The Multidrug Resistance-associated Protein Gene Confers Drug Resistance in Human Gastric and Colon Cancers

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To determine the expression of multidrug resistance-associated protein (MRP) gene and its role in gastric and colon cancers, we analyzed 10 gastric and 10 colon non-drug-selected cell lines and a similar number of tissue samples of these cancers. We compared the expression of MRP and mdr1 mRNA in cell lines and tissues using reverse-transcriptase polymerase chain reaction. In mdr1-negative cells, the relationship between the level of MRP gene expression and sensitivity to anticancer drugs was examined. The effect of verapamil, an MRP-modulating agent, was also examined in these cells. The expression of MRP gene in gastric cancer cell lines varied from a low to a high level, but mdr1 was not detected in any of these cell lines. Colon cancer cell lines expressed low to intermediate levels of MRP gene, and half of the cells co-expressed low to high levels of mdr1. In tissue samples, the expression pattern of the two multidrug resistance (MDR) genes was broadly similar to that described for the cell lines, except that most of the gastric cancer tissue samples did express low levels of mdr1. No significant correlation was observed between the level of MRP gene expression and sensitivity to anticancer drugs in gastric and colon cell lines. However, verapamil significantly increased the sensitivity to etoposide, doxorubicin and vincristine in cells highly expressing MRP gene. Our results indicate that MRP gene may be important in conferring MDR in gastric and colon cancer cells.

Key words: Multidrug resistance — MRP — Gastric cancer — Colon cancer — Modulating agent

Resistance of cancer cells to anticancer drugs is a major problem in chemotherapy,[1] particularly in solid tumors, including gastric and colon cancers. The relatively low response rate (20–50%) of gastric and colon malignancies to chemotherapy[2, 3] suggests the existence of intrinsic drug resistance. The normal colonic mucosa expresses the mdr1 gene encoding human P-glycoprotein (Pgp), an ATP-dependent drug efflux pump, which confers multidrug resistance (MDR).[4] In contrast, normal gastric epithelial cells do not express Pgp,[5–7] and mdr1/Pgp is negative or minimally expressed in most gastric cancers.[7–9] The latter observation strongly suggests the existence of different MDR mechanisms in gastric cancer.

Several MDR cell lines have been selected by exposure to doxorubicin (Dox), etoposide (VP-16) or mitoxantrone, and they display the MDR phenotype in the absence of mdr1/Pgp expression.[10] Cole et al.[11] isolated a 190 kD multidrug resistance-associated protein (MRP) from adriamycin-selected MDR lung cancer cell line. Later studies showed overexpression of the same protein in certain non-Pgp-mediated MDR cell lines.[12–18] Furthermore, MRP gene-transfected cells have been shown to display the MDR phenotype.[19–22] Several studies demonstrated the ability of the calcium channel blocker

verapamil to increase the drug sensitivity of tumor cells overexpressing MRP gene as well as those having mdr1/Pgp.[16–18, 23] MRP gene is expressed in certain normal cells and tissues,[11, 15, 24–27] and several studies have described MRP gene expression in tumor tissues.[27–30] However, the clinical role of the protein in drug resistance remains unknown.

In the present study, we examined the expression of MRP mRNA in gastric and colon cancer cell lines and in clinical samples of gastric and colon cancers using reverse-transcriptase polymerase chain reaction (RT-PCR). We also examined the relationships between the level of MRP gene expression and the *in vitro* sensitivity of mdr1-negative cell lines to various anticancer drugs, and the effects of verapamil on the sensitivity.

MATERIALS AND METHODS

Reagents, cell lines and clinical samples VP-16 and cisplatin (CDDP) were obtained from Nippon Kayaku Co. (Tokyo), Dox and mitomycin C (MMC) from Kyowa Hakko Kogyo Co. (Tokyo), vincristine (VCR) from Sigma Chemical (St. Louis, MO), SN-38 (7-ethyl-10-hydroxycamptothecin), an active metabolite of irinotecan (CPT-11), from Yakult Honsha Co. (Tokyo), and verapamil hydrochloride from Eisai Co. (Tokyo).

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We selected the HL60R cell line overexpressing MRP gene by continuously exposing HL60 human promyelocytic leukemia cell line to increasing concentrations of Dox.[29] The HL60R cells also overexpressed MRP in an immunoblot using rabbit anti-MRP polyclonal antibody (data not shown). Compared with the sensitivity of parental HL60 cells, the HL60R cells were 397-, 15- and 11-fold more resistant to VP-16, Dox and VCR, respectively, but remained relatively sensitive (less than 4-fold more resistant) to MMC, CDDP and SN-38.[29] The MCF7/ADR[31] and HL60R cells were used as positive controls for mdr1 and MRP gene, respectively. All gastric and colon cancer cell lines were obtained from the Japanese Cancer Research Resources Bank (JCRRB; Tokyo) and RIKEN Cell Bank (Ibaraki). All cell lines were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY), supplemented with 10% fetal calf serum (Gibco), 4% L-glutamine, and 80 mg/liter kanamycin sulfate (Meiji Seika, Tokyo) in a humidified incubator (5% CO_2 at 37°C).

Surgically excised gastric and colon adenocarcinomas samples were obtained at Nagasaki University Hospital. The tissue samples were immediately frozen in liquid nitrogen and stored at $-70°C$ until further processing. The samples were obtained from patients who had not received chemotherapy prior to surgery.

RNA extraction and quantitative RT-PCR Total RNA from cells and tissues was isolated using the guanidine isothiocyanate method.[32] RT-PCR for MRP, mdr1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) genes, and semi-quantitation of each MDR mRNA expression were performed as described previously.[29] The relative expression of mRNA of each gene (relative MDR gene expression) was calculated in cells and tissues according to the following formula:

Relative MDR gene expression =

$$\frac{\text{MRP or mdr1 gene in sample}}{\text{MRP or mdr1 gene in positive control}}$$

$$\div \frac{\text{G3PDH gene in sample}}{\text{G3PDH gene in positive control}} \times 100$$

The relative MDR gene expression, calculated using this formula, was classified as low, intermediate or high. "Low" expression was defined as a level less than 30, "intermediate" as between 30 and 70, and "high" as exceeding 70. The relative MRP gene expression in HL60 cells was 15.5 ± 1.3 (mean$\pm$SD) as reported previously.[29]

Drug sensitivity assay The sensitivity of cells to each anticancer drug was determined using the tetrazolium dye assay, as previously described by Mosmann.[33] Assays were performed in quadruplicate at least three times. The drug concentration producing 50% inhibition of growth (IC_{50}) was determined graphically for each drug using

the relative survival curves. The effect of verapamil as an MRP-modulating agent on drug sensitivity was defined as the ratio between the IC_{50} for anticancer drugs and that in the presence of $10 \mu M$ verapamil added just before the anticancer drugs.

Statistical analysis Data were expressed as mean$\pm$SD. Differences between groups were tested for statistical significance using Mann-Whitney's U test, Spearman's rank correlation coefficient or unpaired Student's t test. A two-tailed $P < 0.05$ was taken as the criterion of statistical significance.
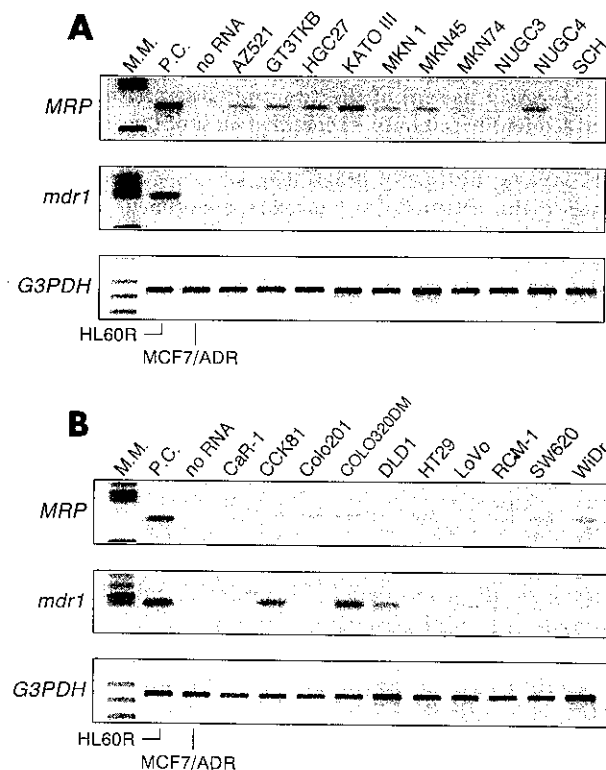

Fig. 1. Analysis of MRP and mdr1 mRNA expression in gastric and colon cancer cell lines using RT-PCR. HL60R and MCF7/ADR represent positive controls for MRP and mdr1 gene, respectively. Relative MRP gene expression was calculated as described in "Materials and Methods." A, Relative MRP gene expression of gastric cancer cells was 23.1 in AZ521, 36.0 in GT3TKB, 32.8 in HGC27, 71.1 in KATOIII, 17.2 in MKN1, 39.9 in MKN45, 15.5 in MKN74, 8.0 in NUGC3, 52.8 in NUGC4, and 10.5 in SCH cells. Relative mdr1 expression was negative in all lines. B, Relative MRP gene expression of colon cancer cells was 9.6 in CaR-1, 27.2 in CCK81, 42.6 in Colo201, 40.7 in COLO320DM, 23.5 in DLD1, 28.1 in HT29, 20.2 in LoVo, 22.4 in RCM-1, 24.7 in SW620, and 32.5 in WiDr cells. Relative mdr1 expression was negative in 50% of the cell lines. M.M., molecular marker; P.C., positive control.

RESULTS

MRP and mdr1 gene expression in cell lines Fig. 1 shows the results of RT-PCR analysis of MRP and mdr1 mRNA expression in gastric ($n = 10$) and colon cancer ($n = 10$) cell lines, while Fig. 2A shows the relative expression of each gene in these cells. The relative MRP

gene expression in gastric and colon cancer cell lines was heterogeneous with a mean value of 33.2 ± 19.5 (median, 34.4) and 27.2 ± 9.7 (median, 26.0), respectively. The difference in the relative MRP gene expression between the two groups of cell lines was not significant. The relative mdr1 expression was also heterogeneous in colon cancer cell lines with a mean value of 24.9 ± 39.5
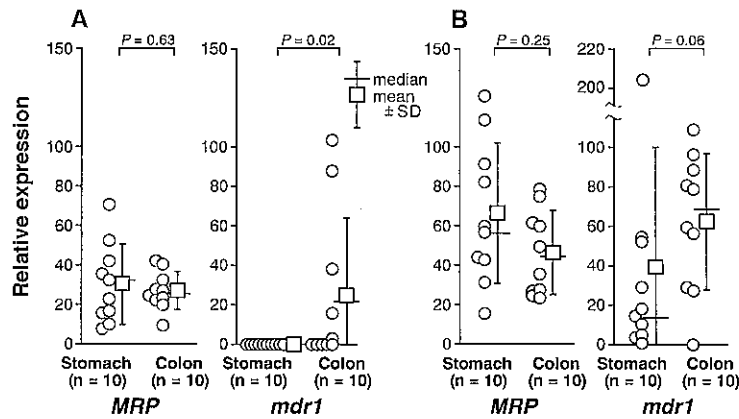

Fig. 2. Relative MRP and mdr1 gene expression in gastric and colon cancer cell lines (A) and tissues (B). The relative expression was calculated as described in "Materials and Methods." The expression of mdr1 in gastric cancer cell lines was significantly different from that of colon cancer cell lines ($P = 0.02$ by the Mann-Whitney's U test). SD, standard deviation.
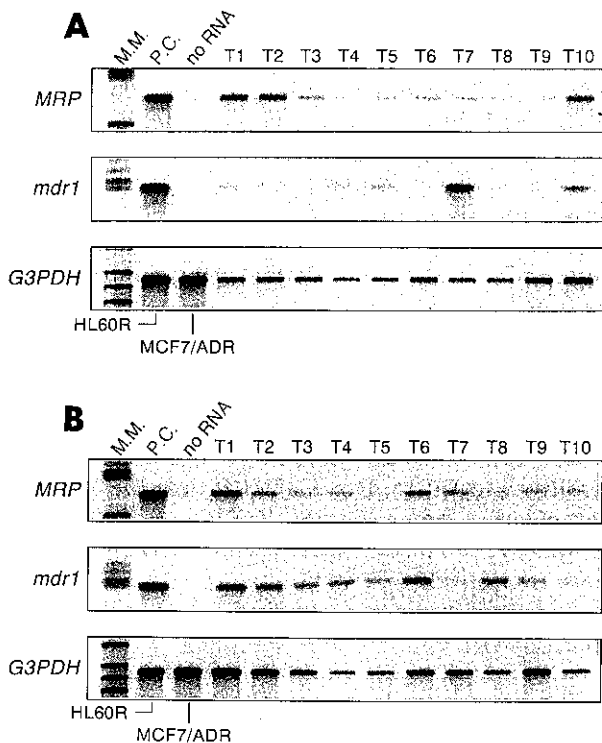

Fig. 3. Analysis of MRP and mdr1 mRNA expression in tissue samples from gastric and colon adenocarcinomas using RT-PCR. HL60R and MCF7/ADR represent positive controls for MRP and mdr1 gene, respectively. The relative MRP gene expression was calculated as described in "Materials and Methods." A, RT-PCR analysis of gastric cancer tissues. Tumor: grade or subtype, relative MRP, relative mdr1 = T1: poorly, 114, 14.4; T2: moderately, 126, 10.3; T3: poorly, 82.6, 18.0; T4: moderately, 43.3, 29.3; T5: moderately, 60.0, 54.4; T6: papillary, 44.3, 1.0; T7: moderately, 56.8, 20.4; T8: poorly, 31.8, 4.8; T9: moderately, 15.9, 3.6; T10: signet-ring, 91.6, 52.0. B, RT-PCR analysis of colon cancer tissues. Tumor: grade, relative MRP, relative mdr1 = T1: moderately, 78.9, 79.1; T2: moderately, 50.1, 80.7; T3: well, 28.2, 59.7; T4: poorly, 62.0, 109; T5: poorly, 35.9, 56.2; T6: moderately, 75.4, 88.4; T7: moderately, 60.2, 27.4; T8: moderately, 28.2, 96.6; T9: moderately, 23.5, 29.0; T10: moderately, 25.1, 0.1. M.M., molecular marker; P.C., positive control; T, tumor tissue; grade: well, moderately, and poorly differentiated adenocarcinomas.
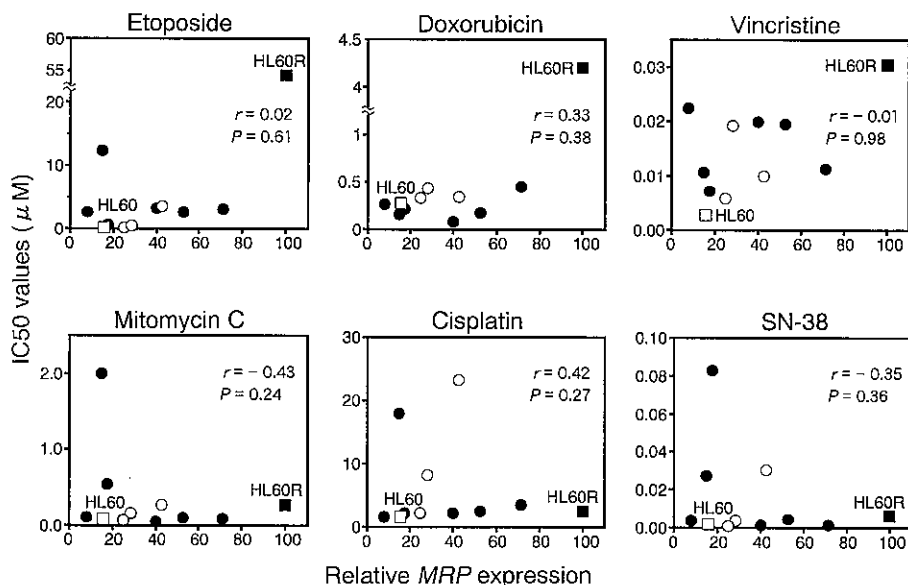
Fig. 4. Relationship between the relative MRP gene expression in gastric (closed circles) and colon (open circles) cancer cell lines and the IC_{50} of anticancer drugs. The mean values of IC_{50} in three independent experiments were plotted; each standard deviation was within 10% of the mean. The correlation was evaluated statistically by using Spearman's rank correlation coefficient. No significant correlation was observed for any of the tested drugs (□, HL60 cells; ■, HL60R cells; these cell lines were excluded from statistical analysis).

(median, 1.6), but all gastric cancer cell lines were negative for mdr1 mRNA. The difference in the relative level of mdr1 between the two groups of cell lines was significant ($P=0.02$). Half of the colon cancer cell lines co-expressed both MDR genes, while the remaining half and all gastric cancer lines expressed MRP gene only.

The expression of DNA topoisomerase II was also determined by immunoblot analysis. The level of topoisomerase II was highly variable in these cancer cell lines (data not shown).

MRP and mdr1 gene expression in tissue samples In the next step, we examined the expression of MRP and mdr1 mRNA in tissue samples of gastric and colon adenocarcinoma (Fig. 3). The relative MRP gene expression in gastric ($n=10$) and colon ($n=10$) cancer tissue samples is shown in Fig. 2B. The relative MRP gene expression in gastric cancer samples (66.7 ± 36.0, median 58.4) was not significantly different from that of colon cancers (46.8 ± 21.3, median 43.0). The relative mdr1 in both tissues varied widely from a low to a high level, but the mean values (gastric: 39.2 ± 61.1, median 16.2, colon: 62.6 ± 34.8, median 69.4) were statistically not different ($P=0.06$). The pattern of expression of the two MDR genes in the tissues was broadly similar to that in the cell lines.

MRP gene expression and drug sensitivity We also examined the relationship between relative MRP gene

expression and the sensitivity to six anticancer drugs, including MDR (VP-16, Dox, VCR, MMC) and non-MDR drugs (CDDP, SN-38). To exclude the contribution of mdr1/Pgp to MDR, six mdr1-negative gastric (KATOIII, MKN1, MKN45, MKN74, NUGC3, and NUGC4 cells) and three mdr1-negative colon (Colo201, HT29, and SW620 cells) cancer cell lines were tested. There was no significant correlation between the level of MRP gene expression and sensitivity to each individual drug (Fig. 4).

Effects of verapamil on drug sensitivity The effects of verapamil, an MRP-modulating agent, on drug sensitivity were examined in four mdr1-negative cancer cell lines; these lines showed the highest (KATOIII and Colo201) and the lowest (NUGC3 and CaR-1) relative MRP gene expression among all gastric and colon cancer cell lines, respectively (Fig. 1). The tested drugs included Dox, VCR, VP-16 and CDDP. Verapamil influenced the sensitivity to VP-16, Dox and VCR, and the effects were particularly strong in KATOIII ($P<0.0005$) and Colo201 ($P<0.02$) compared with NUGC3 and CaR-1 cells, respectively (Table I).

DISCUSSION

The present study demonstrated that gastric cancer cell lines expressed a low to high level of MRP mRNA,

Table I. Effects of Verapamil on Drug Sensitivities of Gastric and Colon Cancer Cells[a]

Treatment	Gastric cancer		Colon cancer	
	KATOIII	NUGC3	Colo201	CaR-1
VP-16	3.66	2.45	3.06	1.08
VP-16+Verapamil	1.01	1.94	1.22	0.837
Modulation ratio	3.62[b]	1.26	2.51[c]	1.29
Dox	0.469	0.248	0.355	0.0862
Dox+Verapamil	0.150	0.161	0.152	0.0668
Modulation ratio	3.13[b]	1.54	2.34[c]	1.29
VCR	0.0126	0.0221	0.0103	0.00639
VCR+Verapamil	0.00251	0.0177	0.00486	0.00429
Modulation ratio	5.02[b]	1.25	2.12[c]	1.49
CDDP	3.54	2.07	23.5	2.56
CDDP+Verapamil	3.25	2.03	25.3	2.33
Modulation ratio	1.09	1.02	0.93	1.10

a) Data represent the mean values of IC_{50} (μM) in three experiments without or with verapamil (10 μM); all standard deviations were within 10% of the mean values (not shown). The relative MRP gene expression was 71.1 in KATOIII, 8.0 in NUGC3, 42.6 in Colo201, and 9.6 in CaR-1 cells. Modulation ratio=IC_{50} for drug ÷ IC_{50} for drug with verapamil.
b) $P<0.0005$ between KATOIII and NUGC3 cells (unpaired Student's t test).
c) $P<0.02$ between Colo201 and CaR-1 cells (unpaired Student's t test).
VP-16, etoposide; Dox, doxorubicin; VCR, vincristine; CDDP, cisplatin.

but did not express mdr1 mRNA. On the other hand, while colon cancer cell lines expressed low to intermediate levels of MRP mRNA, they also expressed mdr1 mRNA at low to high levels. The pattern of expression of the two MDR genes in tissues of the same cancers was broadly similar to that in cell lines, except that mdr1 mRNA was detected in 9 out of 10 gastric cancer tissue samples, though the gastric cancer cell lines were all negative. In mdr1-negative cell lines, the expression of MRP mRNA did not correlate with the sensitivity to a variety of tested anticancer drugs such as VP-16, Dox, VCR and CDDP. However, verapamil, an MRP-modulating agent, significantly increased the sensitivity to VP-16, Dox and VCR in non-drug-selected cells highly expressing MRP. Thus, our in vitro studies indicate that MRP gene may play an important role in MDR of gastric and colon cancer cells.

Our results showed that the level of MRP gene expression in gastric cancer tissues tended to be higher than that of mdr1 expression, although these levels cannot be simply compared with each other. The mdr1 levels detected in the present study were consistent with previous reports showing no or low mdr1/Pgp expression in most gastric cancer cell lines and tissues.[7-9, 34] These results suggest that mdr1/Pgp is not a key factor in MDR of

gastric cancer. Interestingly, our in vitro results showed that all gastric cancer cell lines expressed MRP gene but not mdr1. To date, only one non-Pgp-mediated-MDR gastric cancer cell line has been reported.[35] The MDR mechanism in this cell line is thought to include drug compartmentalization,[35] which is also found in cancer cells overexpressing MRP gene, as previously reported.[16, 23] Thus, compared with mdr1/Pgp, the MRP gene is likely to have a more important role in MDR of gastric cancer.

Colon cancer is a typical mdr1/Pgp-positive cancer.[1, 4] However, an in vitro colon cancer cell line displaying non-Pgp-MDR phenotype has been selected, although the exact MDR mechanism involved clinically remains undetermined.[36] Half of the colon cancer cell lines analyzed in the present study expressed MRP gene only, while the other half and the majority of colon cancer tissues co-expressed MRP and mdr1 gene. These findings suggest that both or either of the two MDR genes may contribute to MDR of colon cancer. In this regard, MDR lung cancer and leukemia cells selected by VP-16 or Dox have recently been shown to co-express two MDR genes in the same cells and to express MRP gene in the early passages of the cells prior to mdr1 expression.[16, 37] However, it is possible that colon cancer tissues are heterogeneous, consisting of cells expressing either MRP gene or mdr1/Pgp. This should be resolved in future studies by immunohistochemical analysis for MRP and Pgp.

Although the role of MRP in intrinsic or acquired MDR in the clinical context remains speculative, recent studies have suggested that MRP may be associated with drug resistance observed clinically in patients with leukemia.[25, 38-40] In the present in vitro study, we found no significant correlation between the expression of MRP gene and sensitivity to various anticancer drugs, even to substrates for MRP, such as VP-16, Dox and VCR. Undoubtedly, other drug resistance mechanisms, including quantitative or qualitative changes in topoisomerase II, may influence sensitivity to VP-16 and Dox.[41] In fact, the expression of topoisomerase II among the tested cell lines varied considerably in the present study (data not shown). Accordingly, it seems that MRP expression alone does not account for all the differences in sensitivity of cell lines to topoisomerase II-targeting drugs. As shown in this study, it may be necessary to establish a drug sensitivity assay in the presence of MRP-modulating agents in order to elucidate the contribution of MRP to MDR in various types of cancer cells.

Interestingly, the MRP-modulating agent used in the present study (verapamil), significantly increased the sensitivity to VP-16, Dox and VCR in non-drug-selected gastric and colon cancer cells that highly expressed MRP gene. Verapamil was thought to inhibit competitively the

MRP function of pumping the drugs out of the cells and thus to increase the sensitivity. To date, there is no evidence indicating a direct interaction of MRP with MDR drugs. It has been reported that MRP is an ATP-dependent glutathione conjugate transporter.[42–45] Accordingly, the ability of individual cancer cells to facilitate the conjugation of the drug, or the intracellular glutathione level, may influence MRP-mediated MDR.

In support of this argument is the recent finding that buthionine sulfoximine increases the drug sensitivity of cancer cells overexpressing the MRP gene.[17, 18, 46] The mechanisms of MDR in cancer cells are obviously multifactorial and additional studies are needed to understand the role of MRP in the development of clinical resistance to cancer chemotherapy.

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