



Diallyl trisulfide reverses drug resistance and lowers the ratio of CD133⁺ cells in conjunction with methotrexate in a human osteosarcoma drug-resistant cell subline

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Abstract. P-glycoprotein (P-gp) overexpression and tumor stem cells are thought to be important factors in the cross-resistance of cancer cells. There have been no studies on whether diallyl trisulfide (DATS) can reverse drug resistance in human osteosarcoma cells. In the present study, we demonstrated that DATS down-regulated P-gp expression and reversed drug resistance. DATS and methotrexate (MTX) decreased the ratio of drug-resistant human osteosarcoma cells positive for CD133 (a tumor stem cell marker). To the best of our knowledge, this is the first evidence that DATS can reverse drug resistance and lower the ratio of CD133⁺ cells in human osteosarcoma cells in conjunction with MTX.

Introduction

Osteosarcoma is the most common primary bone malignancy in children and adolescents. Despite advances in surgery and multi-agent chemotherapy, long-term survival rates have remained at approximately 65%. One perplexing problem for successful chemotherapy is the development of multidrug resistance (MDR) by tumor cells, which is usually related to cross-resistance. P-glycoprotein (P-gp) is a plasma membrane glycoprotein that is an expression product of the MDR gene ABCB1 (ATP-binding cassette, sub-family B, member 1; MDR1). In a way, it functions like a pump, pumping out intracellular anti-cancer drugs and protecting cells from being killed. Overexpression of P-gp is the most common reason

for cross-resistance and MDR. The expression of P-gp has been linked to the development of MDR in human cancer, particularly in leukemia, multiple myeloma, neuroblastoma, soft tissue sarcoma and osteosarcoma (1,2).

On the other hand, the cancer stem cell hypothesis may provide a more interesting interpretation of drug resistance. Tumor stem cells have been described in some solid tumors (3-5). It is believed that regeneration and cancer metastasis after chemotherapy are closely related to residual tumor stem cells. The main reason for this is that tumor stem cells over-express MDR1 and Bcl-2; therefore, they have a low rate of apoptosis and can survive chemical treatment.

Garlic has been demonstrated to reduce cancer risk in experimental and epidemiological studies (6). Fresh garlic contains carbohydrates, proteins, fiber, 33 sulfur compounds, 17 amino acids, and various trace elements. The main components of its volatile oils are sulfur compounds, in particular allicin, diallyl sulfide (DAS), diallyl disulfide (DADS) and diallyl trisulfide (DATS). These have been defined as the main anti-tumor constituents of garlic. Moreover, DAS has been shown to reverse P-gp-mediated MDR in K562 leukemia cells (7). Another study from our laboratory revealed that a minimum dose of 50 μ M DATS had an obvious antiproliferative effect on an osteosarcoma cell line (Saos-2) through the induction of apoptosis (data not shown). However, it has yet to be reported whether DATS can down-regulate the expression of P-gp and reverse P-gp-mediated drug resistance. There have been no studies on the effects of DATS on drug-resistant osteosarcoma cell lines. Here, we evaluated the ability of DATS to reverse MDR in the drug-resistant human osteosarcoma cell subline Saos-2/R *in vitro*.

As a result of the effect of tumor stem cells on drug resistance, we detected changes in the number of CD133⁺ Saos-2/R cells. CD133 is an increasingly important surface protein marker of tumor stem cells that is present in many types of malignancies, including human brain tumors, hepatocellular carcinoma cells and colon cancer cells (4,8,9). Our previous research showed that CD133⁺ Saos-2, a human osteosarcoma cell line, overexpressed MDR1 in comparison with CD133⁻ Saos-2 cells (Fig. 1). This suggests that CD133⁺ Saos-2 cells share some properties with tumor stem cells.

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The search for appropriate methods of suppressing tumor stem cells, particularly in the case of drug resistant malignancy, is an increasingly important area of research. We hypothesized that the tumor stem cell marker ratio of drug resistant malignancy would be lowered by chemical treatment after drug resistance was reversed. Here, we evaluated changes in the CD133⁺ cell ratio of a drug-resistant human osteosarcoma cell subline after drug resistance was reversed.

Materials and methods

Cell culture and drugs. The human osteosarcoma cell line Saos-2 (ATCC) was grown as a monolayer in DMEM (Hyclone) supplemented with 20% fetal calf serum (Gibco) and 2 mM glutamine in a humidified incubator at 37°C with 5% CO₂. DATS was purchased from Zheng-da Tian-qing Pharmaceutical Corp. (Jiangsu, P.R. China). Methotrexate (MTX), diamminedichloroplatinum (DDP), adriamycin (ADM) and epirubicin (EPI) were purchased from Qilu Pharmaceutical Co., Ltd. (Shandong, P.R. China). These products are anti-cancer drugs commonly used in the treatment of osteosarcoma. Stock solutions of DATS were prepared in DMSO and stored at -20°C. For cell treatment, the drugs were further diluted in culture medium to the required concentrations, with a final concentration of DMSO of <0.5%.

Drug-resistant cell subline. A cell line resistant to MTX cytotoxicity was established in our laboratory by the progressive adaptation of sensitive Saos-2 parental cells to increasing concentrations of MTX. Briefly, Saos-2 cells were incubated for 48 h in culture medium with an initial MTX concentration of 0.05 µg/ml. After being washed twice in PBS, the cells were incubated in culture medium without MTX until the population doubling time was similar to that of the control cultures. This procedure was repeated six times, then the MTX concentration was increased to 0.5, 1 and 2 µg/ml. This procedure was repeated using increments of the MTX concentration over the span of a year, until the cells tolerated the continuous presence of 2 µg/ml MTX. The subline selected in this way was termed Saos-2/R. MTX was withdrawn from the medium 1 week before the cells were used for further study. Dead cells were first removed from Saos-2/R cells with the Dead Cell Discrimination Kit (Miltenyi) before analysis of CD133.

Sensitization of resistant cells to anticancer drugs. Saos-2 and Saos-2/R cell suspensions were plated in 96-well culture plates (1×10⁴ cells/well). According to the peak plasma concentration (ppc: MTX 1.5 µg/ml, DDP 2.5 µg/ml, ADM 0.5 µg/ml, EPI 0.8 µg/ml), seven concentration gradient wells were set up of 1000, 100, 10, 1, 0.1, 0.01 and 0.001 ppc. A blank control was also established. After being incubated for 48 h, 20 µl 5% MTT (Sigma) was added to every well, and the plate was placed in a CO₂ incubator. Four hours later, the supernatant was discarded and 150 µl DMSO was added. The plate was oscillated for 10 min. OD₄₉₀ was detected by a microplate reader (Wellscan MK3). The cell suppression ratio was calculated according to the formula: (1 - experimental group OD/control group OD) × 100%. The concentration that inhibited 50% of the cells was recorded as the half-inhibitory concentration (IC₅₀). The

resistance index (RI) was calculated according to the following formula: IC₅₀ (Saos-2/R)/IC₅₀ (Saos-2). RI <5, >15, or =5-15 was defined as low-level, high-level, or mid-level resistance to the drugs, respectively.

Western blot analysis. Cells were harvested and washed twice with ice-cold PBS. Lysates were obtained with TEN-T buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, pH 8.0, 1% Triton X-100, 1 mM PMSF, 2 µg/ml aprotinin) and then subjected to 10,000 × g centrifugation at 4°C for 20 min. The BCA Protein Assay Kit (Beyotime) was used to measure the protein concentration of the supernatants. Per lane, 50 µg of protein was electrophoresed in 8-16% or 10-20% SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Sigma). After blocking with TTBS (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.05% Tween-20) containing 5% (w/v) skimmed milk, the membranes were incubated with first antibodies (anti-P-gp and anti-β-actin; Santa Cruz Biotechnology) at 4°C overnight. Immunoreactive bands were detected by Super ECL Plus Detection Reagent (Amersham).

Total RNA extraction and RT-PCR analysis. Total RNA was isolated from treated cells using TRIzol Reagent (Gibco) and immediately reverse-transcribed using oligo (dT) 12-18 and Superscript II (Invitrogen) according to the manufacturer's protocol. In the same cDNA preparation, MDR1 (P-gp) was amplified by PCR using β-actin as an internal standard. The sequences of the oligonucleotide primers used for the amplification of MDR1 were: upper primer 5'-CAA TGT TTC GCT ATT CAA ATT-3' and lower primer 5'-ATT AAT CTT GGA GAC ATC ATC-3', defining a 436-bp product; and, for β-actin: upper primer 5'-CTC CCT GGA GAA GAG CTA CGA-3' and lower primer 5'-CGA TCC ACA CGG AGT ACT TGC-3', defining a 323-bp product. A 25-µl PCR reaction mixture contained 5 µl cDNA, 1X PCR buffer, 2 µM MgCl₂, 0.2 mM dNTP, 0.5 µM of each primer and 1.25 U Taq DNA polymerase (Takara). After an initial denaturation step at 94°C for 5 min, the PCR profiles for MDR1 and β-actin were amplified with 23 cycles at 94°C for 1 min, 62°C for 45 sec and 72°C for 45 sec. A final extension was performed at 72°C for 8 min. The PCR products were then separated on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV illumination.

MDR1 expression in CD133⁺/ Saos-2 cells. CD133⁺ cells were sorted out from Saos-2 cells to detect MDR1 expression according to the abovementioned procedure for RT-PCR. At least 1×10⁸ Saos-2 cells were collected, and CD133⁺ cells were sorted out from Saos-2 cells using a microbead kit (Miltenyi). MDR1 expression in CD133⁺ and CD133⁻ Saos-2 cells was detected by RT-PCR.

Modulation of MDR1 (P-gp) expression. Saos-2 and Saos-2/R cells were collected to detect MDR1 (P-gp) expression according to the procedure for Western blotting and RT-PCR described above. Saos-2/R cells were incubated in DMEM containing 20% FBS, 0, 10, 20, 30 or 40 µM DATS and 0.05% DMSO at 37°C in 5% CO₂. After incubation for 48 h, MDR1 (P-gp) expression was detected according to the aforementioned procedure for Western blotting and RT-PCR.

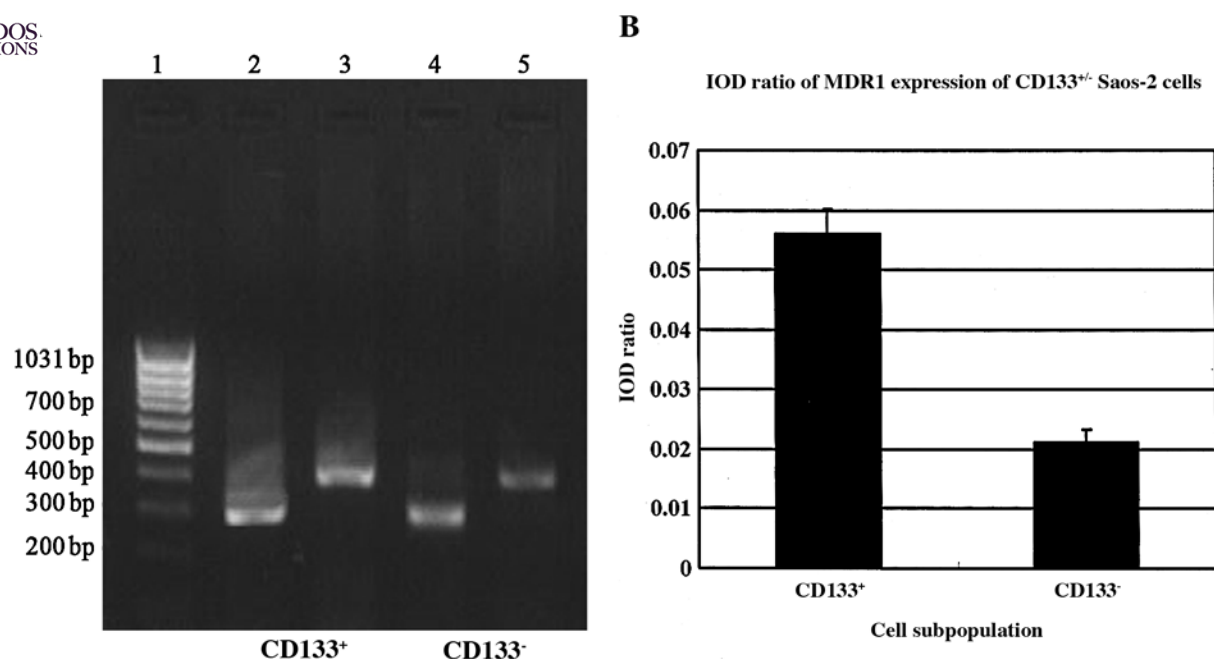


Figure 1. (A) MDR1 (P-gp) expression in CD133^{+/±} Saos-2 cells analyzed by RT-PCR. Lanes 2 and 3, CD133⁺ cells; lanes 4 and 5, CD133⁻ cells. (B) Graphical representation of integral optical density (IOD) ratios of MDR1 (P-gp) expression. The IOD ratios of CD133^{+/±} Saos-2 cells were 0.055±0.004 and 0.021±0.002, respectively. Data from B show that MDR1 (P-gp) expression was significantly greater in the CD133⁺ cells than in the CD133⁻ cells ($P<0.05$).

Table I. Half maximal inhibitory concentration ($\mu\text{g/ml}$) and resistance index of Saos-2 and Saos-2/R cells (mean \pm SD).

Drugs	IC ₅₀ (Saos-2)	IC ₅₀ (Saos-2/R)	P-value	RI
MTX	27.20±1.25	362.57±5.51	0.0000317898 ^a	13.34±0.49
DDP	89.19±0.32	189.50±3.70	0.0001931480 ^a	2.12±0.04
EPI	12.63±0.41	54.87±2.82	0.0007644150 ^a	4.35±0.26
ADM	10.30±0.53	50.39±1.60	0.0002233760 ^a	4.90±0.25

^a $P<0.05$. RI, resistance index; MTX, methotrexate; DDP, diamminedichloroplatinum; EPI, epirubicin; ADM, adriamycin.

Assay for drug resistance reversal. We chose 10 μM DATS as the reversing concentration according to our results for the modulation of MDR1 (P-gp) by DATS. A Saos-2/R cell suspension was plated in a 96-well culture plate (1×10^4 cells/well). The cells were incubated in DMEM containing 20% FBS, 1 ppc of chemotherapeutic drugs and 10 μM DATS at 37°C in 5% CO₂ for 48 h. Subsequently, the MTT tumor chemosensitivity test was performed according to the aforementioned procedure.

Flow cytometric analysis of CD133. Cell suspensions were plated in 24-well culture plates (1×10^6 /well) and incubated in DMEM containing 20% FBS for 36 h until they were fully adherent, then incubated in DMEM containing 20% FBS and increasing concentrations of MTX (0, 0.001, 0.01, 0.1 and 1 ppc) with/without 10 μM DATS at 37°C in 5% CO₂ for 48 h. Aliquots of cultured cells were re-suspended in cold PBS at a volume of 100 μl for antibody labeling. Approximately 0.05-0.1 μg of CD133 monoclonal antibody conjugated to PE (Miltenyi) was added to the cells; the reaction was incubated in the dark for 30 min at 4°C, then terminated by the addition

of 2 ml PBS. Cells were centrifuged at 400 \times g at 4°C for 10 min, then re-suspended in 0.5 ml cold PBS. Flow cytometry was carried out using a FACScan (Becton Dickinson) machine fitted with a 488-nm laser. No-antibody control was analyzed to delineate the unstained and autofluorescent population.

Image and statistical analysis. Integral optical density (IOD) analysis of Western blotting and RT-PCR images was performed with JD801 image analysis software (Jeda Science-Technology Development Co., Jiangsu, P.R. China). The IOD ratio was calculated according to the formula: IOD (P-gp)/IOD (β -actin). All tests were repeated three times. Statistical analysis were carried out using the Student's t-test with SPSS software. $P<0.05$ was considered statistically significant.

Results

Resistance characteristics of Saos-2/R cells. The MTT test results showed that the IC₅₀ of Saos-2 and Saos-2/R cells to MTX was 27.20±1.25 and 362.57±5.51 $\mu\text{g/ml}$, respectively ($P<0.05$). The RI of Saos-2/R cells to MTX was 13.34±0.49.

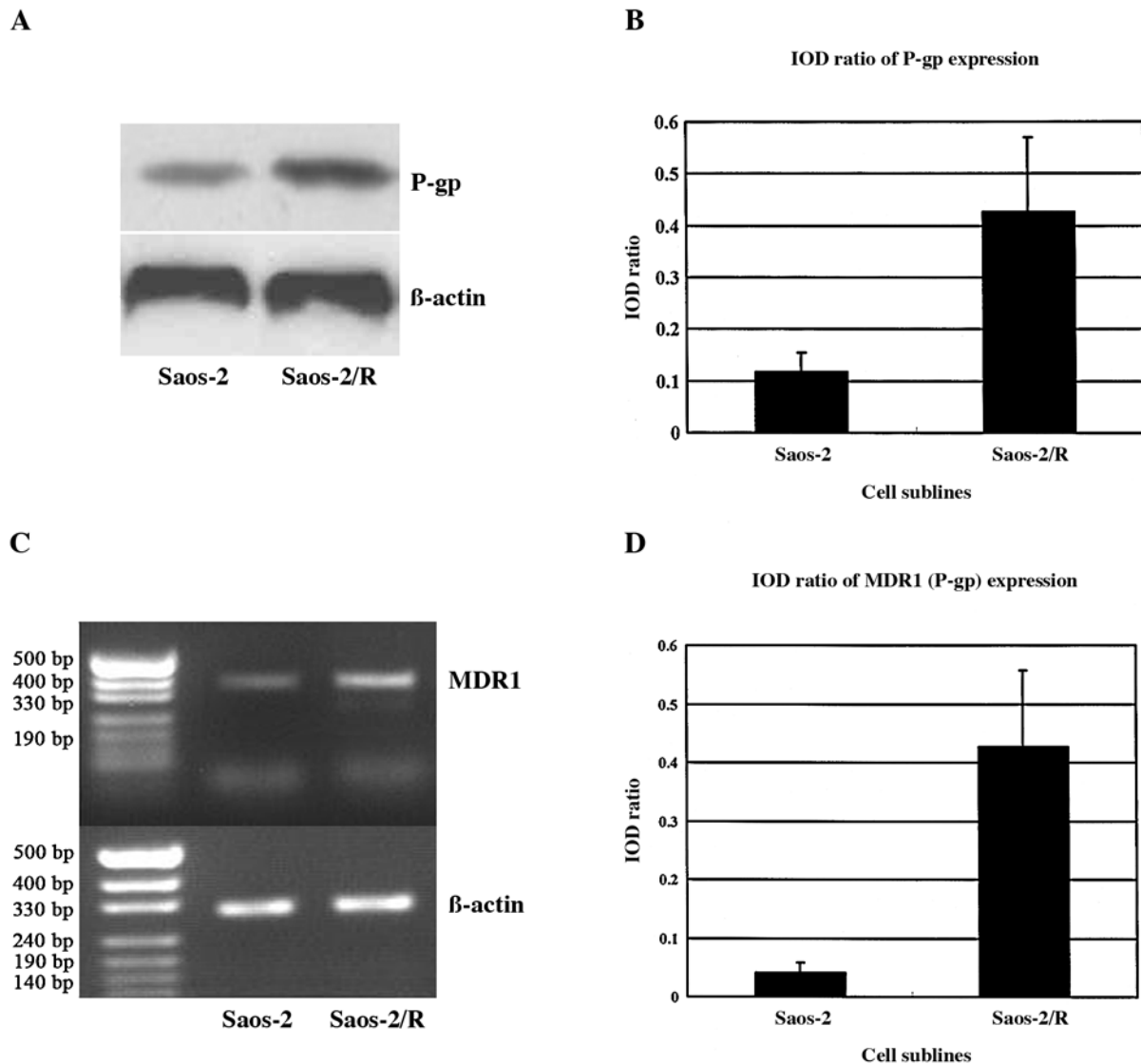


Figure 2. (A) P-gp expression in Saos-2 and Saos-2/R cells, analyzed by Western blotting. (B) Graphical representation of Western blot analysis of the integral optical density (IOD) ratios of P-gp expression. The IOD ratios of the Saos-2 and Saos-2/R cells were 0.11 ± 0.04 and 0.43 ± 0.14 , respectively. (C) Expression levels of MDR1 mRNA for Saos-2 and Saos-2/R cells, analyzed by RT-PCR. (D) Graphical representation of RT-PCR analysis of IOD ratios of MDR1 mRNA expression. The IOD ratios of the Saos-2 and Saos-2/R cells were 0.04 ± 0.02 and 0.41 ± 0.13 , respectively. Data from B and D show that Saos-2/R cells overexpressed MDR1 (P-gp) ($P < 0.05$).

This indicates that Saos-2/R cells had mid-level resistance to MTX. The RI of Saos-2/R cells to DDP, EPI and ADM was 2.12 ± 0.04 , 4.35 ± 0.26 and 4.90 ± 0.25 , respectively. Saos-2/R cells had low resistance to these drugs (Table I). These results suggest that Saos-2/R cells produced cross-resistance to many types of chemotherapeutic drugs.

MDR1 expression in CD133⁺ Saos-2 cells. We detected MDR1 expression in CD133⁺ Saos-2 cells using RT-PCR. Unfortunately, CD133⁺ Saos-2 cells were too rare to be adequately sorted for Western blotting under our laboratory conditions. As shown in Fig. 1, CD133⁺ cells overexpressed MDR1 in comparison with CD133⁻ cells ($P < 0.05$).

MDR1 (P-gp) expression of Saos-2 and Saos-2/R cells. To assess changes in P-gp expression in Saos-2/R cells, protein and mRNA levels were detected by Western blot analysis and RT-PCR using anti-P-gp monoclonal antibody and the

primers we designed. As shown in Fig. 2, Saos-2/R cells were found to overexpress MDR1 (P-gp) in comparison with the parental drug-sensitive Saos-2 cells ($P < 0.05$).

Modulation of MDR1 (P-gp) by diallyl trisulfide. We next investigated whether DATS had the ability to down-regulate P-gp overexpression in Saos-2/R cells. Western blotting and RT-PCR revealed that MDR1 (P-gp) expression experienced a concentration-dependent decline (Fig. 3). Statistical analysis showed that P-gp expression in cells treated with DATS ($10\text{--}40\text{ }\mu\text{M}$) was down-regulated and significantly lower than that of the $0\text{ }\mu\text{M}$ DATS group ($P < 0.05$). This indicates that $10\text{ }\mu\text{M}$ DATS effectively down-regulated MDR1 (P-gp) expression in Saos-2/R cells.

Flow cytometric analysis of the ratio of CD133⁺ cells. We detected the ratio of CD133⁺ Saos-2/R cells in the presence of increasing concentrations of MTX, with/without $10\text{ }\mu\text{M}$

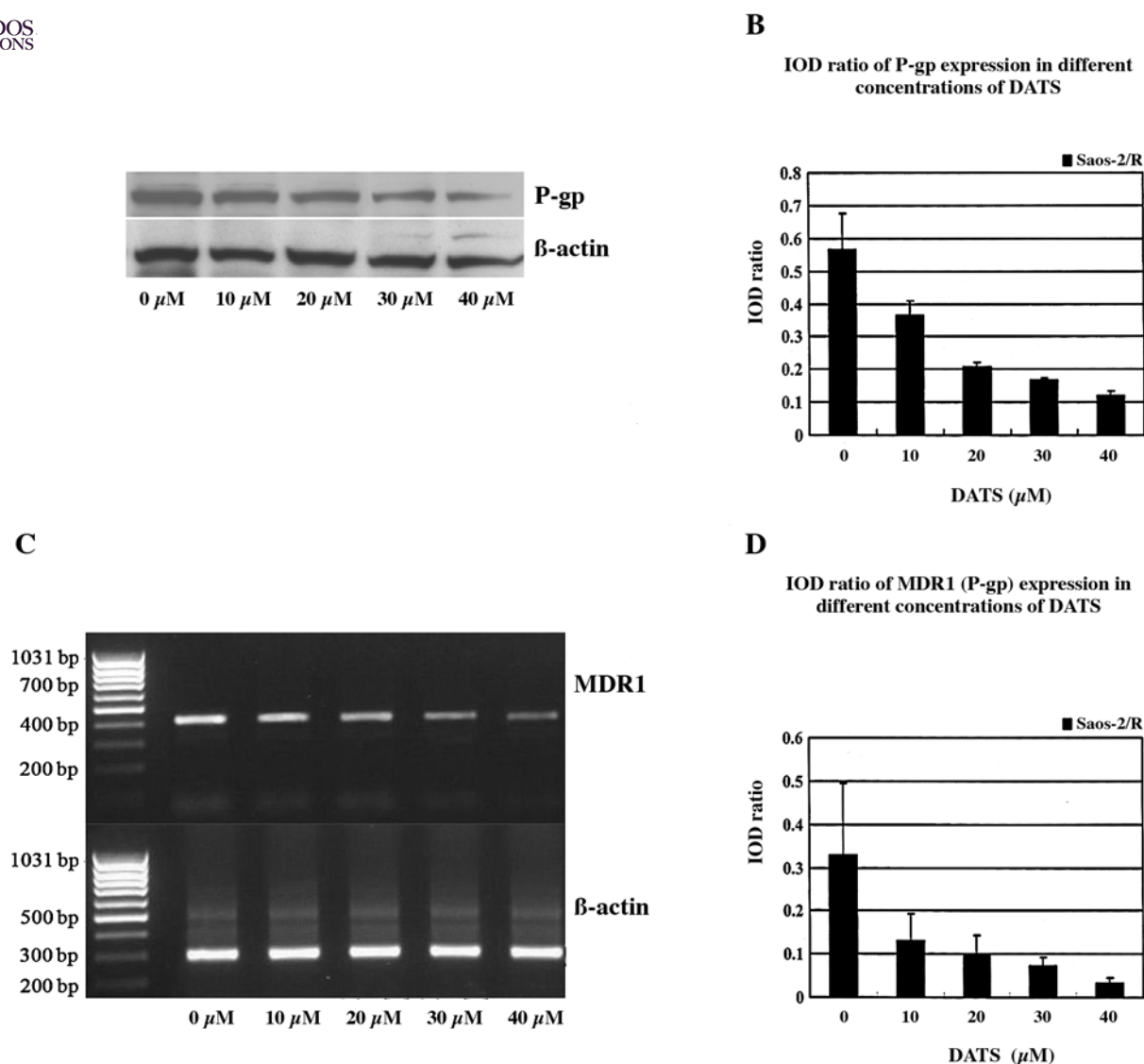


Figure 3. (A) P-gp expression in Saos-2/R cells after incubation with increasing concentrations of diallyl trisulfide (DATS), analyzed by Western blotting. (B) Graphical representation of integral optical density (IOD) ratios of P-gp expression. The IOD ratios of the 0-40 μ M groups were 0.57 ± 0.11 , 0.37 ± 0.04 , 0.21 ± 0.02 , 0.16 ± 0.01 and 0.12 ± 0.01 , respectively. (C) Expression of MDR1 mRNA in Saos-2/R cells after incubation with increasing concentrations of DATS, analyzed by RT-PCR. (D) Graphical representation of IOD ratios of MDR1 mRNA expression. The IOD ratios of the 0-40 μ M groups were 0.33 ± 0.16 , 0.13 ± 0.06 , 0.10 ± 0.05 , 0.07 ± 0.02 and 0.03 ± 0.01 , respectively. Data from B and D show that MDR1 (P-gp) expression experienced a concentration-dependent decline in the presence of DATS ($P < 0.05$).

DATS. As shown in Fig. 4, the ratio of CD133⁺ cells was stable in the group without 10 μ M DATS ($P > 0.05$), but experienced a concentration-dependent decline in the presence of 10 μ M DATS (Fig. 5). In addition, the ratio of CD133⁺ cells in this group was significantly lower than it was in cells without MTX ($P < 0.05$).

Reversal of drug resistance of Saos-2/R cells. After being exposed to 10 μ M DATS and 1 ppc MTX, DDP, EPI and ADM for 48 h, the IC₅₀ and RI of Saos-2/R cells dropped significantly (Table II). The RI of the MTX group decreased from 13.34 ± 0.49 to 4.01 ± 0.04 , and the RI of the other three drug groups also decreased significantly ($P < 0.05$).

Discussion

Cross-resistance to chemotherapeutic agents is a bewildering problem in the treatment of several types of human cancer. The

overexpression of MDR1 (P-gp) is an important mechanism of this resistance. The role of the MDR1 (P-gp) gene in the MDR of osteosarcoma has been confirmed (10,11). We verified that the Saos-2/R cell subline has cross-resistance characteristics. RT-PCR and Western blotting revealed that the MDR1 (P-gp) gene had significantly higher expression in Saos-2/R than in Saos-2 cells. This indicates that the cross-resistance of Saos-2/R cells was related to the overexpression of P-gp.

DATS is a volatile organosulfur compound derived from garlic. Many studies have shown that DATS can suppress the proliferation of various malignancies by inducing cytotoxicity, apoptosis and cell cycle arrest (12-14). Its related compound, DAS, has been shown to reverse P-gp-mediated MDR in K562 leukemia cells. Therefore, it is important to ascertain whether DATS can similarly reverse MTX-induced P-gp-mediated MDR in osteosarcoma cells.

The results of the present study preliminarily demonstrated that DATS is a highly potent modulator of P-gp-mediated

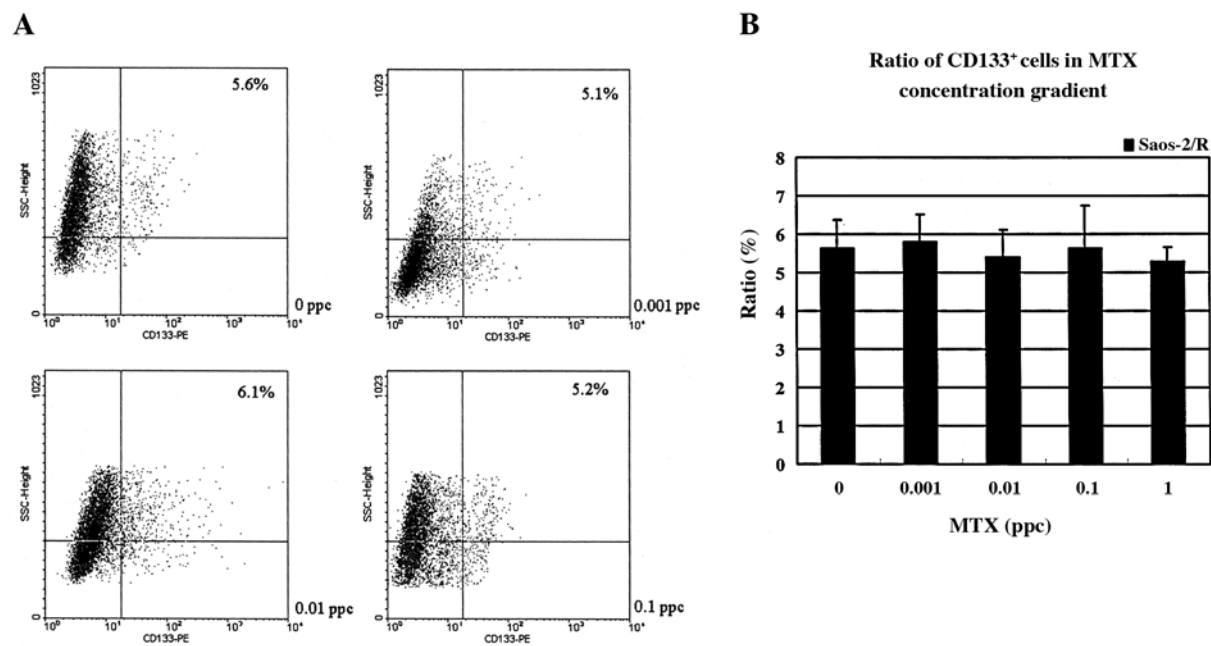


Figure 4. (A) Flow cytometric analysis of the CD133⁺ phenotype in Saos-2/R cells treated with methotrexate (MTX) without diallyl trisulfide (DATS). (B) Graphical representation of CD133⁺ cell ratios. The ratios of the 0-1 ppc groups were 5.67 ± 0.75 , 5.80 ± 0.70 , 5.40 ± 0.70 , 5.63 ± 1.12 and 5.30 ± 0.36 , respectively. The ratio in cells with increasing concentrations of MTX without DATS was stable ($P>0.05$).

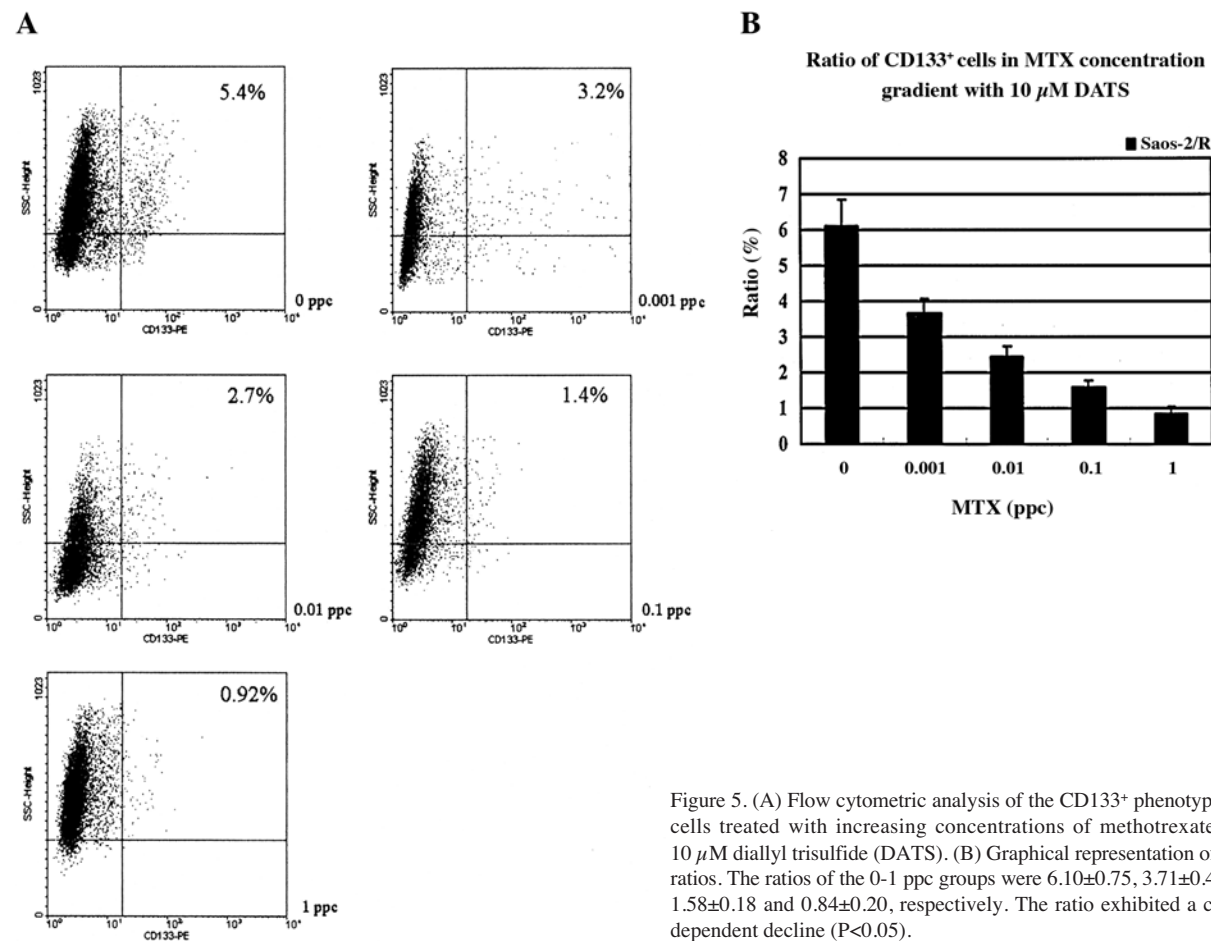


Figure 5. (A) Flow cytometric analysis of the CD133⁺ phenotype in Saos-2/R cells treated with increasing concentrations of methotrexate (MTX) and 10 μ M diallyl trisulfide (DATS). (B) Graphical representation of CD133⁺ cell ratios. The ratios of the 0-1 ppc groups were 6.10 ± 0.75 , 3.71 ± 0.42 , 2.39 ± 0.31 , 1.58 ± 0.18 and 0.84 ± 0.20 , respectively. The ratio exhibited a concentration-dependent decline ($P<0.05$).



Resistance index of Saos-2/R cells after a 48-h incubation without/with 10 μ M diallyl trisulfide (mean \pm SD).

Drugs	IC ₅₀	RI (without DATS)	IC ₅₀	RI (with DATS)	P-value
MTX	362.57 \pm 5.51	13.34 \pm 0.49	109.13 \pm 3.94	4.01 \pm 0.04	0.000390978 ^a
DDP	189.50 \pm 3.70	2.12 \pm 0.04	87.80 \pm 2.25	0.98 \pm 0.03	0.000308990 ^a
EPI	54.87 \pm 2.82	4.35 \pm 0.26	21.13 \pm 1.07	1.68 \pm 0.13	0.000685983 ^a
ADM	50.39 \pm 1.60	4.90 \pm 0.25	12.33 \pm 0.91	1.20 \pm 0.14	0.000579138 ^a

^aP<0.05. RI, resistance index; DATS, diallyl trisulfide; MTX, methotrexate; DDP, diamminedichloroplatinum; EPI, epirubicin; ADM, adriamycin.

MDR in human osteosarcoma cells. We found that MDR1 (P-gp) expression in Saos-2/R cells was significantly down-regulated by 10 μ M DATS after a 48-h culture in DMEM. MDR1 (P-gp) expression was down-regulated by DATS in a concentration-dependent manner (Fig. 3). In the assay of resistant cell sensitivity to anti-cancer drugs, the IC₅₀ and RI of Saos-2/R cells dropped significantly after a 48-h incubation with 1 ppc drugs and 10 μ M DATS.

There are many conflicting views regarding whether or not MDR1 (P-gp) expression plays a key role in the drug resistance of osteosarcoma. Many studies have shown that overexpression of MDR1 may be associated with drug resistance, tumor recurrence and poor outcome in osteosarcoma patients, which suggests that MDR1 overexpression can be used as a prognostic factor (15,16). However, others have failed to confirm this relationship, and the role of MDR1 as a marker of poor prognosis remains controversial (17,18). We found that MTX-resistant Saos-2 human osteosarcoma cells overexpressed MDR1 (P-gp) (Fig. 2), and that Saos-2/R cells showed cross-resistance to many chemotherapeutic drugs (Table I). While DATS down-regulated MDR1 (P-gp) overexpression (Fig. 3), the drug-resistance of Saos-2/R cells was reversed (Table II). These results support the view that MDR1 (P-gp) overexpression is associated with drug resistance in osteosarcoma.

The mechanism by which DATS reverses MDR appears to be mediated through the modulation of P-gp overexpression. Our results show that Saos-2 cells slightly expressed P-gp while, in Saos-2/R cells, P-gp was clearly overexpressed (Fig. 2). DATS decreased the IC₅₀ and RI in Saos-2/R cells, and simultaneously down-regulated expression of MDR1 (P-gp). However, these results were obtained *in vitro*. It is essential to carry out future studies *in vivo* to obtain a complete understanding of the mechanism of the DATS-mediated reversal of MDR.

We hypothesized that the ratio of a tumor stem cell marker would decrease if P-gp overexpression was reversed by DATS. Various important cancer stem cell markers (such as CD133) were detected in Saos-2/R cells after incubation with MTX without/with DATS. We demonstrated that the ratio of CD133⁺ cells was stable in cells incubated with increasing concentrations of MTX without DATS (Fig. 4). Additionally, the ratio of cells was revealed to be significantly reduced in the presence of increasing concentrations of MTX with 10 μ M DATS (Fig. 5).

The contribution of tumor stem cells to hematopoietic cancers has been established for some time, and cells possessing stem cell properties have been described in several solid tumors. Although chemotherapy kills most cells in a tumor, it is believed to leave tumor stem cells alone, which might serve as an important mechanism of resistance. The ATP-binding cassette (ABC) drug transporters have been shown to protect tumor stem cells from chemotherapeutic agents (19-21). The MDR1 gene is an important member of the ABC drug transporter family.

Previous research from our laboratory showed that CD133⁺ Saos-2 cells overexpressed MDR1 (P-gp) in comparison with CD133⁻ Saos-2 cells (Fig. 1). The present study showed that the CD133⁺ Saos-2/R cell ratio was obviously decreased in the presence of increasing concentrations of MTX with DATS, and that MDR1 (P-gp) overexpression was down-regulated by DATS. Unfortunately, CD133⁺ cells were too rare to be sorted from the Saos-2/R cells, which were scarce after being treated by drugs, for MDR1 (P-gp) expression analysis. We can only infer the mechanism in place from P-gp expression changes in the Saos-2/R cells. As a result of MDR1 (P-gp) overexpression, these CD133⁺ cells may have reduced the intracellular concentration of anti-cancer drugs and avoided being killed. Once MDR1 (P-gp) overexpression had been down-regulated, intracellular drug concentrations increased in these CD133⁺ cells until they were killed. The results hint that tumor stem cells of a drug resistant malignancy could not survive chemical treatment following the reversal of drug resistance. It is necessary to conduct further research in future to verify this possibility.

We conclude that low levels of DATS toxicity may contribute to the reversal of P-gp-mediated MDR in Saos-2/R cells *in vitro*. The CD133⁺ cell ratio was obviously decreased by MTX and DATS. We demonstrated that CD133⁺ Saos-2 cells shared various properties with tumor stem cells. This study revealed that DATS lowered the number of tumor stem cells in conjunction with MTX, and simultaneously reversed MDR in a human osteosarcoma drug-resistant cell subline. This has significant implications for the adjuvant chemotherapy of osteosarcoma.

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