Multidrug resistance-associated protein 7 expression is involved in cross-resistance to docetaxel in salivary gland adenocarcinoma cell lines

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Abstract. The aim of the present study was to clarify whether ATP binding cassette transporters are refractory factors in head and neck cancers. For *in vitro* and *in vivo* chemotherapeutic studies, we used the following head and neck cancer cell lines: a mouse oral squamous cell carcinoma (SCC) cell line, Sq-1979; a human SCC cell line, SCCHA; a mouse salivary gland adenocarcinoma (SGA) cell line, NR-PG; and a human SGA cell line, HSY. We used a vinca alkaloid anticancer drug, vincristine (VCR), as a chemotherapeutic anticancer drug. To determine the cause of multidrug resistance, Western blot analysis, reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry of xenografted tumors in nude mice, drug efflux analysis, and drug efflux inhibitory assays were performed. VCR-treated cell lines, Sq-1979/VCR, SCCHA/VCR, NR-PG/VCR, and HSY/VCR, intensively expressed multidrug resistance (MDR) gene 1 mRNA and multidrug resistance associated protein (MRP) 1 mRNA. MRP7 mRNA and protein were expressed in NR-PG/VCR and HSY/VCR cells, but not in Sq-1979/VCR and SCCHA/VCR cells. In each cell clone of NR-PG/VCR and HSY/VCR, MRP7 mRNA was induced by VCR treatment, suggesting an acquired resistance to VCR in the context of MRP7 expression. In the *in vivo* chemotherapeutic nude mice model, VCR-treated xenografted SCCHA and HSY cells expressed MDR1 and MRP1. Moreover, MRP7 expression was immunohistochemically found in xenografted HSY cells of VCR-injected tumor-bearing mice, but not in SCCHA cells. Furthermore, doxorubicin accumulation was increased and drug cross-resistance to docetaxel decreased in HSY/VCR in the presence of a competitive MRP7 inhibitor, 17-ß-estradiol-(17-ß-D-glucuronide). These results indicate that MDR1 expression, MRP1 expression, and MRP7 expression are refractory factors in head and neck cancer chemotherapy and suggest that induction of MRP7 expression is involved in drug resistance to natural products, especially to docetaxel in SGA.

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Introduction

Combined chemotherapy for head and neck cancers has exhibited insufficient clinical outcomes, particularly for salivary gland adenocarcinoma (SGA) as compared with the most prevalent type of head and neck cancer, oral squamous cell carcinoma (SCC). It has been suggested that the sensitivity and/or the mechanism of resistance to anticancer drugs are different between SGA and SCC (1-3).

Various mechanisms are involved in the drug resistance of cancer cells. One mechanism is the multidrug resistance (MDR) phenomenon caused by expression of ATP binding cassette (ABC) transporter in cancer cells (4). Studies of ABC transporters have provided important insights into the cellular resistance mechanisms associated with anticancer drugs. Structurally, multidrug resistance associated protein (MRP) family members can be classified according to whether or not they possess a third (NH_2-terminal) membrane-spanning domain (5). This topological feature is present in MRP1, MRP2, MRP3, MRP6, and MRP7, whereas it is absent in MRP4, MRP5, MRP8, and MRP9. Presumably, there is a difference in the functional properties of MRP proteins based on the presence or absence of this distinctive structure. For example, although all of the characterized MRPs are able to transport amphipathic anions (such as conjugates of glutathione and glucuronic acid), only MRP4, MRP5, and MRP8 are competent transporters of cyclic nucleotides (6-10). In addition, whereas MRP1, MRP2, MRP3, and MRP6 are able to confer resistance to natural product agents (5,11-15), MRP4, MRP5, and MRP8 do not appear to have this ability and instead are able to confer resistance to nucleotide analogs (16-18).

MRP7 has the lowest degree of structural resemblance to the other MRPs on the basis of amino acid alignments (8).

Chen *et al* reported that MRP7 expression is specifically associated with the MgATP-dependent transport of 17ß-estradiol-(17-ß-D-glucuronide) ($E_2$17ßG) (19). In a previous study on the potential for conferring resistance to natural product anticancer agents, vincristine (VCR), paclitaxel (PAC), and doxorubicin (DOX), it was shown that these agents potently inhibited $E_2$17ßG efflux (20). These previous studies suggested that MRP7 can mediate the transport of $E_2$17ßG and that MRP7 might be able to confer cross-resistance to natural product agents.

We previously found that MDR1 expression levels in an SGA cell line, HSY, were 3.7-fold higher than those in an SCC cell line, Hepd, after VCR treatment. Moreover, an MDR1 inhibitor, verapamil, did not completely conquer the drug resistance to VCR in HSY cells after VCR treatment; this finding suggests that a mechanism other than MDR1 is responsible for VCR efflux in SGA cells (21). However, the relationship between the expression of ABC transporters and acquisition of drug resistance in SGA cells is not fully understood.

To investigate the role of ABC transporters in MDR in head and neck cancers, we used both mouse and human head and neck cancer cell lines to evaluate MDR gene expression, and we analyzed gene product levels in *in vitro* and *in vivo* chemotherapeutic models using a natural product agent, VCR, at clinically equivalent doses. Consequently, we found that MRP7, as well as the best-analyzed ABC transporters, MDR1 and MRP1, contributes to drug resistance in head and neck cancer cell lines. Moreover, a unique expression of MRP7 in SGA cell lines affects the drug sensitivities to natural products of anticancer drugs. We discuss the differences between SCC and SGA with regard to the acquisition of the ABC transporter-mediated MDR phenotype, and we issue a warning for the use of natural products in cancer chemotherapy for SGA in conjunction with MRP7 expression.

Materials and methods

Cell culture of head and neck cancer cell lines. For *in vitro* and *in vivo* chemotherapeutic studies, we employed the following mouse and human head and neck cancer cell lines: a mouse oral SCC cell line, Sq-1979; a human SCC cell line, SCCHA; a mouse SGA cell line, NR-PG; and a human SGA cell line, HSY. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine, 44 mM sodium bicarbonate, 50 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, and 10% fetal bovine serum. Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2. DOX-treated HL-60 cells (HL-60/DOX), which were used as a positive control for the expression of MDR1 and MRP1, were maintained with 300 ng/ml of DOX (22). Viable cultured cells were counted after trypan blue exclusion and assayed by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenytetrazolium bromide (MTT) method. Single cells were subcloned in 96-well plates using a limited dilution technique. Single clones were identified after 2 weeks, and were then transferred to 24-well plates for expansion.

MTT assay of drug sensitivity. For the analysis of *in vitro* sensitivities of parental and resistant cells to anticancer drugs, docetaxel (DOC) (Aventis Pharmaceuticals, Bridgewater, NJ) and VCR, cisplatin (CDDP), and DOX (Sigma Chemical Co., St. Louis, MO) were added at various concentrations to the growth medium. Growth was determined with the MTT assay performed after 72 h of incubation in the presence of anticancer drugs (23,24). Briefly, exponentially growing cells ($2x10^3$) in 100 μl of medium were seeded on day 0 in 96-well microtiter plates. On day one, 100-μl aliquots of medium containing graded concentrations of anticancer drugs were added in triplicate to the cell plate. After incubation at 37˚C in a humidified incubator for 3 days, the plate was washed 3 times with PBS, and cell cultures were then incubated with 50 μl of MTT (1 mg/ml in Dulbecco's PBS) for 4 h at 37˚C on day 4. The resulting purple formazan precipitate was solubilized with 200 μl of 0.4 N HCl in isopropanol. The absorbance at 492 nm was quantified using a Bio-Rad Model 3550 UV microplate reader (Bio-Rad, Hercules, CA). Drug sensitivity was expressed as IC_{50}, which was designated as the drug concentration that caused a 50% growth reduction in treated cells relative to untreated cells (controls).

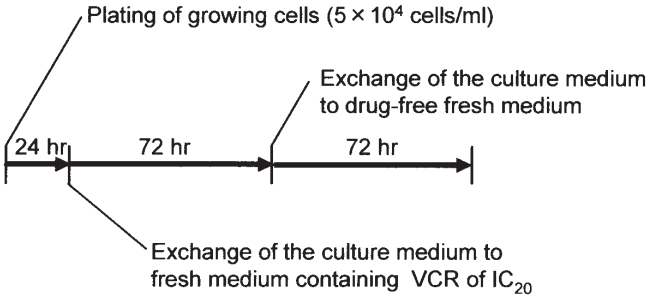

Figure 1. *In vitro* chemotherapeutic model with VCR (1 cycle). For *in vitro* analysis of the change in sensitivity to VCR, we used a continuous incubation with VCR at IC_{20}, in which cultured cells were constantly maintained over 10 cycles. Cell suspensions (10 ml) at a concentration of $5x10^4$ cells/ml in supplemented DMEM were seeded in a 90-mm petri dish and incubated for 24 h. Then, the supernatant was exchanged with fresh medium containing VCR at IC_{20}. After 72-h incubation with VCR, the supernatant was replaced with fresh medium. Cells were incubated an additional 72 h and then subjected to an *in vitro* growth-inhibitory assay.

In vitro model of VCR treatment. To analyze changes in the sensitivity to VCR, we conducted an *in vitro* experiment employing a VCR-treatment model that involves continuous incubation at the 20% inhibitory concentration (IC_{20}) of VCR, in which cultured cells could be constantly maintained over 10 cycles (25). This *in vitro* chemotherapeutic model is based on *in vivo* findings and is equivalent to the *in vivo* chemotherapeutic model. Briefly, 10 ml of cell suspension at a concentration of $5x10^4$ cells/ml in supplemented DMEM was seeded on a 90-mm petri dish and incubated for 24 h. Culture medium was then exchanged with fresh medium containing VCR at IC_{20}. After 72 h of incubation with VCR, culture medium was replaced with fresh medium (Fig. 1). Cells were incubated for an additional 72 h and subjected to an *in vitro* growth-inhibitory assay.

Detection of gene expression by reverse transcription-polymerase chain reaction. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using GAPDH as an

Table I. PCR primers and amplicon sizes.

Genes	Direction	Primer sequences (5'→3')	Amplicon (bp)
Mouse MDR1	Forward	GCCTTTGGAGGACAACAGAA	167
	Reverse	ACCAAGGATGTCCCATACCA	
Human MDR1	Forward	GGTGCTGGTTGCTGCTTACA	291
	Reverse	TGGCCAAAATCACAAGGGT	
Mouse MRP1	Forward	TGAGTGTGCAGAAGGTGGAG	196
	Reverse	ATGAGCAATCGTGAGCACAG	
Human MRP1	Forward	GGACCTGGACTTCGTTCTCA	292
	Reverse	CGTCCAGACTTCTTCATCCG	
Mouse MRP2	Forward	CCTGGCTACCAAGATCAGGA	250
	Reverse	AAATGTGATCACGGACACCA	
Mouse MRP3	Forward	AGCTCACCATCATCCCTCAG	200
	Reverse	CCTCTGGCCAACACTGAGAT	
Mouse MRP4	Forward	AACCTGGTAGACGCTGCTGT	199
	Reverse	CAGCACACCTGGTTTCCTTT	
Mouse MRP5	Forward	TTGAAAGCCATTCGAGGAGT	198
	Reverse	AAACATCTCAGCCTGGAACG	
Mouse MRP6	Forward	GAAGACTCCCAGCCATTGAA	199
	Reverse	TGCAGCTTGTCCTCCATTCT	
Mouse MRP7	Forward	CTGCACGTCTACCGAGCATA	202
	Reverse	GGGAGAAGAGTGCTGTGGAG	
Human MRP7	Forward	TAGGTGGGGTGCCAGCCG	367
	Reverse	TCGGAGGCGCCATCCAGGACT	
Mouse GAPDH	Forward	ACCACAGTCCATGCCATCAC	392
	Reverse	TCCACCACCCTGTTGCTGTA	
Human GAPDH	Forward	CGATGCTGGGCGTGAGTAC	414
	Reverse	CGTTCAGCTCAGGGATGACC	

internal control for normalization. Briefly, total cellular RNA was isolated from $5x10^6$ cells using the standard guanidine isothiocyanate/acid phenol method. First-strand cDNA was then synthesized from 1 μg of total RNA. Aliquots of synthesized cDNA (corresponding to 200 ng of total RNA) were amplified in 50-μl reaction mixtures. The primers are shown in Table I. The final concentration of each primer was 0.2 μM. Deoxynucleoside triphosphates (0.2 μM each) and 1.5 mM $MgCl_2$ were present in the Taq polymerase buffer (Stratagene, La Jolla, CA). PCR cycling was as follows: denaturation at 94˚C for 1 min, primer annealing at 58˚C for 1 min, extension at 74˚C for 4 min, and a final polymerization at 74˚C for 10 min. For each PCR, a blank control without cDNA was included. PCR products were then separated by electrophoresis on a 1% agarose gel. DNA fragments were visualized and photographed under UV light after ethidium bromide staining.

Preparation of plasma membrane. Cells were washed three times in PBS followed by centrifugation (400 x g, for 5 min) at 4˚C. The pellet (~$2x10^7$ cells) was resuspended in lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4 at 25˚C and 2 mM phenylmethyl sulfonyl fluoride added from a 200 mM stock solution in 95% ethanol) for 20 min at 4˚C, and were ruptured using a cell disruption bomb (No. 4639, Parr Instrument Co., Moline, IL). The sample was centrifuged (4,000 x g for 5 min) to remove cell debris. The remaining supernatant was subjected to high-speed centrifugation (100,000 x g for 60 min) to yield a plasma membrane-enriched, microsomal pellet. The final pellet was resuspended in lysis buffer, and protein concentration was determined by bicinchoninic acid assay (26,27).

Generation of MRP7 polyclonal antibody. Commercial monoclonal MRP7 antibody was not available for use in the present study. Therefore, we raised MRP7-specific polyclonal antibodies against synthetic peptides. New Zealand rabbits were immunized independently with purified synthetic peptides PGGPREPWAQEPC coupled with KLH protein. An equal volume of diluted synthetic peptide and Freund's complete adjuvant were emulsified and injected subcutaneously into the rabbits in 3 different sites. On days 15 and 28, the rabbits were immunized again with the same protein emulsified with Incomplete Freund's adjuvant. On day 35 the rabbits were sacrificed, and sera were separated and stored at -20˚C.

Western blot analysis. Immunoblot analysis was performed as described previously (28-30). For analysis of total protein, equal protein concentrations from cell extracts were subjected to 7.5% SDS-PAGE and were then transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Membranes were probed with C219 antibody to detect MDR1 and MRPm5 antibody to detect MRP1. Proteins were visualized using a horseradish peroxidase-labeled secondary antibody (Zymed, S. San Francisco, CA) and light-emitting non-radioactive enhanced chemiluminescence followed by exposure to autoradiographic film.

Xenograft models using cultured carcinoma cell lines. Female nude mice were subcutaneously injected with $5x10^6$ cells from cultured carcinoma cell lines. When tumors grew to ~6x6 mm, which typically takes about 3 weeks, mice were pair-matched into treatment and control groups (day 1). Each group contained 3 tumor-bearing mice that were ear-tagged and followed individually throughout the experiment. Administration of VCR at clinically equivalent doses (0.4 mg/kg) began on day 1 and continued every other day (25). Twice a week the mice were weighed and their tumors were measured with calipers. Tumor weight was calculated as follows: Weight (mg) = Width $(mm)^2$ x Length (mm)/2.

After 5 weeks of VCR administration, the mice were weighed and sacrificed. Then, the tumors were surgically excised and subjected to immunohistochemistry.

Immunocytochemical detection of MDR1, MRP1, and MRP7 in xenograft tumors. For immunohistochemical analysis of inoculated tumors in nude mice, we used one representative 4-μm section from each frozen tumor. After fixation with 4% formaldehyde in PBS for 10 min, preparations were washed 3 times with PBS. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxidase-methanol solution, blocking of non-specific conjugation with 5% normal rabbit serum was performed. Slides were incubated with C219 monoclonal antibody (Centocore, Marvern, PA) (12.5 μg/ml), MRPm5 monoclonal antibody (Alexis Biochemicals, San Diego, CA) (12.5 μg/ml), and MRP7 polyclonal antibody raised against rabbit sera (1:2500) for 60 min at room temperature. After washing 3 times with PBS, slides were incubated at room temperature for 60 min with biotinylated anti-mouse rabbit IgG or anti-rabbit IgG diluted at 1:2,000. After incubation with biotinylated antibody, slides were washed 3 times with PBS and were then incubated with streptavidin coupled to horseradish peroxidase (1:500) for 20 min. All dilutions were in PBS with 1% BSA. Reaction products were visualized by incubating with 4 mg (v/v) of 3,3'-diaminobenzidine-tetrahydrochloride (Sigma, St. Louis, MO) and 0.02% (v/v) hydrogen peroxide in PBS for 5 min. Finally, cover slips were counterstained with 1% methyl-green solution. As a negative control, slides were incubated as described above except that the primary antibody was replaced with 5% normal rabbit serum.

Detection of intracellular DOX concentration by flow cytometry. For DOX efflux studies, cells ($1x10^6$) were incubated with 10 mM DOX at 37˚C for 30 min (substrate-loading phase) and washed twice with ice-cold PBS. Thereafter, cells were resuspended in DOX-free RPMI 1640 in the presence or absence of 5 μM of cyclosporine A or $E_2$17ßG for 60 min. After incubation, cells were centrifuged and washed in ice-cold PBS. Cell pellets were then resuspended in 200 ml of PBS and immediately subjected to flow cytometric analysis for intracellular DOX retention.

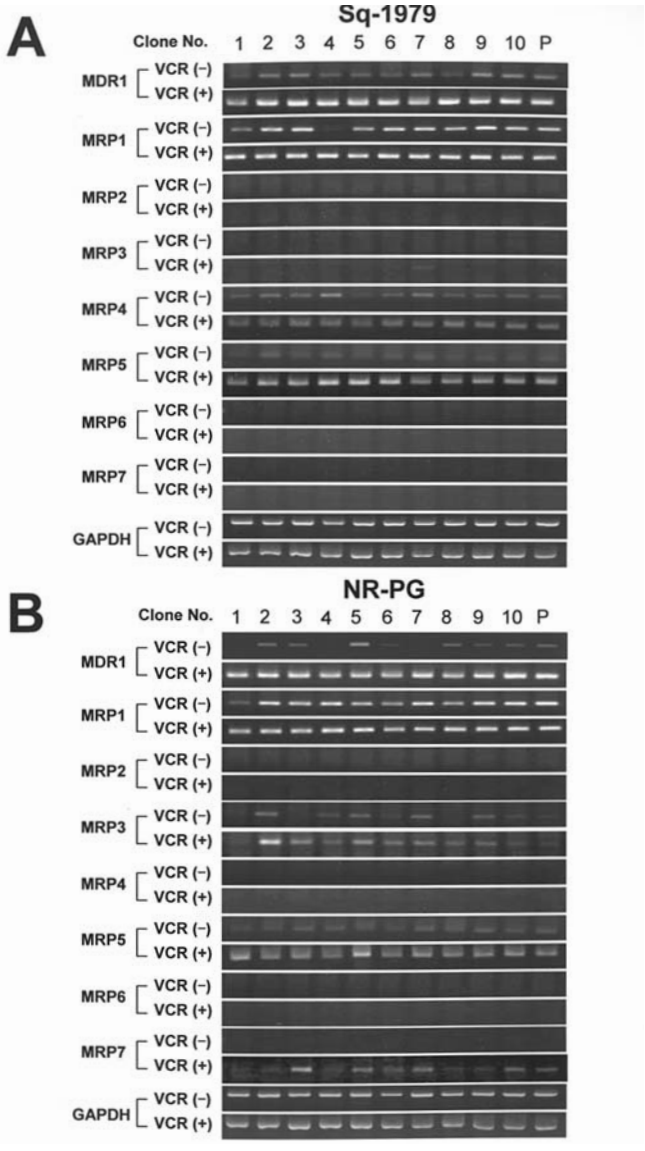

Figure 2. Expression of ABC transporter mRNA in mouse head and neck cancer cell lines. Single cells from the parental cell line were subcloned in 96-well plates using a limited dilution technique. A single clone was identified 2 weeks later, transferred to 24-well plates and subsequently expanded. After isolation of clone cells, clone cells were cultured with VCR for 5 cycles, and then the cDNA template was synthesized. PCR reactions were performed with the primers shown in Table I. Reaction products were separated by 1% agarose gel electrophoresis. 1-10, clone cell number; P, parental cell; (+), cultivation with VCR; (-), cultivation without VCR.

Statistical analysis. Data are shown as mean ± standard deviation (SD) from triplicate samples of a representative experiment. The differences between the means were analyzed by One-way analysis of variance, the Mann-Whitney U test and the Wilcoxon test. SPSS 11.0 software was used to perform the statistical analysis. A $P \leq 0.05$ was considered statistically significant. All statistical tests were two-sided.

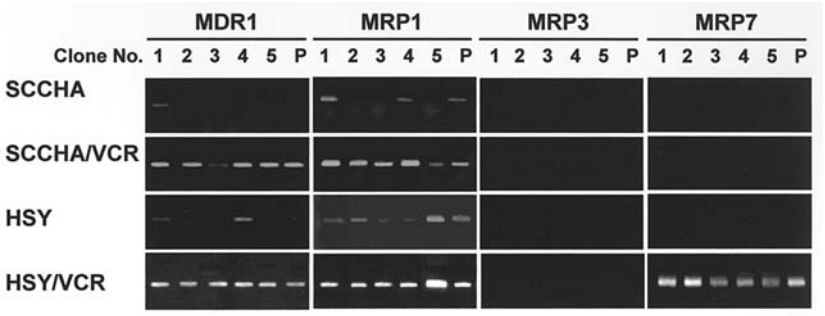

Figure 3. Expression of ABC transporter mRNA in human head and neck cancer cell lines. Single cells from parental cell lines were subcloned in 96-well plates using a limited dilution technique. After isolation of clone cells, clone cells were cultured with or without VCR for 5 cycles, and then the cDNA template was synthesized. PCR reactions were performed with the primers shown in Table I. Reaction products were separated by 1% agarose gel electrophoresis. 1-5, clone cell number; P, parental cell; (+), cultivation with VCR; (-), cultivation without VCR.

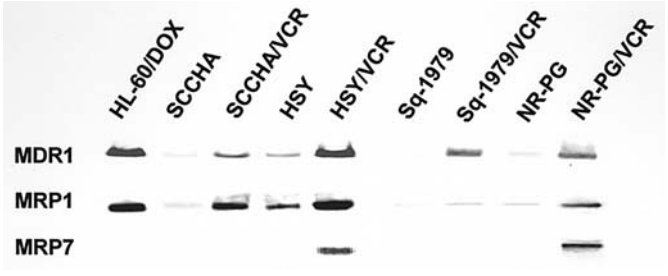

Figure 4. Western blot analysis of mouse and human ABC transporters. Plasma membrane protein (50 μg) from each cell line was separated by SDS-PAGE and then transferred to Immobilon-P membranes. Immunodetection was performed using the enhanced chemiluminescence kit for Western blot detection (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's instructions.

Results

Expression of ABC transporters in mouse and human head and neck cancer cell lines. For *in vitro* analysis, VCR-treated human head and neck cancer cell lines, Sq-1979/VCR, NR-PG/VCR, SCCHA/VCR, and HSY/VCR, were established from their parental cell lines by 5 low-dose VCR (IC_{20}) treatments (Fig. 1). The IC_{20} of VCR for Sq-1979, SCCHA, NR-PG, and HSY was 1.5, 2.2, 2.4, and 3.8 μM, respectively. Then, we used RT-PCR to examine the expression of ABC transporters in the VCR-treated murine cell lines. VCR-treated Sq-1979 expressed MDR1, MRP1, MRP4, and MRP5 but not MRP2, MRP3, MRP6, and MRP7 (Fig. 2A). On the other hand, VCR-treated NR-PG expressed MDR1, MRP1, MRP3, MRP5, and MRP7, but not MRP2, MRP4, and MRP6 (Fig. 2B). Thus, the characteristic findings of ABC transporter expression in mouse cell lines were that NR-PG/VCR expressed MRP3 and MRP7, whereas Sq-1979/VCR expressed MRP4.

Next, we determined if the ABC transporter expression patterns in mouse head and neck cancer cell lines were the same as those in human head and neck cancer cell lines. Consequently, we found that HSY/VCR expressed MRP7 mRNA as well as MDR1 and MRP1 mRNA but not MRP3 mRNA (Fig. 3).

As MDR1, MRP1, and MRP7 mRNA were expressed in the SGA cell lines, we performed Western blot analysis to confirm the expression of the corresponding proteins. DOX-treated HL-60 (HL-60/DOX) cells were used as an MDR1- and MRP1-positive control cell line. Staining intensity for MDR1 and MRP1 was higher in NR-PG/VCR and HSY/VCR than in Sq-1979/VCR and SCCHA/VCR (Fig. 4). Furthermore, MRP7 was expressed only in NR-PG/VCR and HSY/VCR. Thus, the results of Western blot analysis correspond to the results of RT-PCR.

Expression of MDR1, MRP1, and MRP7 in xenograft tumors in nude mice. To confirm the induction of MRP7 as well as MDR1 and MRP1, we used an *in vivo* chemotherapeutic model consisting of nude mice injected with SCCHA and HSY cell lines. Tumors from nude mice that did or did not receive VCR treatments were immunohistochemically examined using an anti-MDR1 mAb (C219), an anti-MRP1 mAb (MRPm5), and an anti-MRP7 polyclonal antibody isolated from rabbit sera. MDR1 and MRP1 were detected in the cell membranes of SCCHA and HSY tumor cells grown in VCR-treated mice, while no expression was observed in SCCHA and HSY tumor cells grown in untreated mice. However, MRP7 was detected only in HSY cells in VCR-treated mice (Fig. 5). Thus, the *in vivo* findings for inoculated culture cells in VCR-treated nude mice were similar to the *in vitro* data on the expression of MRP7, MDR1, and MRP1 in SCCHA/VCR and HSY/VCR cells (as analyzed by Western blotting).

Influence of $E_2$17βG and cyclosporine A on the DOC sensitivity of HSY/VCR. To clarify whether the drug resistance phenotype is caused by ABC transporters, an inhibitory assay of drug efflux in the cell lines was performed using an MDR1 and MRP1 inhibitor (cyclosporine A) and an MRP7 inhibitor ($E_2$17ßG). Cyclosporine A (5 μM) reduced the IC_{50} of DOC and DOX in both SCCHA/VCR and HSY/VCR cell lines, which express MDR1 and MRP1. However, $E_2$17ßG reduced the IC_{50} of DOC and DOX only in HSY/VCR cells, which express MRP7. $E_2$17ßG (5 μM) had a greater effect on the IC_{50} of DOC as compared to DOX. The IC_{50} of DOC and DOX in SCCHA/VCR cells treated with cyclosporine A (5 μM) decreased to similar levels as seen in SCCHA/VCR cells treated with both cyclosporine A (5 μM) and $E_2$17ßG (5 μM). On the other hand, HSY/VCR cells cultured with cyclosporine A (5 μM) and $E_2$17ßG (5 μM) were very sensitive to natural products, especially DOC, as compared with those cultured

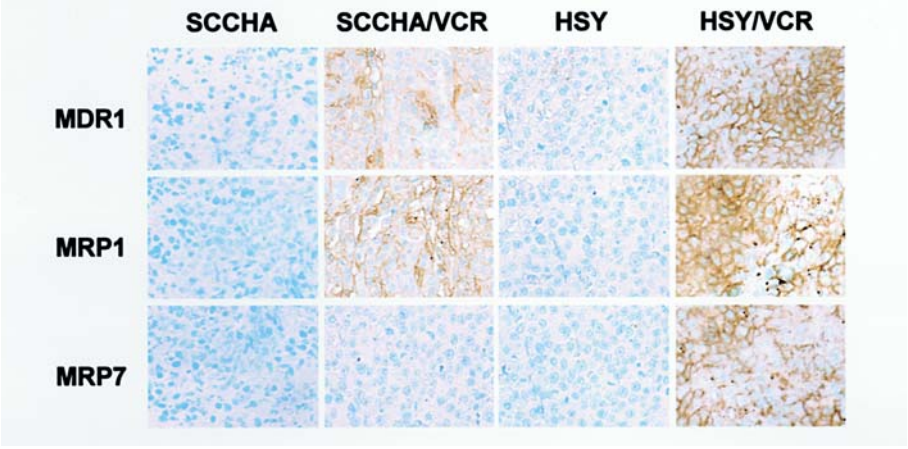

Figure 5. Immunohistochemical detection of MDR1, MRP1, and MRP7 in tumors grown in nude mice. After intraperitoneal administration of VCR (0.4 mg/kg) or PBS, tumors were excised and immunostained with antibodies. The reaction products were visualized by DAB and hydrogen peroxide in PBS. Finally, specimens were counterstained with 1% methyl-green solution.

Table II. Cytotoxicity of anticancer drugs with or without drug efflux inhibitors.

Cells	IC_{50} (nM)[a]		
	DOC	DOX	CDDP
SCCHA			
Inhibitor (-)	1.08±0.3	5.03±1.3	1668±133
+Cs A	1.04±0.2	4.94±0.8	1644±140
+$E_2$17ßG	1.06±0.3	4.89±1.0	1626±136
+Cs A +$E_2$17ßG	1.02±0.4	4.82±1.1	1658±186
SCCHA/VCR			
Inhibitor (-)	5.80±2.3	9.60±2.9	1710±191
+Cs A	1.38±0.8[c]	6.38±1.4[b]	1698±211
+$E_2$17ßG	5.65±0.9	9.35±0.9	1786±188
+Cs A +$E_2$17ßG	1.32±0.7[c]	6.22±0.6[b]	1691±228
HSY			
Inhibitor (-)	2.15±0.4	5.95±1.4	1689±168
+Cs A	1.93±0.6	5.63±1.1	1723±240
+$E_2$17ßG	2.08±0.4	5.58±1.4	1689±220
+Cs A +$E_2$17ßG	1.80±0.4	5.61±0.4	1660±177
HSY/VCR			
Inhibitor (-)	16.80±4.9	11.80±2.1	1689±242
+Cs A	5.60±2.7[c]	7.60±2.7[b]	1721±187
+$E_2$17ßG	8.40±2.8[c]	9.40±1.3[b]	1689±224
+Cs A +$E_2$17ßG	2.46±0.7[d]	6.16±2.8[c]	1682±267

[a]IC_{50} was determined using MTT assay. Values are means ± SD of 6 independent experiments. [b-d]Statistical comparisons were performed by the two-tailed Wilcoxon test between inhibitor (-) and drug conditions ([b]$p<0.05$, [c]$p<0.01$, [d]$p<0.001$). Cs A, cyclosporine A (5 μM), $E_2$17ßG, 17ß-estradiol-(17-ß-D-glucuronide) (5 μM).

with cyclosporine A (5 μM) alone. Thus, the sensitivity to DOC, an MRP7-related anticancer drug, in HSY/VCR cells is restored by treatment with $E_2$17ßG (Table II).

DOX accumulation in SCCHA/VCR and HSY/VCR cells in the presence of $E_2$17ßG and cyclosporine A. Because MRP7 confers resistance to DOC and DOX on the basis of active drug efflux, we confirmed drug accumulation using an auto-fluorescence drug, DOX, with or without transporter inhibitors, cyclosporine A and $E_2$17ßG, by flow cytometric analysis. An inhibitor of both MDR1 and MRP1 (cyclosporine A) markedly increased DOX accumulation in both SCCHA/VCR and HSY/VCR cells. Furthermore, DOX accumulation was increased in HSY/VCR cells, but not in SCCHA/VCR cells, in the presence of cyclosporine A and $E_2$17ßG as compared to cyclosporine A only (Fig. 6). $E_2$17ßG-mediated inhibition of MRP7 function leads to the increased sensitivity of HSY/VCR cells to DOC as well as DOX.

Discussion

The present study demonstrated that VCR-treated SGA cell lines, NR-PG/VCR and HSY/VCR, express MRP7 in addition to MDR1 and MRP1, whereas no expression of MRP7 was observed in VCR-treated SCC cell lines, Sq-1979/VCR and SCCHA/VCR. Furthermore, HSY/VCR exhibited cross-resistance to DOC in association with MRP7 expression. This differential expression of ABC transporters between SGA and SCC may be responsible for the inadequate efficacy of chemotherapy in head and neck cancers.

Recent studies have focused on investigating the role of ABC transporters in acquired drug resistance by measuring levels at diagnosis and at relapse after treatment with MDR-related drugs (31-33). Kelley *et al* reported a correlation between MDR1 expression and response to chemotherapy in patients with SCC of the head and neck (31). They concluded that analyzing MDR1 expression may be useful when planning chemotherapeutic regimens for these patients and that MDR1 expression may be an additional prognostic and diagnostic tool in these patients. Robey-Cafferty *et al* suggested that the

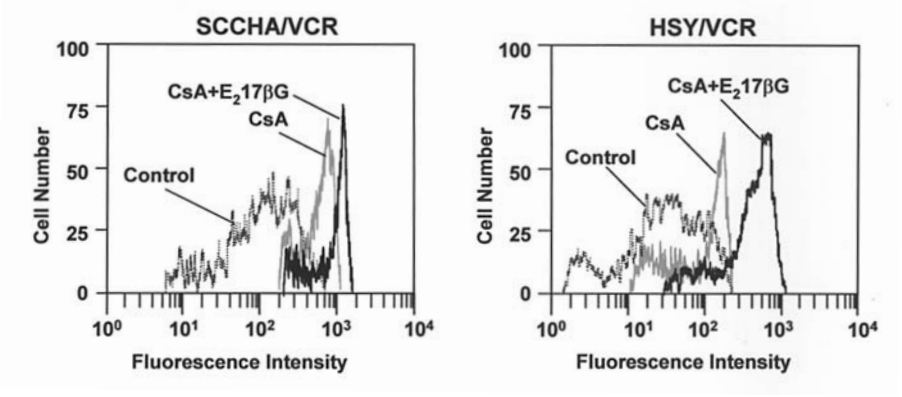

Figure 6. Accumulation of the intrinsically fluorescent anticancer drug, DOX, in SCCHA/VCR and HSY/VCR. SCCHA and HSY were cultured with VCR for 5 cycles to establish VCR-resistant cell lines, SCCHA/VCR and HSY/VCR. For DOX efflux studies, cells ($1x10^6$) were incubated with 10 mM DOX at 37˚C for 30 min (substrate-loading phase) and washed twice with ice-cold PBS. Thereafter, cells were resuspended in DOX-free DMEM in the presence or absence of 5 μM of cyclosporine A as both an MDR1 and MRP1 substrate and/or 17ß-estradiol 17-(ß-D-glucuronide) ($E_2$17ßG) for 60 min. After incubation, cells were centrifuged and washed in ice-cold PBS. Cell pellets were then resuspended in 200 μl of PBS and immediately subjected to flow cytometric analysis for intracellular DOX retention.

clinical response is correlated with MDR1 expression in adenocarcinoma (33). Filipits *et al* reported that MRP1 may be a useful marker for selecting appropriate adjuvant therapy in patients with early-stage breast cancer after prospective confirmatory evaluation (34).

RT-PCR findings from parental cells indicated that the expression of MDR1 and MRP1 is a type of 'inherent MDR', which is derived from clonal selection of intrinsically MDR1- or MRP1-positive cancer cells; in contrast, 'acquired MDR' is caused by induction of MDR1 or MRP1 expression in intrinsically MDR1- or MRP1-negative cells (25). However, both types of clone cells have the potential to exhibit heterogeneous induction and production of MDR1 and MRP1 mRNA. Thus, the interpretation of RT-PCR data in parental cells of each of the 4 cell lines may not be adequate to explain MDR acquisition because clone cells contain a mixture of inherent and acquired expression of ABC transporters.

Our findings support the notion that MDR1 and MRP1 are useful in evaluating potential MDR. However, all clones that inherently did not express MRP7 before VCR treatment expressed MRP7 after VCR treatment. Thus, MRP7 is important for evaluating resistance to natural products, including VCR and DOC, in SGA. Overall, these findings suggest a new approach to combat drug resistance using MDR1, MRP1, and MRP7 inhibitors with natural product agents.

MRP7 expression was observed in mouse and human SGA cell lines but not in SCC cell lines. Acquired expression of MRP7 was found in all SGA clones. Thus, the expression phenotype of MRP7 is different from that of MDR1 and MRP1 in SGA cells. On the other hand, MRP4 was weakly expressed in SCC cell lines and intensively expressed in VCR-treated SCC cell lines, whereas SGA cell lines did not express MRP4. These findings suggest that MRP4 expression is a characteristic of SCC cell lines. MRP1, MRP2, MRP3, and MRP6 confer resistance to natural product agents (12,35,36), in contrast to MRP4 and MRP5, which confer resistance to nucleotide analogs (17,37). Thus, MRP7 appears to confer resistance to natural product agents in SGA whereas MRP4 acts as transporter of nucleotide analogs in SCC. The significance of VCR-induced MRP4 expression in SCC and MRP7 expression in SGA remains to be elucidated.

Previous research has identified some similarities in the most downstream regions of the promoters for MRP1, MRP3, and MRP7. These regions are GC-rich and bear multiple Sp1 *cis* elements. This is consistent with the lack of a TATA box or transcription initiator element in the most proximal promoter region. Thus, co-expression of MRP1, MRP3, and MRP7 could occur in the transcription level, as seen in the results for NR-PG cells. In the present study, we did not analyze MRP3 function in SGA because parental NR-PG cells expressed MRP3 mRNA, but HSY/VCR cells did not. The reason for the discrepancy between MRP3 and MRP7 expression in different animal species is not clear and should be examined using additional head and neck cell lines.

Taxanes and vinca alkaloids are among the most active classes of drugs in the treatment of cancer. CDDP or 5-fluorouracil-based vincristine chemotherapy has been applied to head and neck cancers including SGA (38,39). The addition of a taxane, DOC or PAC, to standard platinum plus 5-fluorouracil induction chemotherapy has been shown to improve response rates and survival outcomes in head and neck cancers (39). At the same time, the continuous exposure of tumors to antimicrotubule drugs often results in the emergence of drug-resistant tumor cells with altered expression of several ß-tubulin isotypes (40). Thus, from a clinical standpoint, it is important to analyze the cross-resistance between natural product agents including DOC and PAC.

A previous study found that the transcriptional status of p53 determines the sensitivity to antimicrotubule drugs and that this effect is mediated through regulation of the microtubule-associated protein (MAP) 4 (40). MAP4 expression is transcriptionally repressed by wild-type p53. Increased MAP4 expression, which occurs when p53 is transcriptionally inactive, increases microtubule polymerization, PAC binding, and sensitivity to PAC, a drug that stabilizes polymerized microtubules (41). In contrast, MAP4 overexpression decreases microtubule binding and sensitivity to vinca alkaloids, which promote microtubule depolymerization (40,41). Thus, these findings suggest that changes in drug

sensitivity following increased binding of vinca alkaloids were associated with decreased binding of paclitaxel. In the present study, we identified ABC transporter-mediated cross-resistance between VCR and DOC, a mechanism that is separate from wild-type p53 induction-mediated drug resistance. These findings may have implications for the design of clinical trials of multidrug chemotherapy designed to control the multidrug resistance.

In conclusion, the present study indicates that MDR1- and MRP1-related MDR in SGA is an inherent phenotype caused by high levels of MDR1 and MRP1 induction and activated production based on clonal selection during VCR treatment. Moreover, VCR-treated SGA cell lines expressed MRP7 (in addition to MDR1 and MRP1) and exhibited cross-resistance to DOC. In contrast, MRP7 expression was not seen in SCC cells. This differential expression of ABC transporters may influence MDR. These findings are consistent with the previous clinical finding that SGA exhibits severe cross-resistance to anticancer drugs when compared with SCC.

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