to 1.281×10^{-7}
ACC-LC-176	ad	A/A	4.435×10^{-7}	3.138×10^{-7} to 6.274×10^{-7}	1.593×10^{-9}	9.976×10^{-10} to 2.543×10^{-9}
NCU-LC-201	ad	A/A	5.118×10^{-6}	3.203×10^{-6} to 8.187×10^{-6}	5.824×10^{-7}	3.853×10^{-7} to 8.802×10^{-7}
ACC-LC-94	ad	A/A	5.376×10^{-6}	3.222×10^{-6} to 8.911×10^{-6}	2.016×10^{-8}	1.261×10^{-8} to 3.220×10^{-8}
PC-14	ad	A/A	4.663×10^{-6}	3.406×10^{-6} to 6.380×10^{-6}	1.587×10^{-7}	1.058×10^{-7} to 2.382×10^{-7}
RERF-MC-MT	ad	A/A	1.317×10^{-4}	2.944×10^{-5} to 5.896×10^{-4}	6.391×10^{-6}	4.155×10^{-6} to 700×10^{-6}
PC-9	ad	A/A	1.049×10^{-5}	7.873×10^{-6} to 1.397×10^{-5}	1.592×10^{-6}	1.066×10^{-6} to 2.377×10^{-6}
ACC-LC-174	ad	A/A	6.132×10^{-6}	4.689×10^{-6} to 8.017×10^{-6}	1.418×10^{-6}	2.878×10^{-7} to 7.465×10^{-6}
RERF-LC-AI	sq	A/A	1.106×10^{-5}	1.012×10^{-5} to 1.208×10^{-5}	1.372×10^{-6}	1.186×10^{-6} to 1.588×10^{-6}
PC-10	sq	A/A	1.600×10^{-5}	1.362×10^{-5} to 1.880×10^{-5}	1.133×10^{-7}	7.670×10^{-8} to 1.675×10^{-7}
QG56	sq	A/A	5.096×10^{-6}	3.203×10^{-6} to 8.187×10^{-6}	2.764×10^{-6}	2.170×10^{-6} to 3.523×10^{-6}

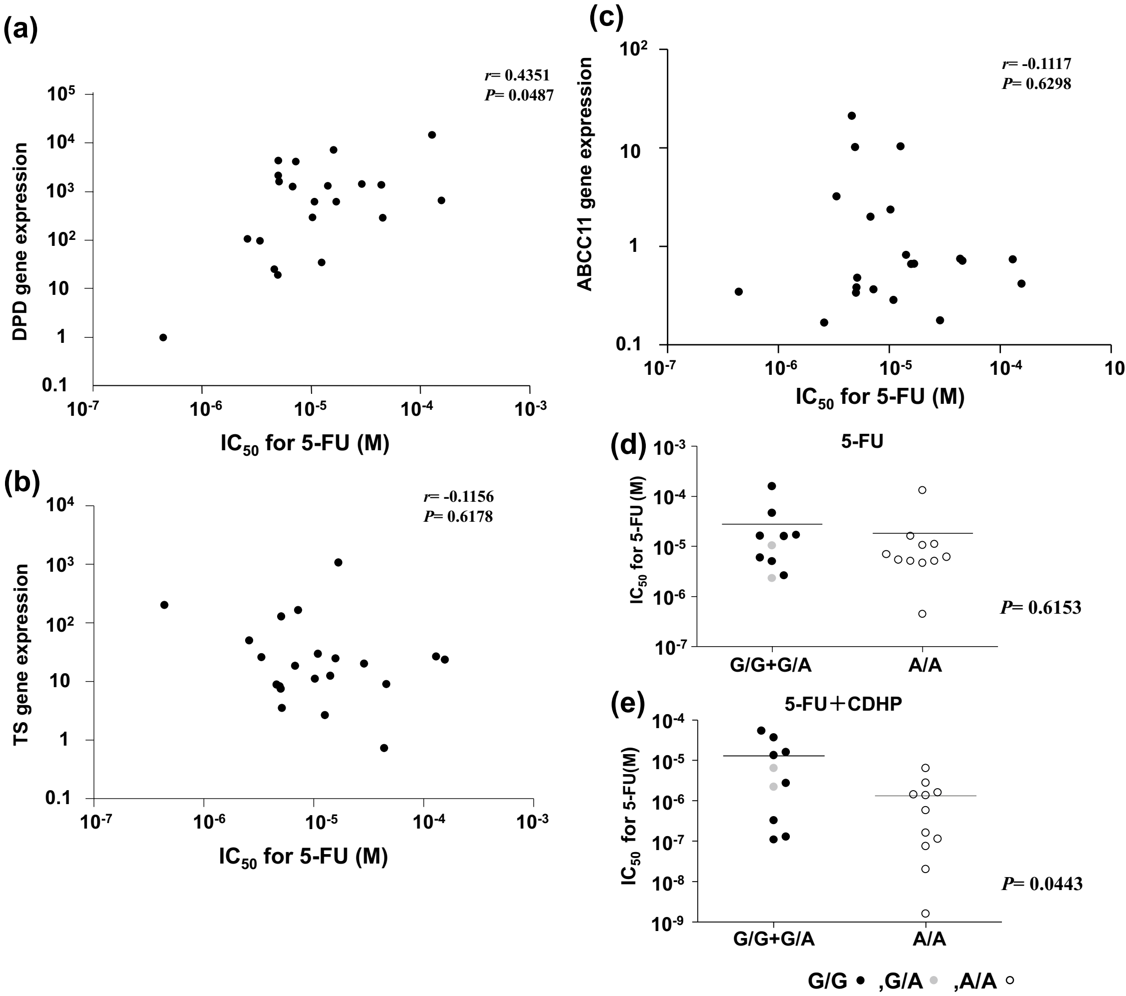

Fig. 1 Relationships between IC_{50} values for 5-FU and each gene expression or genotype status of ABCC11 in 21 NSCLC cell lines. **a** Relationships between IC_{50} values for 5-FU and *DPD* gene expressions ($n=21$, $r=0.4351$, $P=0.0487$), **b** for 5-FU and *TS* gene expression ($n=21$, $r=-0.1156$, $P=0.6178$), **c** for 5-FU and *ABCC11* gene expression ($n=21$, $r=-0.1117$, $P=0.6298$) in 21 NSCLC cell lines. **d** Distribution of IC_{50} values for 5-FU and **e** combination of 5-FU and CDHP in 21 NSCLC cell lines classified according to *ABCC11* 538(G > A) SNP types. Black circles, 538G/G; gray circles, 538G/A; white circles, 538A/A

HeLa cells had G/G genotypes in ABCC11 SNP538. Their expression levels of the ABCC11 protein were confirmed by real-time PCR and western blotting, respectively, (Fig. 2a, b). Real-time PCR indicated that the gene expression of HeLa/ABCC11-538G and HeLa/ABCC11-538A cells was increased compared with that in the HeLa/mock cells (Fig. 2a ; 6.4-fold increase, $P < 0.0001$ and 6.6-fold increase, $P < 0.0001$, respectively), whereas the expression of HeLa cells was not different from that of HeLa/mock cells.

Compared with the HeLa, HeLa/mock, and HeLa/ABCC11-538A cells, the level of ABCC11 protein was up-regulated in HeLa/ABCC11-538G cells (Fig. 2b). The distributions of the IC_{50} values for MTX, 5-FU, and 5-FU in combination with CDHP were also compared in the HeLa, HeLa/ABCC11-538G, HeLa/ABCC11-538A, and HeLa/mock cells (Fig. 2c, d, Supplementary Fig. 2). The IC_{50} for 5-FU was 6.17, 0.83, and 0.85 times higher, the IC_{50} for 5-FU plus CDHP was 9.75, 0.64, and 0.93 times higher, and the IC_{50} for MTX was 7.00, 0.356, and 1.57 times higher in the HeLa/ABCC11-538G, HeLa/ABCC11-538A, and HeLa cells, respectively, than in the HeLa/mock cells (Table 2).

Intracellular MTX concentration in ABCC11-transfected cells

The intracellular accumulation of MTX, which is known to be a substrate for ABCC11 [15] similar to FdUMP [10] and PEM [11] was assayed in HeLa, HeLa/ABCC11-538G, HeLa/ABCC11-538A, and HeLa/mock cells using a fluorescence polarization immunoassay (FPIA). After treatment

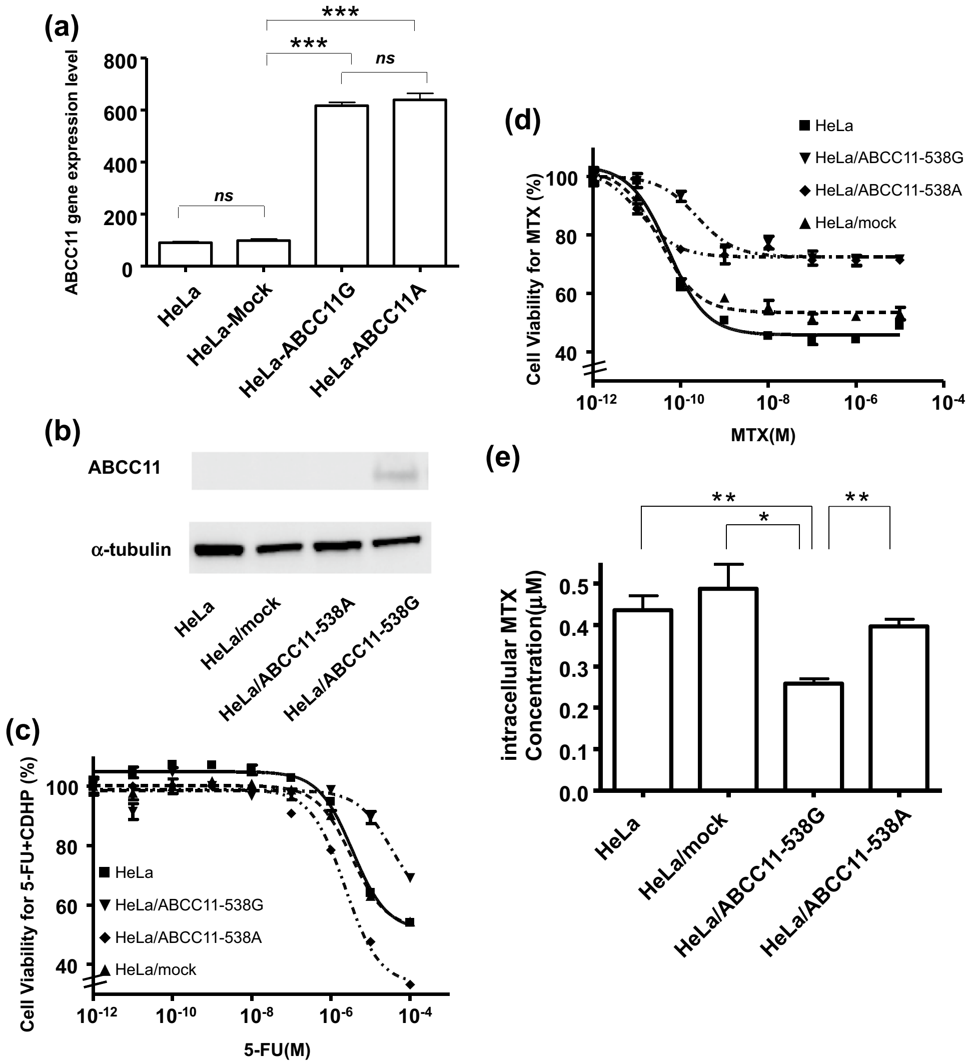

Fig. 2 Establishment of ABCC11 transfected cells and their changes of cell viability and intracellular concentration for cytotoxic agents. **a** Comparisons of ABCC11 gene expression and **b** protein levels between HeLa, HeLa/ABCC11-538G, HeLa/ABCC11-538A, and HeLa/Mock cells using real-time PCR and western blotting, respectively. α-Tubulin was assayed as a loading control. ***$P < 0.001$, *ns* no significance. **c** Cytotoxicities of 5-FU plus CDHP and **d** MTX in HeLa, HeLa/ABCC11-538G, HeLa/ABCC11-538A, and HeLa/Mock cells. **e** Intracellular MTX concentration was measured in HeLa, HeLa/ABCC11-538G, HeLa/ABCC11-538A, and HeLa/Mock cells after exposure to MTX for 2 h. *$P < 0.01$, **$P < 0.001$

Table 2 IC_{50} drug concentrations for 5-FU, 5-FU plus CDHP and MTX in HeLa, HeLa/Mock, HeLa/ABCC11-538G and HeLa/ABCC11-538A cells

	5-FU			5-FU + CDHP			MTX		
	IC_{50} (μM)	95% CI	RR	IC_{50} (μM)	95% CI	RR	IC_{50} (nM)	95% CI	RR
HeLa	13.42	7.533–23.90	0.85	3.33	2.416–4.591	0.93	0.05275	0.03714–0.07492	1.57
HeLa/Mock	15.73	7.123–34.73		3.558	2.481–5.104		0.03358	0.02259–0.04991	
HeLa/ABCC11-538G	97.2	18.63–507.1	6.17	34.7	12.49–96.41	9.75	0.2352	0.1259–0.4393	7
HeLa/ABCC11-538A	13.13	6.195–27.82	0.83	2.286	1.884–2.774	0.64	0.01196	0.006515–0.02195	0.356

RR resistance ratio (IC_{50} in resistant subline)/(IC_{50} in the control cells)

with 80 M MTX over a period of 2 h, intracellular MTX accumulation was lower in the HeLa/ABCC11-538G cells than in the other cells (Fig. 2e).

Clinical data of NSCLC patients who were treated with S-1

The characteristics of the patients from which the 106 NSCLC blood samples were obtained are listed in Table 3. There were 83 male and 23 female patients with a median

Table 3 Characteristics of the 106 NSCLC patients treated with S-1 monotherapy or combination therapy of S-1 and a platinum-containing drug

Characteristic	G/G(n=1)+G/A(n=31)		A/A(n=74)		*P* value
	No.	%	No.	%	
Age, years					
Median	69		67		0.344
Range	45–83		46–83		
Sex					
Male	25	78	58	78	1
Female	7	22	16	22	
Histology					
Adenocarcinoma	12	38	29	39	0.912
Squamous cell carcinoma	17	53	36	49	
Other	3	9	9	12	
S-1 chemotherapy					
Single agent	22	69	52	70	1
Platinum doublet	10	31	22	30	
Stage					
III	7	22	15	20	0.639
IV	25	78	57	77	
Recurrence	0	0	2	3	
Treatment line					
1	9	28	22	30	0.969
2	3	9	6	8	
3-	20	63	46	62	

P values were calculated using the Chi-square test (Sex, Histology, S-1 chemotherapy, Stage and Treatment line) or Student's *t* test (Age, years) in categorical variables or continuous variables, respectively

Table 4 Response rate (RR) and disease control rate (DCR) of the 106 NSCLC patients treated with S-1

	G/G(n=1)+G/A(n=31)		A/A(n=74)		*P* value
	No.	%	No.	%	
Response					
CR	0	0	1	1	0.135
PR	2	6	8	11	
SD	12	38	41	55	
PD	18	56	24	33	
Response rate	–	6	–	12	0.569
Disease control rate	–	44	–	68	0.037

P values were calculated using the Chi-square test

age of 68 (range 45–83) years. All patients were treated with S-1 monotherapy or combination therapy of S-1 and a platinum-containing drug (cisplatin or carboplatin) with 31, 9, and 66 patients receiving this as a first-, second-, and after third-line therapy, respectively. As shown in Table 4, there was a significant difference in the disease control rate (DCR; proportion of CR, PR plus SD to all patients) between the G/G and G/A genotype group and the A/A genotype group. Similarly, we also analyzed the 74 NSCLC patients treated with S-1 monotherapy. The characteristics of the patients treated with S-1 are listed in Supplementary Table 1. There was also a significant difference in the DCR of the patients treated with S-1 between the G/G and G/A genotype group and the A/A genotype group (Supplementary Table 2).

Discussion

In this study, we analyzed the relationship between *ABCC11* genes and the sensitivity to 5-FU of NSCLC cell lines. We found that the *ABCC11* SNP538(G > A) affected the 5-FU sensitivity of NSCLC cells when 5-FU treatment was combined with CDHP, a potent inhibitor of DPD. ABCC11-538G or ABCC11-538A-transfected cells were also established, and it was confirmed that the IC_{50} for 5-FU plus CDHP was higher in ABCC11-538G-transfected cells than in the other cells. Furthermore, intracellular MTX accumulation was lower in ABCC11-538G-transfected cells than in the other cells. Finally, in patients treated with S-1 monotherapy or combination therapy of S-1 and a platinum-containing drug (cisplatin or carboplatin), the DCR was significantly better in patients harboring ABCC11-538A than in patients harboring ABCC11-538G.

DPD activity is important as a sensitivity determinant factor of 5-FU [16–18]. We previously found that the basal level of DPD expression was significantly correlated with 5-FU sensitivity in NSCLC cells [12]. In this present study, we confirmed these previous results again. NSCLC was characterized by high DPD activity [5], indicating that DPD levels may dominantly affect the sensitivity to 5-FU in NSCLC [19]. Recent clinical trials showed that a combination of platinum drugs with a novel oral fluorouracil S-1 containing a prodrug of 5-FU and CDHP exhibited promising results against advanced NSCLC [20, 21]. According to these results, combination therapy of S-1 and a platinum drug is one of the important options in lung cancer treatment. Furthermore, in respect to second-line treatment, it was shown that S-1 is equally as efficacious as docetaxel and offers a treatment option for patients with previously treated advanced NSCLC in the latest East Asia S-1 Trial in Lung cancer [22]. Now, anti-PD-1/PD-L1 therapy is the new standard second-line therapy for patients who have failed first-line doublet chemotherapy [23–26], but not all patients are eligible or able to afford the novel but costly immunotherapies. Single-agent cytotoxic chemotherapy still remains one of the options for second-line treatment, and S-1 is an oral alternative to standard docetaxel. In these clinical trials, carboplatin and S-1 showed a survival advantage especially for squamous cell carcinoma patients [27]. This may be a reflection of the fact that DPD activity is lower in squamous cell carcinoma than in adenocarcinoma [28]. DPD was also highly expressed on epithelial mesenchymal transition (EMT) induction and was necessary for cells to acquire mesenchymal characteristics [29]. EMT cells acquire traits associated with high-grade malignancy, including resistance to apoptosis and chemotherapy, dedifferentiation, and invasiveness, which can lead to metastatic dissemination from primary tumors [30]. These results also support the view that DPD is most important as a sensitivity determinant factor of 5-FU in NSCLC.

In contrast, the previous reports showed that TS, similar to DPD, is also a predictive biomarker for 5-FU and carboplatin plus S-1 therapy [21, 27]. We also showed that TS expression was increased in PEM-resistant cells and that the resistant cells showed cross resistance to 5-FU [31]. These results indicate that TS activity is also a determinant factor for TS-targeting drugs such as 5-FU and PEM. Because DIFs inhibit only DPD, TS activity may also be important for the efficacy of S-1 treatment. Further analysis of DPD, ABCC11, and TS activity in clinical samples of NSCLC patients treated with S-1 is required. While there are no predictive biomarkers for the use of DIFs, the focus in the present study was on ABCC11, because ABCC11 is directly involved in 5-FU resistance by the efflux transport of its active metabolite FdUMP [12].

At first, we thought that any one or all of TS, DPD, or ABCC11 may be a determinant factor of the pharmacokinetics/pharmacodynamics of 5-FU. A genome-wide association study or next-generation sequencer would be suitable to examine this, and there may be other important polymorphisms or mutations in other genes. However, no other biomarker for 5-FU other than DPD, TS, and ABCC11 has been reported to date, so this study focused on these genes. Therefore, the relationships between the expression levels of these genes and the IC_{50} for 5-FU were checked in cell lines, and it was found that DPD expression was strongly correlated with the IC_{50} of 5-FU. However, this had already been known, and DPD was suppressed by CDHP as part of S-1 in clinical lung cancer treatment, so an attempt was made to identify the biomarker for S-1 to make it available for clinical use. Based on these reasons, an in vitro experiment under DPD suppression was then performed. Nonetheless, ABCC11 expression was not related with the IC_{50} in NSCLC cells treated with 5-FU plus CDHP. ABCC11 has a functional SNP (538G > A), and we previously showed that this *ABCC11* SNP is important as a sensitivity determinant factor for PEM, which is also a substrate for ABCC11 [11]. We, therefore, classified the NSCLC cell lines into two groups based on the phenotype of SNP538 (G > A) in ABCC11 (a combined G/G and G/A group and an A/A group) and examined the IC_{50} for NSCLC cell lines treated with 5-FU plus CDHP. We found that the IC_{50} for cells treated with 5-FU plus CDHP was significantly different between the A/A group and the combined G/G and G/A group. From these results, it was thought that not *ABCC11* gene expression, but the SNP538 of *ABCC11* is important as a determinant of sensitivity for 5-FU plus CDHP.

Next, ABCC11-538G or ABCC11-538A-transfected cells were established. In these transfected cells, mRNA expression levels were not different between Hela/ABCC11-G and HeLa/ABCC11-A cells, whereas the protein levels differed between these transfected cell lines. It has already been reported that the expression level of protein corresponding to SNP538A genotype of ABCC11 is much lower than that corresponding to the SNP538G genotype, because variant protein that lacks N-linked glycosylation is recognized as misfolded protein in the endoplasmic reticulum and readily undergoes ubiquitination and proteasomal degradation [32]. Therefore, only normal protein is synthesized by mRNA of SNP538G, but misfolded protein by mRNA of SNP538A is not synthesized. Finally, we confirmed that ABCC11-538G or ABCC11-538A determine the sensitivity for 5-FU plus CDHP by changing the efflux of anti-cancer agents. These results were similar to previous results, showing that the SNP (538G > A) in the *ABCC11* gene is an important determinant of PEM sensitivity [11]. Based on these basic findings, the efficacy of S-1 treatment according to the *ABCC11* SNP genotype of NSCLC patients was also examined clinically, and it was found that the DCR for the A/A group was significantly better than that for the G/G and G/A group. The SNP538 in the *ABCC11* gene is known to determine the characteristics of human earwax type and axillary osmidrosis [14, 33, 34], which show geographical gradient distributions. For example, the dry type is seen frequently (80–95%) among East Asians, but is uncommon (0–3%) among Europeans and people of African origin, and it is observed at intermediate (30–50%) frequencies in native North Americans. It is easy to check the genotype of *ABCC11,* because it can be analyzed using a blood sample; therefore, the SNP538 in *ABCC11* may be considered an important and useful biomarker not only for PEM, but also for S-1 treatment.

There are several limitations to our study. Tsuchiya et al. have already reported that *ABCC11* SNP538 A/A patients had significantly shorter relapse-free survivals than 538G/G plus G/A patients in patients with curatively resected pathological stage IB-to-IIIA NSCLC who were treated with S-1 postoperatively [35]. This result was the opposite of the present one. Although a definitive reason for the differences in the results between these two reports has yet to be clarified, the report by Tsuchiya et al. differed in many ways. For example, the patients in their report received S-1 chemotherapy as adjuvant therapy after complete resection surgery, whereas the present patients had advanced or recurrent NSCLC. In our view, ABCC11 has two important roles in cancer patients: one is drug efflux in the cancer environment and the other is drug metabolism. The ABCC11 role in the patients reported by Tsuchiya et al. may be intensely focused on drug efflux in drug metabolism, because the patients were all post resection of cancer, whereas the present patients had advanced lung cancer, and both roles may have been involved. Thus, our hypothesis would have to be proven by checking the PK/PD and the distribution of S-1 to each organ in the patients in each of the trials. The patients treated with S-1 in the present study were mainly second-line or later setting, because the other anti-cancer drugs tended to be administered as first-line treatment prior to the use of S-1, making it difficult to analyze only patients administered S-1 as first-line treatment. Furthermore, a significant difference was only shown in the DCR. Nevertheless, the present study is valuable, because S-1 is usually used in second-line or later chemotherapy for relapsed NSCLC and is expected to bring about disease stabilization rather than tumor shrinkage. According to Hotta et al., in the second-line or later setting with advanced NSCLC treatment involving systemic chemotherapy, the median MST (6.6 months) was not associated with the overall response rate (ORR; proportion of CR plus PR to all patients) (6.8%), but was associated with the DCR (42.4%) in an analysis of 4318 randomized patients in 19 phase III trials [36]. This suggests that not the ORR but the DCR might be a good candidate as a surrogate marker for anti-cancer agents such as S-1. The clinical study in this report also had a small sample size and was retrospective. The pharmacokinetic parameters of 5-FU and CDHP may also differ between the patient groups. We have been planning to conduct a prospective study to confirm our hypothesis.

In conclusion, our results indicate that the SNP538 in *ABCC11* may be a candidate biomarker for S-1 treatment of NSCLC. S-1 and a platinum drug combination is now used as a first-line treatment for NSCLC only in Japan; therefore, prospective clinical trials are required to determine whether *ABCC11* genotype is a candidate biomarker for S-1 treatment.

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Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

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