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. Our findings suggest that ABCB6 is involved in 5-FU, SN-38 and Vcr resistance.

Arsenite is an environmental pollutant related to cancers, endocrine and neuropsychological disorders (1-4). Arsenic trioxide (As2O3) is a therapeutic agent for acute promyelocytic leukemia (PML) (5, 6). As2O3 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 epidermoid carcinoma KB-3-1 cells, termed KAS (10). KAS cells are highly resistant not only to sodium arsenite but also cis-diamminedi-chloroplatinum (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.
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-diaminedichloroplatinum(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 MRP6 antibody against ABCCI/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 (DMEM) (Nissui Seiyaku Co., Tokyo, Japan) containing 10% calf serum, under 5% CO2 at 37°C. KM12C cells 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).


ting 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 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 3×103 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 ABCCI/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 ABCCI/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 ABCCI/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.
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 ABCC1/MRP1 and KAS-KD35 cells, by knockdown of ABCC1/MRP1 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 ABCC1/MRP1 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).

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).
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 ABCC1/MRP1 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.

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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.

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 $ABCB6$ conferred 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 upregulation 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/ABCC1, 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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References


