Tohoku J. Exp. Med., 2016, **240**, 295-301

ABCB1 Is Upregulated in Acquisition of Taxane Resistance: Lessons from Esophageal Squamous Cell Carcinoma Cell Lines

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Esophageal cancer is one of the common malignancies worldwide, particularly in eastern African and Asian countries including Japan. Taxane (paclitaxel or docetaxel) is one of the effective chemotherapeutic reagents for patients with esophageal cancer, but acquisition of chemoresistance frequently occurs; this is one of the most frequent causes for therapeutic failure. In this study, we established three taxane resistant esophageal squamous cell carcinoma cell lines and explored possible mechanisms for the acquisition of chemoresistance. Microarray analyses indicated that the *ABCB1* (ATP binding cassette subfamily B member 1) gene was significantly upregulated in taxane resistant esophageal cancer cell lines. Moreover, we found that siRNA mediated *ABCB1* knockdown successfully restored drug sensitivity in both paclitaxel and docetaxel resistant esophageal cancer cell lines. In conclusion, we propose that ABCB1 might play a pivotal role in acquisition of taxane resistance and could be a promising target for treatment of patients with esophageal cancer after acquisition of taxane resistance.

Keywords: *ABCB1*; chemoresistance; docetaxel; esophageal squamous cell carcinoma; paclitaxel
Tohoku J. Exp. Med., 2016 December, **240** (4), 295-301. © 2016 Tohoku University Medical Press

Introduction

Esophageal cancer is one of the most common malignancies worldwide. In Japan, it is the sixth leading cause of cancer-related mortality with the percentage of four times higher in males than females (Ferlay et al. 2015). Esophageal cancer has two major histological types; esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) (Napier et al. 2014). ESCC is the predominant type in eastern African and Asian countries including Japan (Matsuda et al. 2014); incidence of esophageal cancer among Japanese population could be resulted from synergistic effect of genetic, food and preference factors including alcohol and smoking (Sakata et al. 2005; Sawada et al. 2016).

According to the recent guidelines proposed by Japan Esophageal Society, combination of neoadjuvant chemotherapy and radical surgery was positioned as a standard treatment for resectable esophageal cancer among Japanese patients (Kuwano et al. 2015), meanwhile the use of chemotherapy as a single treatment is applied to patients with unresectable tumor (Ilson 2008; Kuwano et al. 2015). Taxanes such as paclitaxel and docetaxel are generally administered as a second-line combination therapy with other chemotherapeutic agents, most commonly cisplatin (Ilson 2008; Jimenez et al. 2011; Thallinger et al. 2011). Both paclitaxel and docetaxel share similar chemical structure and mechanism of actions by binding to β-subunit of microtubule and disrupt spindle microtubule dynamics, eventually inhibit microtubule de-polymerization. Those events lead to cell cycle arrest and cell apoptosis (Ringel and Horwitz 1991; Gligorov and Lotz 2004; Jordan and Wilson 2004).

Despite their therapeutic potentials, acquisition of resistance against paclitaxel and docetaxel in esophageal cancer has been observed in a number of patients (Rohatgi et al. 2005; Izzo et al. 2006; Piro et al. 2015). Therefore, in order to elucidate potential mechanisms contributing to acquisition of taxane resistance in esophageal cancer, we first established taxane resistant cell lines derived from human esophageal cancer cell lines and conducted comprehensive expression analyses to find key molecules for taxane resistance.

Received September 21, 2016; revised and accepted October 31, 2016. Published online December 9, 2016; doi: 10.1620/tjem.240.295.
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Subjects and Methods

Establishment of taxane resistant esophageal cancer cell lines

Esophageal cancer cell lines TE1 and TE2 were used as the starting materials (Nishihira et al. 1979). We examined specific *TP53* mutations of these cells by direct sequencing to confirm the cell origins before starting the experiments; results are shown in Table 1 (Flaman et al. 1995; Jia et al. 1997), and primers used in this study are listed in Table 2.

TE1- and TE2-derived taxane resistant cell lines were established in this study by the methods described previously (Ogawa et al. 2010; Saiki et al. 2012). In brief, each individual cell line at the subconfluent density was exposed to either paclitaxel or docetaxel at the concentration for respective IC50 for more than one month. When surviving cell colonies were observed, concentration of each drug was gradually increased in stepwise manner. Each taxane-resistant cell line was grown without taxane for more than one month and then exposed to taxane again to confirm the acquisition of resistance. Established cell lines were then named with R (Resistant) as the prefix, and D or P as the suffix for docetaxel and paclitaxel, respectively.

Cell proliferation assay

A total of 5 × 10^3 cells were seeded in each well of flat-bottomed 24-well plates in quadruplicate and cultured in 1 ml of medium with the different concentrations of taxanes as indicated. Cultures were then allowed to proceed for the indicated time. At the end point, medium was replaced with 500 μl of 5% alamarBlue (Thermo Fisher SCIENTIFIC, Waltham, MA), and after 3 hours of incubation, absorption at 590 nm was measured. At least three independent proliferation assays were performed in each experiment.

Microarray analysis

Total RNAs isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) were labeled with Cy-3 using Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA), and each aliquot of 1.65 μg of cRNA was assembled and hybridized to an Agilent Whole Human Genome Microarray (4 × 44K). Microarray slides were scanned with the Agilent G2565BA microarray scanner (Agilent Technologies). Intensity data from microarray images were extracted with Feature Extraction Software 9.5.1 (Agilent Technologies) at the Biomedical Research Core of Tohoku University School of Medicine. These experiments were performed in duplicate. Microarray data are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under the accession number of GSE 86099. Obtained results were analyzed using the Gene Spring software (Silicone Genetics, Redwood City, CA).

Quantitative reverse transcription PCR (qRT-PCR)

Each aliquot of 2 μg total RNA was reverse transcribed to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. mRNA expression levels were determined using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Expression of the *B2M* (β-2 microglobulin) gene was used as the internal control. Amplifications were carried out in 15 μl reaction mixtures in triplicate using ABsolute qPCR Mixes (Thermo Scientific, Waltham, MA) according to manufacturer's recommendations. Expression levels of target genes relative to *B2M* were calculated as follows: 2^{-dCT} (dCT = CT [target] − [B2M]).

Knockdown of ABCB1 in resistant cell lines

Each aliquot of 5 × 10^5 cells of a resistant cell line was cultured

Table 1. Mutation status of the *TP53* gene in cell lines used in this study.

Cell line	Mutation
TE1	V272M (exon 8)
RTE1D	V272M (exon 8)
RTE1P	V272M (exon 8)
TE2	c.375+1G>A (splicing donor site of intron 4)
RTE2D	c.375+1G>A (splicing donor site of intron 4)

Table 2. Oligonucleotide sequences for PCR, qRT-PCR, and knockdown experiments.

Experiment	Gene	Name	Sequence (5' to 3')
PCR	*TP53*	p53-E4F Primer	TCC TGA AAA CAA CGT TCT GG
		p53-E4R Primer	GCC AGG CAT TGA AGT CTC AT
		p53-E8F Primer	CTT CTC TTT TCC TAT CCT GAG[a]
		p53-E8R Primer	CCT CCA CCG CTT CTT GTC CT[a]
qRT-PCR	*ABCB1*	ABCB1 2807F Primer	CTT CAG GGT TTC ACA TTT GGC
		ABCB1 2941R Primer	GGT AGT CAA TGC TCC AGT GG
		ABCB1 Probe	CCT CAC CAA GCG GCT CCG ATA CA
	B2M	B2M-common F Primer	TTT CAG CAA GGA CTG GTC TTT[b]
		B2M-R3 Primer	CCA AAT GCG GCA TCT TCA AAC[b]
		B2M Probe	CTG AAA AAG ATG AGT ATG CCT GCC GTG TG[b]
knockdown	*ABCB1*	ABCB1 siRNA-S	GGG AUA AAG AAA GCU AUU ACA GCC A
		ABCB1 siRNA-AS	UGG CUG UAA UAG CUU UCU UUA UCC CAA
	GL2	GL2 siRNA-S	CGU ACG CGG AAU ACU UCG AAA UGT C[c]
		GL2 siRNA-AS	GAC AUU UCG AAG UAU UCC GCG UAC GUG[c]

Some nucletides were reported as followings: [a]Cuny et al. (2000), [b]Ogawa et al. (2006), and [c]Saiki et al. (2012).

in 10 cm dish for 24 h and transfected with siRNA against ATP binding cassette subfamily B member 1 (*ABCB1*) at 16 nM. After 24 h, an aliquot of 5 × 10^3 cells was transferred to each well of flat-bottomed 24-well plates for the proliferation assay. siRNA against the *GL2* (luciferase) gene was used as the negative control (Saiki et al. 2012). These siRNAs were purchased from Integrated DNA Technologies (Coralville, IA). Efficiencies of *ABCB1* knockdown were confirmed by Western blotting analyses.

Western blotting analysis

Cells were harvested and lysed in a buffer consisting of 65 mM Tris-HCl (pH 6.8) and 3% sodium dodecyl sulfate. Protein concentrations of the cell lysates were measured using the DC protein assay kit (Bio-Rad, Hercules, CA). Each aliquot (40 μg protein) was subjected to immunoblotting using a 5-20% polyacrylamide gradient gel (Wako, Osaka, Japan). Antibodies used were rabbit anti-ABCB1 polyclonal antibody (Abcam, Cambridge, UK) and mouse anti-β-actin monoclonal antibody (Sigma, St Louis, MO). We also used anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Amersham Biosciences Corp., Piscataway, NJ). After blocking with 5% skim milk in phosphate-buffered saline containing Tween 20, membranes were incubated over night with respective primary antibody at 4°C and then washed with PBS and incubated with relevant secondary antibody for 60 min at room temperature. Signals were visualized by reaction with ECL Western Blotting Detection System (GE Healthcare, Uppsala, Sweden) and digitally processed using Image Quant LAS 4000 (Fuji Photo Film, Minamiashigara, Japan).

Statistical analyses

All experiments were performed in triplicates or more. A two-tailed Student's *t*-test was used for statistical analyses of comparative data using Microsoft Excel software (Microsoft Corporation, Redmond, WA). Values of $P < 0.05$ were considered as significant.

Results

Establishment of taxane resistant cells

A total of three taxane resistant cell lines, two originated from TE1, RTE1P (paclitaxel resistant) and RTE1D (docetaxel resistant), and one originated from TE2, RTE2D (docetaxel resistant), were generated. Mutation status for the TP53 gene was analyzed to confirm that these resistant cell lines are really originated from respective parental cell lines (see Table 1). After culturing with drug-free medium for 4 weeks, sensitivities to taxanes were measured at the 3 doubling time (3DT) point. As shown in Fig. 1A, proliferations of taxane-resistant esophageal cancer cell lines were

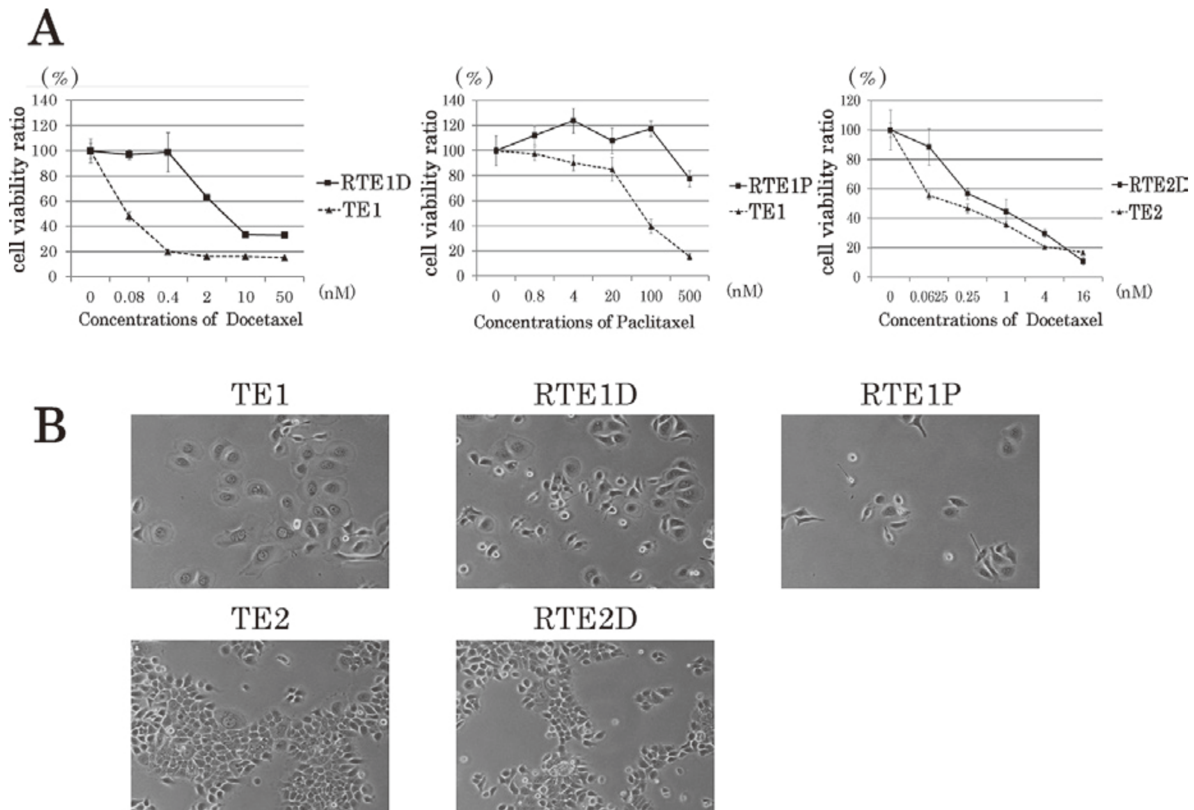

Fig. 1. Establishment and morphological analyses of three taxane resistant esophageal cancer cell lines.
(A) Cell viabilities at the 3 doubling time (3DT) after addition of different concentration of taxanes are shown. Cell viabilities of TE1 and RTE1P 93 h after paclitaxel addition (left), TE1 and RTE1D 93 h after docetaxel addition (center), and TE2 and RTE2D 84 h after docetaxel addition (right) are shown. These experiments were performed in quadruplicate. Data are presented as mean ± S.D.
(B) Morphological appearance of parental and taxane resistant esophageal cancer cell lines. TE1 and TE2 are parental cell lines, and RTE1P, RTE1D and RTE2D are established taxane resistant cell lines. (magnification ×200 for TE1, RTE1D, and RTE1P, and ×100 for TE2 and RTE2D).

Table 3. Comprehensive expression analysis of taxane resistant esophageal cancer cells.

Gene symbol	Gene name	TE1*	RTE1P*	Fold change
ABCB1	ATP binding cassette subfamily B member 1	39.3	2223.5	5.7×10^{1}
SEPP1	selenoprotein P, plasma, 1	N/D	215.9	N/A
SPDYE1	speedy/RINGO cell cycle regulator family member E1	2.3	184.6	8.0×10^{1}
CLIC5	chloride intracellular channel 5	14.8	504.6	3.4×10^{1}
SRGN	serglycin	3931.6	138.8	3.5×10^{-2}
HEATR7B1	HEAT repeat containing 7B1	548.8	22.0	4.0×10^{-2}
COL4A6	collagen type IV alpha 6 chain	744.5	32.6	4.4×10^{-2}
LAPTM5	lysosomal protein transmembrane 5	153.0	5.8	3.8×10^{-2}
SCD	stearoyl-CoA desaturase	116.4	2867.2	2.5×10^{1}
SESN3	sestrin 3	4.0	132.7	3.3×10^{1}

*Average date are shown; N/D, not detectable; N/A, not applicable.

suppressed only at higher doses of taxanes than the respective parental cells. These results demonstrate that taxane-resistance is sustainable. Morphologic features of the established resistant cell lines as well as those of the parental cell lines are shown in Fig. 1B; there are no prominent differences between parental and established resistant cell lines.

Comprehensive expression analysis

Using a set of parental and resistant cell lines TE1 and RTE1P as the representatives, we studied comprehensive mRNA expression analyses by microarray in order to find any of the key molecules associated with development of taxane resistance. Ten genes in which expression levels were significantly up- or down-regulated in RTE1P than its parental TE1 cells were picked-up as shown in Table 3. Among these 10 selected genes, we further analyzed *ABCB1* because this molecule was significantly upregulated in a paclitaxel resistant esophageal cancer cell line (Wang et al. 2013). mRNA expression was determined by qRT-PCR, and results were shown in Fig. 2A; expression increased nearly 2 orders in magnitude in TE1-derived resistant cells, and significant upregulation was also evident in TE2-derived docetaxel resistant cells.

ABCB1 expression in taxane resistant cell lines

To investigate ABCB1 protein expression in parental and taxane resistant cells, we performed Western blotting analyses. As shown in Fig. 2B, the ABCB1 protein are markedly increased in all the three taxane resistant cell lines when compared to respective parental cell lines; these results suggested that increased expression of ABCB1 is a common feature to acquisition of taxane resistance in esophageal cancer cells.

Knockdown of ABCB1

To determine the importance of *ABCB1* expression in the acquisition of taxane resistance, we attempted siRNA-mediated knockdown of *ABCB1* using resistant cell lines. Efficient knockdown of ABCB1 was evident as shown in Fig. 3A. Then we investigated effects of siRNA-mediated knockdown of *ABCB1* to taxane resistance. Cell viability was assessed at the scheduled time point as shown in Fig. 3B in the upper column, and the cell survival curves are

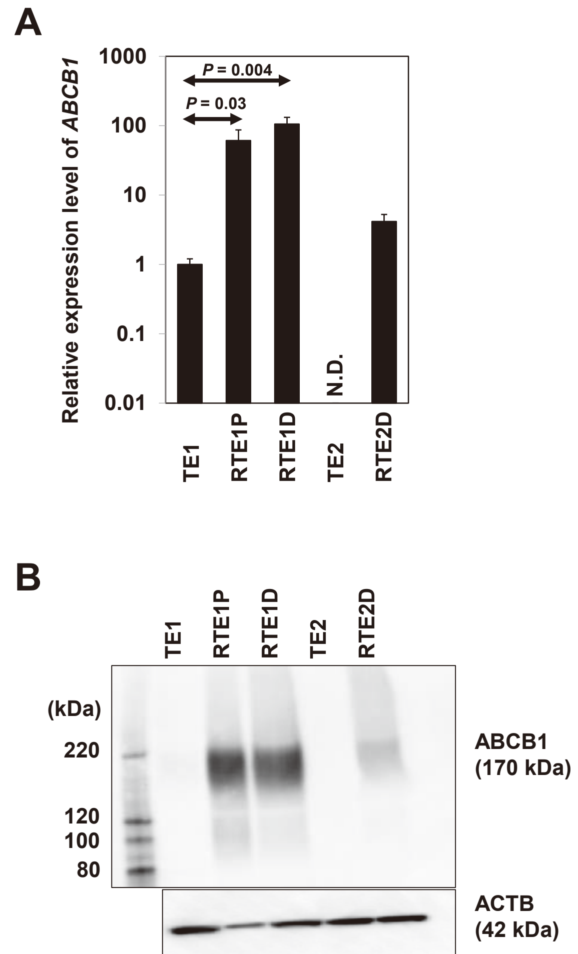

Fig. 2. Results of expressional analyses of ABCB1 in analyzed cancer cells.
(A) Expression levels of *ABCB1* mRNA were determined by qRT-PCR. Data are presented as the mean ±S.D. These results represent that the expressions of *ABCB1* in taxane resistant cell lines were significantly increased; RTE1P and RTE1D are nearly two orders in magnitude higher than that of parental cell line (TE1), and RTE2D showed expression of ABCB1 whereas that of TE2 was not detectable (N.D.).
(B) ABCB1 protein expression levels in parental cell lines and taxane resistant cell lines were determined by Western blotting analyses. Parental cell lines TE1 and TE2 did not show ABCB1 expression at the detectable level. However, all the established resistant cell lines did express ABCB1. Expression levels of beta-actin (ACTB) were monitored as the control.

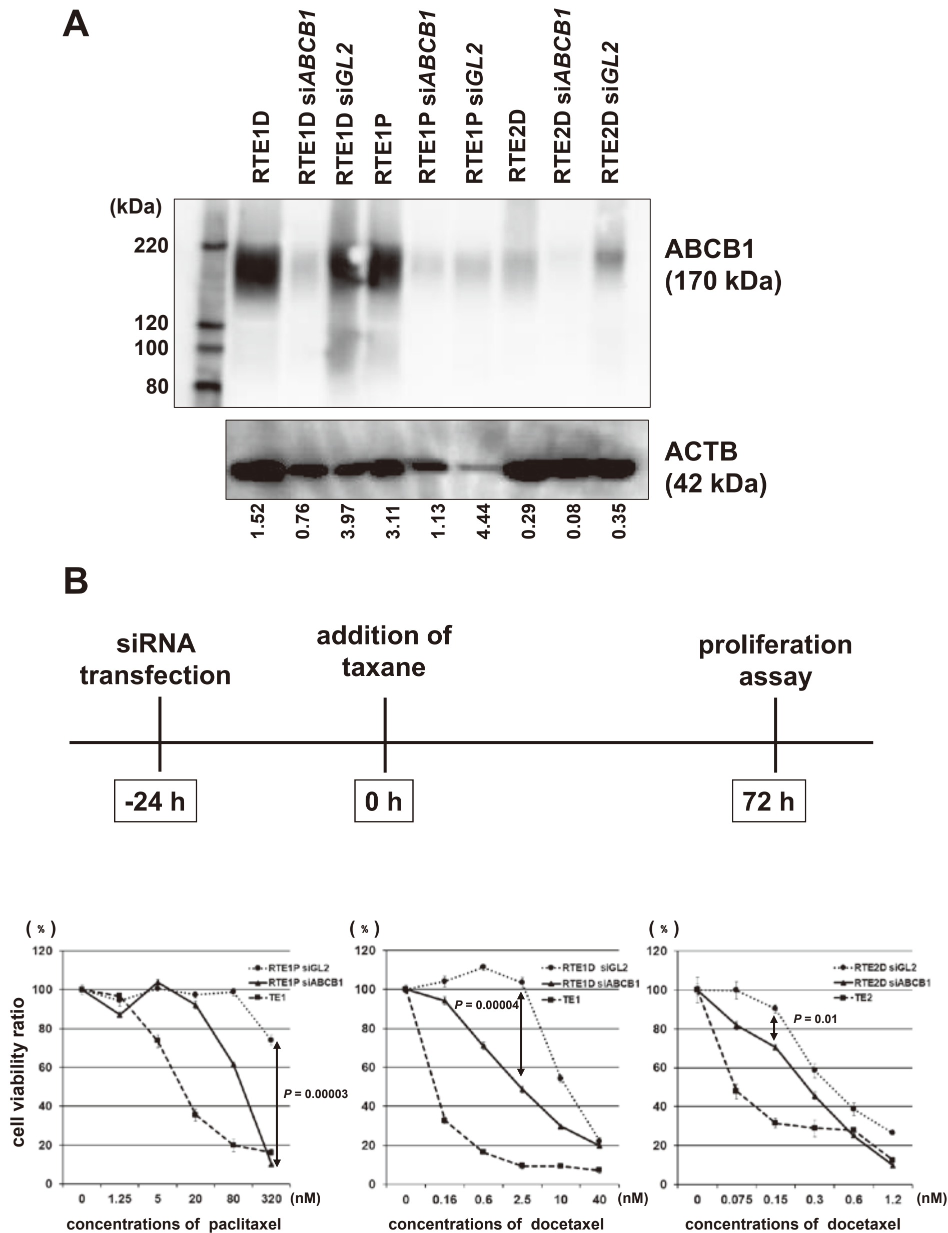

Fig. 3. siRNA-mediated knockdown of *ABCB1* causes loss of taxane resistance.
(A) Results of Western blotting analyses of the ABCB1 protein after siRNA-mediated knockdown against *ABCB1* in esophageal cancer cell lines are shown. siRNA against the luciferase gene *GL2* was monitored as the negative control. Expression levels of beta-actin (ACTB) were monitored as the control, and ABCB1/ACTB ratio from the densitometry analysis is shown in the lower panel.
(B) Taxane sensitivities of resistant cell lines after siRNA-treatment against *ABCB1* are shown. Schedule of knockdown experiments are summarized at the upper column. A total of 5×10^5 cells were transfected with siRNA against *ABCB1* under the concentration of 16 nM at the time point of 24 h previous (−24 h) from taxane addition. At the time point of 0 h, 5×10^3 cells were transferred to each well of 24-well plates for the proliferation assay, and each concentration of taxane was added to each cell line. Cell proliferation levels were analyzed at the time point of 72 h. Cell viabilities are summarized at the lower column; those of TE1 and RTE1P at 72 h after Paclitaxel addition (left), TE1 and RTE1D at 72 h after Docetaxel addition (center), and TE2 and RTE2D at 72 h after Docetaxel addition (right). These experiments were performed in quadruplicate, and data are presented as the mean ± S.D. *P* values between cell viability ratios after si*GL2*- and si*ABCB1*-mediated knockdown (indicated by arrows) are also shown.

shown in the lower column; siRNA-mediated knockdown of *ABCB1* in all the resistant cell lines significantly resumed taxane sensitivity.

Discussion

We have established one paclitaxel resistant and two docetaxel resistant human esophageal cancer cell lines.

Several taxane resistant cell lines have been generated in lung, ovarian, prostatic, breast, and gastric cancers (Chu et al. 2000 ; Okugawa et al. 2004; Duan et al. 2005; Takeda et al. 2007; Zhang et al. 2010). However, establishment of esophageal cancer is limited; only a single study has been reported (Wang et al. 2013).

By comprehensive expression analyses, we found that the expression of *ABCB1* was significantly upregulated in taxane resistant esophageal cancer cell line, RTE1P, when compared to parental cell line, TE1. The ABCB1 protein is a member of the ATP binding cassette transporter proteins that is located on chromosome 7q21 and participate in the elimination of various substances by active transportation (Scotto 2003; Katayama et al. 2014). Detailed characterizations for overexpression of *ABCB1* in these resistant cell lines are still elusive.

Several studies have reported that *ABCB1* expression is significantly upregulated in drug resistant cancers and associated with chemotherapeutic failure (Choi 2005; Holohan et al. 2013; Katayama et al. 2014). Wang et al. (2013) established a paclitaxel resistant esophageal cancer cell line and demonstrated the overexpression of *ABCB1*. Our study is the first one to investigate thoroughly and confirm more concrete involvement of *ABCB1* in acquiring taxane resistance. We demonstrated that siRNA mediated *ABCB1* knockdown successfully restored drug sensitivity in all the three established taxane resistant esophageal cancer cell lines; these results indicate that (1) increased expression of ABCB1 plays a crucial role for acquisition of taxane resistance, and (2) there can be possible applications for patients with esophageal cancer who acquired taxane resistance.

Taken together, we propose that ABCB1 might play a pivotal role in acquisition of taxane resistance and could be a promising target for esophageal cancer treatment. Nevertheless, further studies are needed to investigate more detailed mechanisms responsible for taxane resistance using clinical samples.

Acknowledgments

We are grateful to Dr. B.L.S. Pierce (University of Maryland University College) for editorial work in the preparation of this manuscript and to Biomedical Research Core (Tohoku University School of Medicine) for technical support. This work was supported in part by Grant-in-Aid from JSPS KAKENHI Grant Numbers JP15K10084, 26460468, 26462074.

Conflict of Interest

The authors declare no conflict of interest.

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