Refractory Factors in Head and Neck Cancer: ATP Binding Cassette Transporters Expressed in Head and Neck Cancer 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 cancer chemotherapy. For in vitro and in vivo chemotherapeutic studies, we employed a human salivary gland adenocarcinoma cell line (HSY) and a human oral squamous cell carcinoma cell line (SCCSK) with vincristine (VCR) at clinically equivalent doses. Western blot analysis, reverse transcription-polymerase chain reaction, in vivo evaluation in xenograft models inoculated with cultured carcinoma cell line and drug efflux analysis were performed. VCR-treated SCCSK and HSY cells, as well as xenografted SCCSK and HSY cells in tumor-bearing nude mice, were found to express MDR1/ABCB1 and MRP1/ABCC1. In addition to MDR1 and MRP1 mRNA, HSY/VCR and its cloned cells expressed MRP7/ABCC10 mRNA, but SCCSK/VCR did not express MRP7. Furthermore, drug resistance to VCR and docetaxel decreased in HSY/VCR in the presence of a competitive MRP7 inhibitor, 17-beta-estradiol-(17-beta-D-glucuronide). These results indicate that MDR1 and MRP1 expression are refractory factors in head and neck cancer chemotherapy and suggest that induction of MRP7 expression is involved in drug resistance in salivary gland adenocarcinomas.

Key words: multidrug resistance, squamous cell carcinoma, salivary gland cell adenocarcinoma, ABC transporter, MDR1, MRP1, MRP7

Introduction

Combined chemotherapy for head and neck cancers has exhibited insufficient clinical outcomes, particularly for salivary gland adenocarcinoma (SGA) as compared with oral squamous cell carcinoma (SCC). 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. The ABC
transporter is responsible for active efflux of anticancer drugs out of resistant cells, resulting in significantly decreased intracellular drug accumulation and efficacy.

Investigations into ABC transporters have provided important insights into the cellular resistance mechanisms associated with anticancer drugs. Some of the functional properties of the ABC transporters MDR1/ABCB1, multidrug resistance-associated proteins (MRPs) 1-6/ABCC1-6 and MRP7/ABCC10 have been characterized. MRP1, MRP2 (cMOAT) and MRP3 are MgATP-energized transporters of glutathione S-conjugates, such as leukotriene C4 (LTC4) and S-(2,4-dinitrophenyl)glutathione. MRP1, which is widely expressed and localized at the basolateral surfaces of hepatocytes, where it functions in the extrusion of endogenous organic anions, such as bilirubin glucuronide and certain anticancer agents, and in the provision of the biliary fluid constituent glutathione. MRP3 mediates the transport of monoanionic bile acids.

MRP4 and MRP5 have the capacity to mediate the transport of cyclic nucleotides; this capacity implicates the two pumps in the regulation of intracellular levels of second messengers and in the extrusion of cAMP involved in intercellular signaling. Due to their capacity for transporting cyclic nucleotides, MRP4 and MRP5 are able to confer resistance against certain antiviral and anticancer nucleotide analogs, but are apparently unable to extrude "natural" agents. MRP6, a main factor in pseudoxanthoma elasticum, is capable of transporting glutathione conjugates. However, the physiological transport substrate involved in the pathogenesis of pseudoxanthoma elasticum is unknown. MRP7 was recently analyzed and found to catalyze the MgATP-energized transport of 17-beta-estradiol (E2)-17beta-glucuronide (E217G). The expression of these ABC transporters may also contribute to MDR in head and neck cancers, but the relationship between MDR and the ABC transporters is not fully understood.

To investigate the role of ABC transporters in MDR in head and neck cancers, we studied a human SCC cell line (SCCSK) and a human SGA cell line (HSY) originating from the parotid glands. We evaluated MDR gene expression and gene product levels in in vitro and in vivo chemotherapeutic models using an MDR-related drug (vincristine; VCR) at clinically equivalent doses (CED). Furthermore, we discuss the differences between SCC and SGA with regard to acquisition of the ABC transporter-mediated MDR phenotype.

Materials and Methods

1. Cell culture of head and neck cancer cell lines

A human SCC cell line (SCCSK), a human SGA cell line (HSY), and an MDR1- and MRP1-inducible cell line (HL-60) 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% CO2. Doxorubicin-treated HL-60 (HL-60/DOX), which was used as a positive control for the expression of MDR1 and MRP1, was maintained with 300 ng/ml of DOX. Viable cultured cells were counted after trypan blue dye exclusion and then assayed by the 3-(4,5-dimethylthiazyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Single cells were subcloned in 96-well plates using a limited dilution technique (single cell cloning). Single clones were identified after 2 weeks. Then, each single colony was isolated by penicillin syringe with trypsinization and subjected to additional single cell cloning twice. Then, the cloned cells were transferred to 24-well plates for expansion.

2. 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 (IC20) of VCR, in which cultured cells could be constantly maintained over 10 cycles. 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 5 × 104 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 IC20. After 72 h of incubation with VCR, culture medium was replaced with...
drug-free fresh medium and cells were incubated for an additional 72 h. This 1-week continuous cultivation was used as 1 cycle of in vitro chemotherapy (Fig. 1). Cells were then subjected to in vitro growth-inhibitory assay.

3. In vitro growth-inhibitory activity

The 50% growth inhibitory concentration (IC₅₀) and IC₂₀ of VCR were determined by cultivation in medium supplemented with various concentrations of VCR for 72 h.

Exponentially growing cells (2 × 10⁵) 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 VCR 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. Then, cell cultures were 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. Absorbance was quantified using a Bio-Rad Model 3550 UV microplate reader (Bio-Rad, Hercules, CA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

4. Preparation of plasma membrane

Cells were washed 3 times in PBS followed by centrifugation (400 × g, for 5 min) at 4°C. The pellet (~2 × 10⁷ cells) was resuspended in lysis buffer (10 mM KCl, 1.5 mM MgCl₂, 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 was ruptured using a cell disruption bomb (No. 4639, Parr Instrument Co., Moline, IL). The sample was centrifuged (4,000 × g for 5 min) to remove cell debris. The remaining supernatant was subjected to high-speed centrifugation (100,000 × 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 assay²⁹,³⁰.

5. Western blot analysis

Immunoblot analysis was performed as described previously³⁰. For analysis of total protein, equal protein concentrations from cell extracts were subjected to 7.5% SDS-Urea PAGE and were then transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Membranes were probed with C219 antibody to detect P-glycoprotein or MRPm5 antibody to detect MRPl. Proteins were visualized using a horseradish peroxidase-labeled secondary antibody (Zymed, S. San Francisco, CA) and light-emitting nonradioactive enhanced chemiluminescence followed by exposure to autoradiographic film.

6. Xenograft models using cultured carcinoma cell lines

Female nude mice were subcutaneously injected with 5 × 10⁶ cells from cultured carcinoma cell lines. When tumors grew to approximately 6 × 6 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 CED (0.4 mg/kg) began on day 1 and continued every other day³¹. Twice a week the mice were weighed and their tumors were measured with calipers. Tumor weight was calculated as follows:

\[ \text{Weight (mg)} = \frac{\text{Width (mm)}^2 \times \text{Length (mm)}}{2} \]

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

7. Immunohistochemical detection of MDR1 and MRPl in xenograft tumors

For immunohistochemical analysis of inoculated tumors in nude mice, we used 1 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, nonspecific conjugation was blocked with 5% normal rabbit serum. Slides were incubated with C219 monoclonal antibody (Centocore, Marvern, PA) (12.5 μg/ml) or MRPm5 monoclonal antibody (Alexis Biochemicals, San Diego, CA) (12.5 μg/ml) 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 IgG rabbit antibody diluted at 1:2,000. After incubation with biotinylated anti-rabbit antibody, slides were washed 3 times with PBS and then were 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, slides were counterstained with 1% methylgreen solution. As a negative control, slides were incubated as described above except that the primary antibody was replaced with 5% normal rabbit serum.

8. DOX accumulation and efflux assay
For DOX efflux studies, cells (1 × 10⁶) 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 RPMI1640 in the presence or absence of 5 μM of cyclosporine or E₂17βG 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.

9. PCR amplification
Total cellular RNA was isolated from 5 × 10⁶ 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 1. The primer sequences for MDR1, MRP1-5, and MRP6 were used in previous studies. The primers for MRP7 were designed and synthesized in our laboratory using Primer 3 Release 1.0 (Howard Hughes Medical Institute, Chevy Chase, MD). The final concentration of each primer was 0.2 μM. Deoxynucleoside triphosphates (0.2 μM each) and 1.5 mM MgCl₂ were present in the Taq polymerase buffer (Stratagene, La Jolla, CA). PCR cycling for 30 cycles 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 po-
lymerization 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.

10. Statistical analysis

Data are presented as mean ± S.D. Analysis of variables was measured using Student’s t-test. Statistical significance was set at *p*<0.05.

Results

1. VCR-treated HSY cells strongly express MDR1 and MRP1 when compared with VCR-treated SCCSK cells

To clarify whether MDR1 and MRP1, the 2 best-characterized ABC transporters, are involved in resistance to anticancer drugs, we analyzed their expression in cultured cell lines by Western blot analysis. For *in vitro* analysis, the VCR-treated human head and neck cancer cell lines SCCSK/VCR and HSY/VCR were established from SCCSK and HSY cells by 5 low-dose VCR (IC20) treatments (Fig. 1). The IC20 and IC50 values of anticancer drugs in cultured cells are shown in Table 2. The IC50 of SCCSK/VCR and HSY/VCR was significantly increased as compared to parental cells. In the Western blot analysis, DOX-treated HL-60 (HL-60/DOX) cells were used as an ABC transporter-positive control cell line that strongly expresses both MDR1 and MRP1. The staining intensities for MDR1 and MRP1 were higher in HSY/VCR cells than in SCCSK/VCR cells (Fig. 2).

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC20 (nM)a</th>
<th>IC50 (nM)a</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>VCR</td>
<td>DOC</td>
</tr>
<tr>
<td>SCCSK</td>
<td>2.8 ± 0.5</td>
<td>4.2 ± 1.5</td>
</tr>
<tr>
<td>SCCSK/VCR</td>
<td>-</td>
<td>8.4 ± 1.1b</td>
</tr>
<tr>
<td>HSY</td>
<td>5.1 ± 1.6</td>
<td>13.8 ± 2.6</td>
</tr>
<tr>
<td>HSY/VCR</td>
<td>-</td>
<td>26.3 ± 3.8d</td>
</tr>
</tbody>
</table>

*a* Growth inhibitory assay was determined using MTT assay. Values are means ± SD of 6 independent experiments. b–dStatistical comparisons were performed by the two-tailed Wilcoxon test between parental cell line and VCR-treated parental cell (*p*<0.05, *p*<0.01, *p*<0.001).

2. Xenograft tumors in VCR-treated nude mice express MDR1 and MRP1

On immunohistochemical analysis of inoculated culture cells in nude mice, SCCSK and HSY cells (with or without VCR treatment) were examined using an anti-MDR1 mAb (C219) and an anti-MRP1 mAb (MRPm5). MDR1 and MRP1 expression was observed in the cell membranes of SCCSK and HSY tumor cells from VCR-treated mice, while no expression was observed in tumors from untreated mice (Fig. 3). 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 MDR1 and MRP1 in SCC/VCR and HSY/VCR cells as analyzed by Western blots (Fig. 2).

3. HSY/VCR cells express MRP7 mRNA in addition to MDR1 and MRP1 mRNA

Because specific monoclonal antibodies against MRP2...
through MRP7 were not available, the expression of MDR1 and MRP1-7 in cultured cells was analyzed by RT-PCR (Fig. 4). MDR1 mRNA and MRP1 mRNA were detected in SCCSK, SCCSK/VCR, HSY and HSY/VCR cells. Furthermore, MRP5 mRNA was detected in SCCSK/VCR and HSY/VCR cells, but was barely detectable in SCCSK and HSY cells. Interestingly, MRP7 mRNA was expressed in HSY/VCR cells, but was not detected in the other cell lines. SCCSK/VCR cells expressed MRP4 mRNA at slightly higher levels than SCCSK cells. Thus, after VCR treatment, SCCSK cells expressed MRP4 and MRP5, and HSY cells expressed MRP5 and MRP7.
4. MRP7 mRNA is expressed in VCR-treated HSY clones

Additional experiments were performed to determine whether ABC transporter expression is caused by the induction of ABC transporter mRNA in intrinsically ABC transporter-negative or -undetectable cells or if activated production is caused by clonal selection of intrinsically ABC transporter-positive cancer cells. Seven single cells from the SCCSK and HSY cell lines were cloned and subjected to heterogeneity analysis of MDR1, MRP1 and MRP7 gene expression by RT-PCR (Fig. 5). Six SCCSK and 5 HSY clones exhibited induction of MDR1 mRNA, while the remaining cells showed activated production of MDR1 mRNA. Four SCCSK clones exhibited induction of MRP1 mRNA, while the remaining cells showed activated production of MRP1 mRNA. All HSY clones weakly expressed MRP1 mRNA and exhibited activated production after VCR treatment. MRP7 mRNA was heterogeneously induced in VCR-treated HSY clone cells. No expression of MRP7 was observed in SCCSK clone cells.

5. Sensitivity of HSY/VCR to docetaxel is increased by E217G and cyclosporine A

To clarify whether the drug resistance phenotype is caused by ABC transporters, an inhibitory assay of drug efflux in the cultured cell lines was performed using an MDR1 and MRP1 inhibitor (cyclosporine A) and an MRP7 inhibitor (E217G). Although cyclosporine A and E217G are not specific inhibitors for ABC transporters, they do have comparatively high affinity to MDR1 and MRP1, and MRP7, respectively. Because MRP confers resistance to vinca alkaloids and docetaxel, cell cytotoxicity was measured for cross-resistance using docetaxel. Survival of SCCSK/VCR cells cultured with cyclosporine A (5 μM) decreased to similar levels as seen in SCCSK cells cultured with both cyclosporine A (5 μM) and E217G (5 μM). On the other hand, HSY/VCR cells cultured with cyclosporine A (5 μM) and E217G (5 μM) were very sensitive to docetaxel, as compared with cells cultured with cyclosporine A (5 μM) alone (Table 3). Thus, the sensitivity to an MRP7-related anticancer drug, docetaxel, in HSY/VCR cells is restored by treatment with E217G (Fig. 6).

6. DOX accumulation is increased in SCCSK/VCR and HSY/VCR in the presence of E217G and cyclosporine A

Because MRP7 confers resistance to taxanes (docetaxel and paclitaxel) and DOX, we confirmed DOX accumulation in SCCSK/VCR and HSY/VCR cells by flow cytometric analysis. An inhibitor of both MDR1 and MRP1 (cyclosporine A) increased DOX accumulation in both SCC/VCR and HSY/VCR cells (Figs. 7A, B). However,
DOX accumulation was markedly increased in HSY/VCR cells, but not in SCCSK/VCR cells, in the presence of both CsA and E217βG as compared to only CsA (Figs. 7C, D). Thus, MRP7 is involved in drug efflux in HSY/VCR cells.

**Discussion**

The present study has demonstrated that a VCR-treated SGA cell line, HSY/VCR, expressed MRP7 in addition to MDR1 and MRP1, whereas no expression of MRP7 was observed in an oral VCR-treated SCC cell line, SCCSK/VCR. Furthermore, HSY/VCR exhibited cross-resistance to docetaxel. This differential expression of ABC transporters in SGA and SCC may be responsible for the inadequate efficacy of chemotherapy in head and neck cancer.

### Table 3

Cytotoxicity of anticancer drugs with or without inhibitors.

<table>
<thead>
<tr>
<th>Cells</th>
<th>IC₅₀ (nM)ᵃ</th>
<th>VCR</th>
<th>DOC</th>
<th>DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCSK/VCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inhibitor (−)</td>
<td>8.4 ± 1.1</td>
<td>4.8 ± 0.9</td>
<td>9.6 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>+ CsA</td>
<td>4.9 ± 1.8ᵇ</td>
<td>1.1 ± 0.2ᵇ</td>
<td>6.4 ± 1.4ᵇ</td>
<td></td>
</tr>
<tr>
<td>+ CsA + E₂₁₇βG</td>
<td>4.6 ± 1.2</td>
<td>1.0 ± 0.2</td>
<td>6.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>HSY/VCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>inhibitor (−)</td>
<td>26.3 ± 3.8</td>
<td>10.3 ± 2.4</td>
<td>15.8 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>+ CsA</td>
<td>19.4 ± 2.2ᶜ</td>
<td>5.9 ± 1.8ᶜ</td>
<td>8.6 ± 1.6ᶜ</td>
<td></td>
</tr>
<tr>
<td>+ CsA + E₂₁₇βG</td>
<td>13.8 ± 1.8ᶠ</td>
<td>2.8 ± 0.4ᶠ</td>
<td>6.2 ± 1.8ˢ</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ IC₅₀ 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 (ᵇp<0.05, ᵇᵖ<0.01, ᵇᵖ<0.001) or between CsA and drug conditions (ᶜᵖ<0.05, ᵇᵖ<0.01). CsA: cyclosporin A (5 μM), E₂₁₇βG: 17β-estradiol-(17-β-D-glucuronide) (5 μM)

**Fig. 6** Restoration of cytotoxic activity in SCCSK/VCR and HSY/VCR cells by modulators, cyclosporine A (CsA) and E₂₁₇βG.

After cultivation of SCCSK/VCR and HSY/VCR cells with several concentrations of VCR and modulators for 72 h, cytotoxic activity in vitro was determined by trypan blue dye exclusion. * Significantly different from inhibitor (−), as assessed by Student’s t test (p<0.05). ** Significantly different from cyclosporin A, as assessed by Student’s t test (p<0.05).
Our RT-PCR findings for SCCSK/VCR, HSY/VCR, and their parental cell lines suggested that the MDR1 and MRP1 phenotype is a type of inherent MDR, which is derived from clonal selection of intrinsically MDR1- or MRP1-positive cancer cells, rather than acquired MDR, which is caused by induction of MDR1 or MRP1 expression in intrinsically MDR1- or MRP1-negative cells. However, both clones have the potential to exhibit heterogeneous induction and production of MDR1 and MRP1 mRNA. Thus, the interpretation of the RT-PCR data may not be adequate to explain the acquisition of MDR. Moreover, expression of MRP7 was observed in HSY/VCR cells but not in SCCSK/VCR cells. Thus, when compared with SCC, SGA exhibits induction and activated production of MDR1, MRP1 and MRP7, leading to potent MDR against anticancer drugs. These suggestive results were based on the study of only 7 cloned cells derived from SCCSK and HSY cells. A fluctuation test should be performed with more cloned cells and other head and neck cell lines to validate this evidence.

In the present study, MRP4 was weakly expressed in SCCSK cells and strongly expressed in SCCSK/VCR cells. However, HSY and HSY/VCR cells did not express MRP4, while both cell types expressed MRP5. These results suggest that MRP4 expression is a characteristic of oral SCC cells. MRP members can be structurally classified according to the presence of an N-terminal membrane-spanning domain. This topological feature is expressed in MRP1, MRP2, MRP3, MRP6 and
MRP7 has 3 membrane-spanning domains and possesses the cardinal biochemical feature of the MRP family, the ability to transport amphipathic anions such as E217G, an inhibitor of MRP3, MRP6 and MRP7. In the present study, HSY/VCR did not express MRP3 or MRP6, suggesting that E217G-mediated inhibition of MRP7 is involved in the increased sensitivity of HSY/VCR to docetaxel.

The majority of salivary gland tumors contain 2 distinct populations of tumor cells, luminal cells and abluminal cells. Abluminal cells demonstrate the most heterogeneous cellular differentiation. Hence, the different types of salivary gland tumors can exhibit variable levels of MDR1 and MRP1 expression, as well as cellular differentiation. Disparities in the expression of MDR1 and MRP1 could also be derived from the heterogeneous populations of clone cells as a consequence of inherited, acquired or activated production of MDR1 and MRP1. However, it has been difficult to determine whether clonal selection or the acquisition or activated production of MDR1 and MRP1 causes the observed MDR phenotype.

Because previous findings from in vivo experiments were subsequent to clonal selection of drug-resistant phenotypes, it is difficult to confirm which MDR1 or MRP1 expression process is involved in drug resistance. On the other hand, the stepwise sequential exposure to increasing concentrations of anticancer drugs in vitro has previously been used to establish MDR1- and MRP1-expressing cells using variant cell lines that express high levels of MDR1 and MRP1. Despite the importance of characterizing transporter function, these experiments are unsuitable for clinical settings and for clarifying whether ABC transporters play a functional role in the failure of initial chemotherapy or whether MDR is caused by the development of intrinsic or acquired resistance. Clinically, two-fold resistance to anticancer drugs suggests a difficulty in overcoming chemotherapeutic cancer treatments due to their toxicity in a living body. Thus, it is necessary to analyze the progression of ABC transporter mediated-MDR phenotypes with CED in both in vivo and in vitro studies. In the present study, we determined VCR concentrations for in vitro drug-resistance analysis based on an in vivo chemotherapeutic model using CED. The combination of in vitro and in vivo studies was useful in determining drug concentrations for clinical observations and is suitable for in vitro drug-resistance assays.

Recent studies have focused on investigating the role of MDR1 and MRP1 in acquired drug resistance by measuring levels at diagnosis and at relapse after treatment with MDR-related drugs. Kelley et al. reported a correlation between MDR1 expression and response to chemotherapy in SCC of the head and neck. They concluded that analyzing the expression of MDR1 may be useful when planning the chemotherapeutic regimen for patients with head and neck cancer and that MDR1 expression may be an additional prognostic and diagnostic tool in these patients. Robey-Caffery et al. suggested that clinical response is correlated with expression of MDR1 in adenocarcinoma. 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. As in previous studies, the present study demonstrated the expression of MDR1 and MRP1 in SCC and SGA. The IC50 of VCR, DOC and DOX was also higher in the presence of MDR1 and MRP1 expression. Our findings support the notion that MDR1 and MRP1 are useful in evaluating potential MDR. However, inherently MDR1- and MRP1-negative clone cells capable of inducing high levels of MDR1 and MRP1 expression were barely detected by immunohistochemistry and RT-PCR; these results suggest that MDR1 and MRP are not sufficient predictive markers of MDR phenotypes, such as SGA.

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, HSY/VCR cells expressed MRP7, in addition to MDR1 and MRP1, and exhibited cross-resistance to docetaxel. In contrast, MRP7 expression was not seen in SCCSK/VCR 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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