Expression of ABCB6 Is Related to Resistance to 5-FU, SN-38 and Vincristine

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Abstract. *A previously established arsenite-resistant cell line, KAS, is also resistant to a variety of anticancer drugs. In order to understand responsible molecules for the multidrug resistance phenotype of KAS cells, we examined the expressions of ATP-binding cassette (ABC) transporters and found that the ABCB6 and ABCC1/ multidrug resistance protein 1 (ABCC1/MRP1) were increased. ABCC1/MRP1 was not completely responsible for the drug resistance spectrum of KAS cells and several reports have suggested that ABCB6 is related to anticancer drug and metal resistance. We, therefore, established and examined ABCB6-expressing KB cells and ABCB6-knockdown KAS cells. ABCB6 expression enhanced resistance to 5-fluorouracil (5-FU), SN-38 and vincristine (Vcr) but not to arsenite. Conversely, down-regulation of ABCB6 in KAS cells increased the sensitivity of KAS cells to 5-FU, SN-38 and Vcr, but not to arsenite. Our findings suggest that ABCB6 is involved in 5-FU, SN-38 and Vcr resistance.*

Arsenite is an enviromental polutant related to cancers, endocrine and neuropsychological disorders (1-4). Arsenic trioxide (As_2O_3) is a therapeutic agent for acute promyelocytic leukemia (PML) (5, 6). As_2O_3 has been reported to have pro-apoptotic effects of *in vivo* and also *in vitro* esophageal carcinoma, ovarian carcinoma and prostate carcinoma cells (7, 8) and is a promising agent for the treatment of solid cancers. However, arsenite resistance has been reported following the treatment (9). To understand the molecular basis of arsenite toxicity and resistance, we established arsenite-resistant human epiermoid carcinoma KB-3-1 cells, termed KAS (10). KAS cells are highly resistant not only to sodium arsenite but also cis-diamminedichloroplatinum (II) (CDDP), antimony potassium tartrate (antimony) and doxorubicin (Dox). Although increased expression of multidrug resistance protein 1 (ABCC1/MRP1) and high level of glutathione (GSH) in KAS cells were involved in the aforementioned type of resistance, these molecules were not fully-responsible for the resistance of KAS cells (10).

Some members of the ATP-binding cassette (ABC) transporter superfamily function as ATP-dependent active transporters of a broad variety of substrates including metals and drugs.

To know attribution of ABC transporters to metal and drug resistance in KAS cell, we examined and analyzed ABC transporter expression.

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Key Words: ABC transporters, ABCB6, anticancer agents, drug resistance, arsenite.

Materials and Methods

Chemicals and antibodies. Sodium arsenite (arsenite) and antimony potassium tartrate (antimony) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). cis-diamminedichloroplatinum(II) (CDDP), 5-Fluorouracil (5-FU), doxorubicin (Dox), cyclophosphamide (CPA), vincristine (Vcr) and paclitaxel (Taxol) were purchased from Sigma-Aldrich Life Science (St. Louis, MO, USA). 7-ethyl-10-hydroxy-camptothecin (SN-38) was kindly donated by Yakult Honsha Co., Ltd. (Tokyo, Japan) and Daiichi-Sankyo Co., Ltd. (Tokyo, Japan).

We used the anti-ABCB6 rabbit antibody from Rockland Immunochemicals, Inc. (Gilbertsville, PA, USA) and the MRPm6 antibody against ABCC1/MRP1 from Abcam (Cambridge, UK).

Cell lines and cell culture. Arsenite-resistant KAS cells were isolated from KB-3-1 cells, as described previously (10). KB-3-1, KAS, KB-B6N8 and KASKD35 cells were cultured in Dulbecco's modified Eagle's medium (DMED) (Nissui Seiyaku Co., Tokyo, Japan) containing 10% calf serum, under 5% CO_2 at 37˚C. KM12C cells and their 5-FU resistant subline, KM12C/5-FU cells, were cultures in DMED with 10% fetal calf serum (11).

RT-PCR analysis. Total RNA was extracted using TRIzol (Life technologies, Carlsbad, California, USA). For RT-PCR, 1 μg of total RNA was used as a template for cDNA synthesis with ReverTra Ace-α (Toyobo, Japan), according to the manufacturer's protocol. Expression of *ABCC1/MRP1*, *ABCC2/MRP2* and *ABCB6* genes was detected using reverse transcription - polymerase chain reaction (RT-PCR). The forward (F) and reverse (R) sequences of the primer sets used for this analysis were as follows: ABCC1/MRP1: F 5'-GCTTCTCTGGCCAAAGTCTG-3', R 5'-GCCACATGTCAGC ATACACC-3', ABCC2/MRP2: F 5'-CGGAAACCATCCACG ACCCTA-3', R 5'-ACCTCCTCATTCGCATCCACC-3', ABCB6: F 5'-GCTTCTCTGGCCAAAGTCTG-3', R 5'-GCCACATGTCAGC ATACACC-3'.

Quantitative real-time PCR. Real-time PCR assays were performed using an ABI PRISM 7900HT (Applied Biosystems Japan Ltd., Tokyo, Japan) and the Taqman PCR method. The probe was labeled with FAM (6-carboxyfluorescein) at the 5' end and with TAMRA (6-carboxy-tetramethylrhodamine) at the 3' end. Relative quantification of *GAPDH, ABCC1/MRP1, ABCC2/MRP2* and *ABCB6* was calculated using the comparative cycle threshold method. The thermal cycling conditions comprised an initial incubation at 50˚C, Ampli Taq Gold Activation at 95˚C for 15 s, then annealing and extension at 60˚C for 1 min. Experiments were performed in duplicate for each data point. Each sample was quantified using the standard curve method. The sequences of the primer/probe sets used for this analysis were as follows: ABCB6: F 5'-TGAAAGAGGAGACAGAAGTGAAGGA-3', R 5'-AAAC TCAATACGGCCCTTCTGA-3', P 5'-FAM-CTGGAG CAGGGCCCCTTCCGCT-TAMRA-3'; ABCC1/MRP1: F 5'-AGGCG AGTGTCTCCCTCAAA-3', R 5'-TCCTCACGGTGATGCTGTTC-3', P 5'-FAM-TGACAGCATCGAGCGACGGCC-TAMRA-3'; ABCC2/MRP2: F 5'-GAAGACGATGACTATGGGCTGATAT-3', R 5'-GTG TTCGACGAAAGCTGTTCTCT-3', P 5'-FAM-AGAGATCCCCGA AGATGCAGCCTCC-TAMRA-3'.

ABCB6 cDNA transfection. Human full length *ABCB6* cDNA was kindly provided by Prof. S. Seino, Kobe University. This cDNA was inserted into p3xFLAG-CMV-14 (Sigma-Aldrich Life Science, St. Louis, MO, USA) and transfected into KB-3-1 cells. After selection with G-418, a FLAG-tagged ABCB6 expressing clone, KB-B6N8, was used for further analysis.

ABCB6 knockdown in KAS cells. shRNA expression plasmids against *ABCB6* mRNA were purchased from Sigma-Aldrich life Sciences (NM_005689.1_1109s1c1). KAS cells were transfected with the plasmid and selected with puromycin. KAS-KD35 cells, whose ABCB6 protein expression was decreased, was used for analysis.

Isolation of membrane fractions. Membrane fractions were prepared using a nitrogen cavitation method, as described previously (12). Protein concentrations of the membrane fractions were determined and the fractions were stored at –80˚C prior to use.

Immunoblotting. Immunoblotting was performed as described elsewhere using enhanced chemiluminescence ECL plus Western blotting detection system (GE Healthcare, Buckinghamshire, UK) (12). The blotted membranes were immediately exposed to X-ray film or were analyzed using the Chemi Doc XRS System for various periods. The intensities of fluorescent bands of ABCB6 or ABCC1 were quantified using the Chemi Doc XRS System and Quantity One 1D Analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

Cell survival and chemosensitivity assay. Chemosensitivity was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay as described previously in triplicate using 96-well plates with 3×10^3 each kind of cells *per* well (12).

Statistical analysis. Differences between groups were analyzed by the Student's *t*-test. A *p*-value of <0.05 was considered significant.

Results

Profiling of the ABC transporter gene expression in arsenite-resistant cells. To investigate the responsible molecules for the acquired arsenite and drug resistance in KAS cells, we examined 40 ABC transporter genes expression profiles of the parental and resistant cells with RT-PCR.

RT-PCR analysis indicated increased expression of *ABCC1/MRP1* and *ABCB6* in KAS cells, while the expression of *ABCC2/MRP2* was found to be similar in the resistant and parental cells (Figure 1A). Using real-time PCR assays, the expression levels of *ABCC1/MRP1* and *ABCB6* mRNA in KAS cells were 2.5- and 2.4-fold higher than in KB-3-1 cells, respectively (Figure 1B).

Since we previously showed that the expression of *ABCC1/MRP1* in KB-3-1 cells did not fully reconstitute the resistance phenotype (13), and previous reports suggested that ABCB6 expression is related to resistance to several drugs and metals (14-16), we focused on the potential role of ABCB6 as a cause of metal and drug resistance in KAS cells.

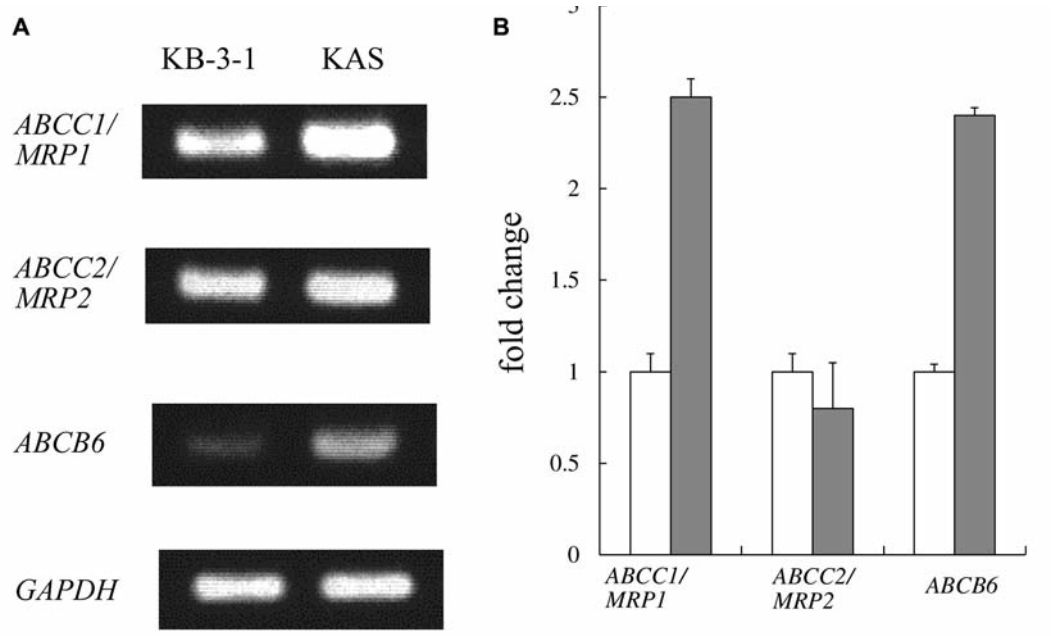

Figure 1. *Expression of ABCC1/MRP1, ABCC2/MRP2 and ABCB6 in KB-3-1 and KAS cells. The relative levels of ABCC1/MRP1, ABCC2/MRP2 and ABCB6 mRNA in KB-3-1 and KAS cells were determined using PCR (A) and real-time PCR (B). GAPDH mRNA was used as a loading control in A. The open and grey bars in B indicate KB-3-1 and KAS cells respectively. The level of ABCC1/MRP1 and ABCB6 mRNA significantly increased in KAS cells compared to KB-3-1 cells ($p<0.01$).*

Expression of ABCB6 in cells transfected with ABCB6 cDNA and in KAS cells. To determine if expression of ABCB6 was responsible for conferring metal and drug resistance to KAS cells, we established KB-B6N8 cells stably-expressing FLAG-tagged human *ABCB6* and KAS-KD35 cells, by knockdown of *ABCB6* in KAS cells.

Expression of the ABCB6 protein in KB-B6N8 cells was about 20-times higher than that in KAS cells whereas expression of the ABCB6 protein in KAS-KD35 cells was one-fifth of that in KAS cells. In contrast, expression of the ABCC1/MRP1 protein in KAS-KD35 cells was similar to that in KAS cells (Figure 2).

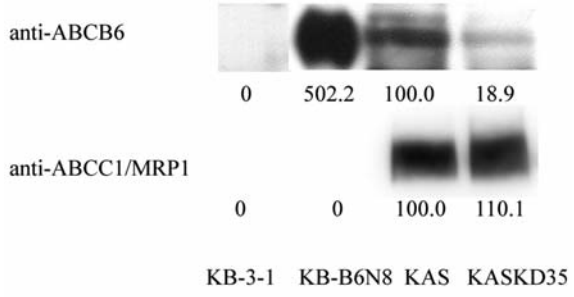

Figure 2. *Expression of ABCB6 and ABCC1/MRP1 in KB-3-1, KB-B6N8, KAS and KASKD35 cells. The membrane fractions of the indicated cells were subjected to immunoblotting with specific antibodies. Protein loading: KB-B6N8 cells, 5 μg; other cells, 20 μg (upper blots); and 50 μg of all cells (lower blots). The expression levels of ABCB6 and ABCC1/MRP1 in the cells are indicated relative to the levels in KAS cells, which were assigned a value of 100.*

Sensitivity of KB cells to metals and anticancer agents. We next examined the sensitivity of KB-3-1, KB-B6N8, KAS and KASKD35 cells to metals and anticancer agents. The present analysis revealed, for the first time, that KAS cells are highly resistant to SN-38, an active form of CPT-11 (Table I). The KB-B6N8 cells were significantly more resistant to 5-FU, SN-38, Vcr and Dox (2.9-, 1.74-, 1.47- and 1.41-fold more resistant, respectively), but not to arsenite, than KB-3-1 cells. In contrast, KASKD35 cells were more sensitive to 5-FU, SN-38 and Vcr (1.6-, 3.0- and 1.6-fold more sensitive respectively), but not to Dox or arsenite, than KAS cells (Table I). These results imply that ABCB6 is involved in 5-FU, SN-38 and Vcr resistance, but not in the arsenite resistance, of KAS cells.

To test the generality of the relation between ABCB6 expression and 5-FU resistance, the expression of ABCB6 in 5-FU resistance KM12C/5-FU cells was compared to that of KM12C cells. The expression of *ABCB6* was up-regulated in 5-FU-resistant cells (Figure 3).

Table I. *Relative resistance against metals and anticancer agents. IC_{50} and relative resistance values to indicated materials of each cell line are showed. The detailed procedures were described in materials and methods.*

Cells	KB	KBB6N8		KAS		KASKD35	
Agent (conc unit)	IC_{50} SD	IC_{50} SD	RR	IC_{50} SD	RR	IC_{50} SD	RR
Arsenite (μM)	13.20±3.80	14.70±2.20	1.10	247.40±3.80[a]	18.70	245.90±5.90	18.60
Antimony (μM)	2.19±0.14	2.57±0.14	1.17	83.68±10.73[a]	38.21	100.15±3.55	45.73
CDDP (μM)	1.24±0.10	0.84±0.03	0.68	8.56±0.57[a]	6.91	9.01±0.64	7.28
CPA (mM)	6.17±0.11	6.20±0.35	1.01	7.39±0.73	1.20	8.67±0.26	1.40
5-FU (μM)	20.50±4.03	61.50±8.75[a]	2.99	43.28±2.99[a]	2.11	26.93±1.17	1.31[b]
Dox (nM)	31.40±6.24	44.30±4.52[a]	1.41	2256.49±79.41[a]	71.86	2136.42±128.50	68.04
SN-38 (nM)	1.65±0.001	2.86±0.079	1.74	7470.99±261.17[a]	4507.55	2482.5±567.3	1497.8[b]
Vcr (nM)	14.37±0.53	21.20±0.74[a]	1.47	1080.51±41.45[a]	74.85	671.52±21.99	46.51[b]
Taxol (nM)	9.20±0.74	6.69±0.52	0.73	40.09±2.20[a]	2.86	36.44±3.66	2.60

IC_{50} values are means±SDs from triplicate determinations with MTT assay. RR: Relative resistance (IC_{50} of the agents for KAS, KB-B6N8 and KASKD35 was divided by the IC_{50} for KB-3-1 cells). a, The value is significantly higher than that of KB cells ($p<0.05$), b, The value is significantly lower than that of KAS cells ($p<0.05$).

Discussion

To investigate the molecular basis of acquired metal resistance, we previously isolated arsenite-resistant KAS cells. KAS cells were also resistant to CDDP, antimony and Dox. Based on previous studies, we concluded that an active efflux pump for arsenite, that is different from ABCB1/P-gp and ABCC1/MRP1, is expressed in KAS cells (10).

Arsenite resistance has been linked to several metabolic changes, which include differential synthesis of heat-shock proteins (17), inorganic arsenic methylation (18), glutathione metabolism (19) and decreased intracellular arsenite accumulation (19-22) with increased arsenite efflux (21) or attenuation of active uptake (22). ABCC1/MRP1 and ABCB1/P-gp have been reported as putative arsenic efflux pumps (19, 20, 23, 24).

ABCC1/MRP1 has been reported to be an arsenite efflux pump that effluxes arsenite in coordination with GSH and glutathione transferase (GST) (19, 24). However, the ABCC1/MRP1-overexpressing cell line C-A120 was only 2.6-fold more resistant to sodium arsenite (13) and ABCC1/MRP1 is not involved in CDDP resistance. These data suggested that ABCC1/MRP1 might not be completely responsible for the phenotype of KAS cells.

ABCC2/*MRP2* was reported to be related to arsenite resistance (25), however *ABCC2*/*MRP2* mRNA in KAS cells was comparable to that in KB-3-1 cells.

Although ABCB6 has been reported to transport coproporphyrin III from the cytoplasm to mitochondria (26), it is dispensable for erythropoiesis from a human genetic study (27). *ABCB6* has also been reported to be a causative gene of several diseases (28-30). ABCB6 shares a high degree of similarity with the yeast proteins *Arabidopsis thaliana* half-molecule ABC transporter (ATM1) and heavy metal tolerance 1 (HMT1). ATM1 plays a role in the biogenesis of iron-sulfur (Fe/S) clusters by transporting a precursor from mitochondria to cytosol (31, 32) and HMT1 confers cadmium resistance through sequestration of cadmium into vacuoles (33, 34). ABCB6 is present at mitochondria (26, 31, 35), the direct target of arsenite (36-38). Thus, ABCB6 appeared to be the best candidate involved in arsenite resistance.

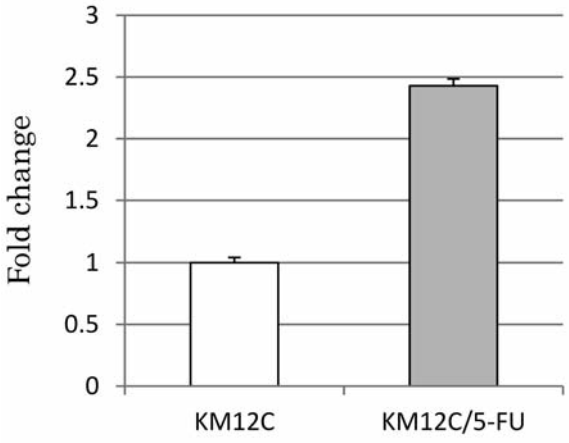

Figure 3. *Expression of ABCB6 in KM12C and KM12C/5-FU. The relative levels of ABCB6 mRNA in KM12C and KM12C/5-FU cells were determined using real-time PCR. The open and grey bars indicate KM12C and KM12C/5-FU cells respectively. The level of ABCB6 mRNA significantly increased in KM12CFU cells compared to KM12C cells (p<0.01).*

However, KB-B6N8 cells, that express high levels of ABCB6, did not exhibit resistance to arsenite. Furthermore,

knockdown of *ABCB6* in KAS cells did not affect their sensitivity to arsenite. These data indicate that ABCB6 is not responsible for the arsenite resistance of KAS cells. However, it is still possible that ABCB6 may play a role in arsenite resistance under certain conditions. It has been reported that ABCB6 confered arsenite resistance to HepG2 and Hep3B cells (39). The discrepancy of our result might be due to cellular background between epidermoid cancer and hepatocarcinoma cells. Arsenite has been reported to induce reactive oxygen species (ROS) production and Lynch recently suggested that ABCB6 plays an indirect role in cell survival under peroxide stress conditions through up-regulation of heme proteins (40). Resistance to arsenite in KAS cells is probably a result of complex cellular responses that involve multiple factors.

Several reports have suggested that ABCB6 is related to resistance against anticancer drugs and metals but not arsenite. The *ABCB6* gene was reported as a gene overexpressed during hepatocarcinogenesis (41). Comparative genomic hybridization (CGH) analysis indicated that the *ABCB6* gene was amplified in CPT-11-resistant A549 cells in comparison with the parental cells (14). It has also been shown that the expression of ABCB6 in anticancer drug-resistant human breast cancer cells after weekly treatment with neoadjuvant chemotherapy with Paclitaxel/FEC (5-fluorouracil, epirubicin and cyclophosphamide) was higher than that in sensitive cells, as assessed by microarray analysis (15). Rat Abcb6-overexpressing cells were resistant to copper (16).

These results suggested that ABCB6 might play important roles in metal and drug resistance. In this study, we examined the effect of ABCB6 expression on cellular metal and drug resistance. ABCB6 may not be solely responsible for the high resistance of KAS cells to SN-38, since SN-38 resistance is likely to be also partly due to the expression of ABCC1/MRP1(42). Regarding the contribution of ABCB6 to Dox resistance, although the ABCB6-overexpressing KB-B6N8 cells were more resistant to Dox than the KB-3-1 cells, the *ABCB6*-knockdown KAS-KD35 cells were as resistant as KAS cells. These inconsistent results could be explained, at least in part, by the effect of MRP1/ABCC1 in KAS cells. Thus, the effect of ABCB6 on Dox resistance might be weak and masked by that of ABCC1/MRP1, which strongly contributes to Dox resistance in KAS cells.

The selection for arsenite resistance activates regulatory systems that turn on expression of multiple stress resistance genes. Some of the genes protect cells against arsenite directly while the others confer resistance to other toxic agents. In turn, the multidrug resistance phenotype of KAS cells seems due to the multiple-gene regulatory system.

In the present study, we found that ABCB6 was overexpressed in KAS cells and demonstrated that the ABCB6 transporter plays an important role in 5-FU, SN-38 and Vcr resistance, but not in the arsenite resistance, of KAS cells. Further studies are required to characterize the unknown transporter that effluxes arsenite from KAS cells and the mechanisms of ABCB6-mediated 5-FU, SN-38 and Vcr resistance.

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