

## Relationship between RFC gene expression and intracellular drug concentration in methotrexate-resistant osteosarcoma cells

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**ABSTRACT.** Osteosarcoma is a primary malignant tumor in adolescents, associated with high mortality and morbidity. The high-dose methotrexate (MTX) chemotherapy used to treat this disease may induce primary or secondary drug resistance, resulting in a reduced effect of comprehensive treatment. In this study, the relationship between reduced folate carrier (RFC) gene expression and intracellular drug concentration in MTX-resistant osteosarcoma cells (Saos-2) was investigated. MTX-resistant human osteosarcoma cells (Saos-2/MTX2.2, Saos-2/MTX4.4) were prepared. The sensitivities of Saos-2 (primary cells), Saos-2/MTX2.2, and Saos-2/MTX4.4 cells to MTX, diamminedichloroplatinum (DDP), ifosfamide (IFO), epirubicin (EPI), adriamycin (ADM), theprubicin (THP), and paclitaxel (PTX) were detected by MTT. The median inhibitory concentration (IC<sub>50</sub>) and resistance index were measured. Semi-quantitative RT-PCR was

used to evaluate the expression of RFC gene in cells. The intracellular  $^3\text{H}$ -MTX concentration was determined. Results showed that  $\text{IC}_{50}$  of Saos-2/MTX2.2 and Saos-2/MTX4.4 was 4.87 and 12.73 times that of Saos-2, respectively. Both Saos-2/MTX2.2 and Saos-2/MTX4.4 had resistance to IFO, ADM, EPI, THP, and PTX, but not DDP. Compared to Saos-2/MTX2.2 and Saos-2/MTX4.4, the expression of RFC mRNA in Saos-2 was significantly higher. The intracellular  $^3\text{H}$ -MTX concentration reached a peak at 50 min. After 70 min, the concentration was maintained at a plateau. During this phase, the  $^3\text{H}$ -MTX concentration in Saos-2 cells was 2.15 times higher than the concentration in Saos-2/MTX4.4 cells. The reduced RFC mRNA expression in PTX-resistant osteosarcoma cells may be related to the decrease in intracellular  $^3\text{H}$ -MTX concentration.

**Key words:** Osteosarcoma; Methotrexate; Reduced folate carrier; Drug resistance

## INTRODUCTION

Osteosarcoma is a primary malignant tumor in the bone, affecting a patient population of adolescents of 10-30 years of age. About 3 quarters of osteosarcoma lesions are in the distal femur, proximal tibia, femur, and pelvis, and the ratio of the diseased parts is 4:2:1:1. The clinical features of osteosarcoma are rapid progression and early lung metastasis. It has the highest juvenile mortality and morbidity of all bone cancers. Before the 1970s, the standard treatment of osteosarcoma was amputation, but the 5-year survival rate was only 10-20%. In the past 30 years, with the application and development of adjuvant chemotherapy, the 5-year survival rate has reached 60-70%. Now, comprehensive therapy including local surgery and adjuvant chemotherapy is the main treatment method for osteosarcoma. The improvements in adjuvant chemotherapy have not only improved the prognosis of patients with osteosarcoma, but have also improved the patients' limb salvage rate and quality of life.

Adjuvant chemotherapy is an important part of the comprehensive treatment of osteosarcoma. The early neoadjuvant chemotherapy drugs used were doxorubicin and cisplatin. Jaffe (1972) used high-dose methotrexate (MTX) for the treatment of osteosarcoma, and it was considered to be the most effective of single-drug chemotherapy drugs. Delepine et al. (1988) found that the MTX peak concentration in blood was related to tumor necrosis reaction via multifactorial regression analysis. Graf et al. (1994) and Bacci et al. (1998) also found that high-dose MTX chemotherapy could significantly improve the 5-year survival rate. However, in clinical practice, there is the fear that high-dose MTX chemotherapy may induce primary or secondary drug resistance, resulting in reduced effect of comprehensive treatment. Therefore, research on the MTX resistance of osteosarcoma is a hot topic for many scholars. In this study, the relationship between reduced folate carrier (RFC) gene expression and intracellular drug concentration in MTX-resistant osteosarcoma cells (Saos-2) was investigated, and the mechanism of resistance in osteosarcoma cells is discussed.

## MATERIAL AND METHODS

### Cell culture

Human osteosarcoma cells (Saos-2) (Chinese Academy of Sciences, Shanghai Biological Cells Institute, Shanghai, China) were cultured in RPMI-1640 medium with 10% fetal bovine serum. The shock therapy and gradual increased drug concentration method were carried out. When the cells had grown to 60-70% confluence in a logarithmic phase, MTX (Shanghai Hualian Pharmaceuticals, Shanghai, China) was added as an initial concentration. After 24 h, the cells were washed with 1X PBS at 37°C twice, and the medium free from drugs was added. After the proliferation of cells returned to a normal state, the drug impact process was repeated 3 times for each concentration. The increasing concentrations of MTX were used: 1.1, 2.2, and 4.4  $\mu\text{M}$ , respectively. This resistance induction process lasted for 7 months. Cell treated with different concentrations of MTX were grouped as Saos-2/MTX1.1, Saos-2/MTX2.2, and Saos-2/MTX4.4. Saos-2/MTX2.2 and Saos-2/MTX4.4 were selected for comparison with primary Saos-2 cells.

### MTT test

The sensitivities of Saos-2, Saos-2/MTX2.2, and Saos-2/MTX4.4 to MTX, ifosfamide (IFO), diamminedichloroplatinum (DDP), adriamycin (ADM), epirubicine (EPI), theprubicin (THP), and paclitaxel (PTX) were detected by MTT method. Cells were suspended with RPMI-1640 medium in logarithm phase, and were seeded on 96-well plates (200  $\mu\text{L}$ /well with  $1 \times 10^4$  cells) for 24 h of culture. Based on the clinical drug peak plasma concentrations, different concentrations of MTX, IFO (Jiangsu Henrui Pharmaceuticals, Lianyungang, China), DDP (Jiangsu Haosen Pharmaceuticals, Nanjing, China), ADM (Zhejiang Haizheng Pharmaceuticals, Taizhou, China), EPI (Pfizer Pharmaceuticals, USA), THP (Shenzhen Wanle Pharmaceuticals, Shenzhen, China), and PTX (Bristol-Myers Squibb Company, USA) (7 concentration gradients: 1000, 100, 10, 1, 0.1, 0.01, and 0.001 ppc) were added and the cells were incubated for 24 h. Then, 20  $\mu\text{L}$  5% MTT was added to each well. Four hours later, the culture supernatant was removed and 150  $\mu\text{L}$  dimethylsulfoxide was added to each well, followed by shaking for 10 min at room temperature. A Wellsan MK3 enzyme mark instrument was used to measure light absorption at 490 nm. Each drug concentration was repeated in triplicate, and the cells not treated with drugs were regarded as the control. The inhibitory rate of cells was calculated as  $(1 - \text{mean absorption value}_{\text{resistance group}} / \text{mean absorption value}_{\text{control group}}) \times 100\%$ , and the median inhibitory concentration ( $\text{IC}_{50}$ ) was identified. The resistant index of cells was calculated as  $\text{IC}_{50 \text{ Saos-2/MTX4.4}} / \text{IC}_{50 \text{ Saos-2}}$ .

### Expression of RFC mRNA of Saos-2, Saos-2/MTX2.2, and Saos-2/MTX4.4 cells

The extraction of total RNA was performed as follows: Saos-2, Saos-2/MTX2.2, and Saos-2/MTX4.4 cells were digested by trypsin in the logarithm growth phase, followed by centrifugation. After the removal of supernatant and resuspension, 1 mL cell suspension ( $5 \times 10^6$  cell/mL) was collected. After addition of 1 mL TRIZOL for cell lysis, we ensured full-cell lysis by pipetting, and then collected the liquid in a 1.5-mL Eppendorf tube. Then, 0.2 mL

chloroform was added to each tube and after 15 s of vortexing, all tubes were kept static for 2 min. All tubes were centrifuged (4°C, 12,000 rpm, 15 min), and the supernatant was retained. Then, 0.5 mL isopropanol was added to each tube, followed by gentle mixing at room temperature. Tubes were then kept static for 10 min. After centrifugation (4°C, 12,000 rpm, 10 min), the supernatant was removed. The sediments in each tube were gently washed by 1 mL 75% ethanol and then centrifuged (4°C, 12,000 rpm, 15 min). The retained sediments in the tubes were dried at room temperature and dissolved in DEPC water.

The reverse transcription reaction synthesis of cDNA was performed in a 0.5-mL Eppendorf tube, 1-5 µg RNA was added and the volume was adjusted to 11 µL by using DEPC water. Then, 1 µL 10 µM Oligo (dT) was added to each tube, mixed gently, and centrifuged. All tubes were put in 70°C for 10 min and immediately kept on ice. Then, 2 µL 10X PCR buffer, 2 µL 25 mM MgCl<sub>2</sub>, 1 µL 10 mM dNTPmix, and 2 µL 0.1 M DTT were added. After gentle mixing and centrifugation, samples were incubated at 42°C for 2-5 min. Then, 1 µL reverse transcriptase (AMV) was added, and samples incubated in a 42°C water bath for 50 min. The termination reaction consisted of incubation at 70°C for 15 min. Each tube was inserted in ice and 1 µL RNase added. The residual RNA in the tubes was degraded via incubation (37°C for 20 min). The cDNA was harvested and then stored at -20°C.

The PCR amplification was as follows: 12.5 µL RNase water, 5 µL 10X PCR buffer, 2 µL dNTPmix (2 mM), 2 µL MgCl<sub>2</sub> (25 mM), 0.5 µL upstream primer (10 µM), 0.5 µL downstream primer (10 µM), 0.5 µL Taq enzyme (5 U/µL), and 2 µL cDNA were added to each 0.5-mL Eppendorf tube. The amplification conditions of target genes and β-actin were as follows: 95°C for 5 min; 95°C for 30 s; 56°C for 30 s; and 72°C for 45 s. After 32 cycles, an extending process (72°C for 6 min) was performed. The final product was stored at 4°C. For electrophoresis of the amplification products a 5-µL sample was added to each lane of 2% agarose gel. The results were observed, saved, and analyzed by Bio-Rad gel imaging instrument. The image results of strips were statistically analyzed via the ImageMaster VDS Software 2.0 and the system software. The absorbance ratio of RFC/β-actin was used as the relative value of RFC mRNA. The above experiment was repeated 3 times, and the average value of each time was used for statistical analysis.

The primer sequences of RFC and β-actin genes were as follows: 1) RFC gene: F: 5'-CGTTCTTTGCCACCATCGTCA-3'; R: 5'-AGTCGCTGTGCTGCCTTCTCC-3', 203 bp. 2) β-actin gene: F: 5'-ACACTGTGCCCATCTACGACC-3'; R: 5'-AGGGGCCGGACTCGTCATAGA-3', 242 bp.

### Determination of <sup>3</sup>H-MTX in Saos-2 and Saos-2/MTX4.4 cells

We diluted 250 µCi [<sup>3</sup>,<sup>5</sup>,<sup>7</sup>-<sup>3</sup>H]-methotrexate sodium salt (<sup>3</sup>H-MTX) powder (GE Healthcare, USA) was diluted with 500 µL saline to a concentration of 0.5 µCi/µL, and the liquid was stored at -20°C. About 200,000 Saos-2 and Saos-2/MTX4.4 cells were seeded on 24 plates in the logarithmic phase, with 3 duplicated wells. After 48 h, 1 µL <sup>3</sup>H-MTX (0.5 µCi/µL) was added to each well, and the time of action was recorded. Cells in each well were washed with cold PBS 3 times at 5, 10, 30, 50, 70, and 100 min. Then, 100 µL Tween 80 was added to each well to rupture the cells, followed by shaking at 4°C for 24 h. The liquid in each well was removed and put into the liquid scintillation tube; 3 mL scintillation determination fluid was added. The intracellular β-ray was determined by the fluid scintillation analyzer. The experiment was repeated 3 times.

## Statistical analysis

The SAS 6.12 statistical analysis software was used for statistical analysis. All data are reported as means  $\pm$  SD. The *t*-test was used to analyze interclass difference, and  $P < 0.05$  was considered to be statistically significant. The differences in the RFC gene mRNA among the groups was explained via ANOVA.

## RESULTS

### Sensitivities of Saos-2, Saos-2/MTX2.2, and Saos-2/MTX4.4 cells to MTX and multidrug resistance

The results of MTT assay showed that the  $IC_{50}$  of MTX for Saos-2, Saos-2/MTX2.2, and Saos-2/MTX4.4 cells was  $25.78 \pm 0.29$ ,  $125.56 \pm 0.24$ , and  $328.24 \pm 0.29$   $\mu\text{g/mL}$ , respectively. The  $IC_{50}$  for Saos-2/MTX2.2 was 4.87 times that of Saos-2, with a low resistance to MTX. The  $IC_{50}$  of Saos-2/MTX4.4 was 12.73 times that of Saos-2, exhibiting moderate resistance to MTX. Both Saos-2/MTX2.2 and Saos-2/MTX4.4 had low resistance to 5 kinds of chemotherapeutic drugs (IFO, ADM, EPI, THP, and PTX), with no obvious resistance to DDP (Table 1).

**Table 1.** Sensitivities of Saos-2, Saos-2/MTX2.2 and Saos-2/MTX4.4 cells to chemotherapeutic drugs.

Drug	$IC_{50}$ (ng/mL)			$IC_{50}$ (ng/mL)	
	Saos-2	Saos-2/MTX2.2	RI	Saos-2/MTX4.4	RI
MTX	$25.78 \pm 0.29$	$125.56 \pm 0.24$	4.87	$328.24 \pm 0.29$	12.73
IFO	$583.23 \pm 0.14$	$2045.46 \pm 0.68$	3.5	$2346.52 \pm 0.37$	4.02
DDP	$127.67 \pm 0.21$	$148.69 \pm 0.45$	1.16	$156.56 \pm 0.87$	1.23
ADM	$8.80 \pm 0.45$	$32.58 \pm 1.26$	3.7	$34.67 \pm 0.23$	3.94
EPI	$10.68 \pm 0.35$	$36.89 \pm 1.38$	3.45	$43.67 \pm 0.46$	4.09
THP	$12.29 \pm 0.72$	$32.89 \pm 4.23$	3.19	$38.72 \pm 0.25$	3.15
PTX	$5.24 \pm 0.64$	$10.23 \pm 1.45$	1.95	$13.24 \pm 0.26$	2.44

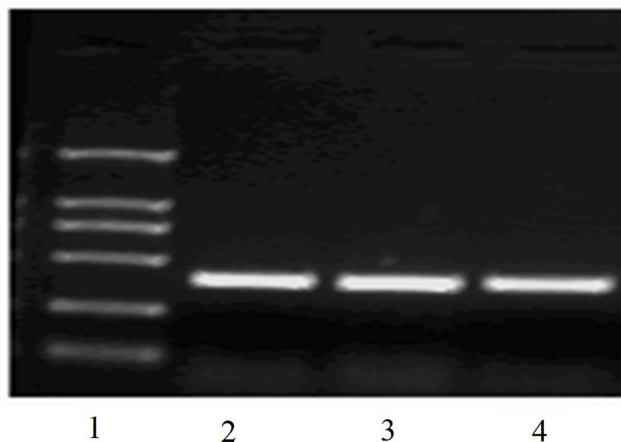
MTX = methotrexate; IFO = ifosfamide; DDP = diamminedichloroplatinum; ADM = adriamycin; EPI = epirubicin; THP = theprubicin; PTX = paclitaxel; RI = resistance index.

### Expressions of RFC mRNA in Saos-2, Saos-2/MTX2.2, and Saos-2/MTX4.4 cells

The expression of the reference gene ( $\beta$ -actin) mRNA is shown in Figure 1. The luminescence ratios of Saos-2, Saos-2/MTX2.2, and Saos-2/MTX4.4 to  $\beta$ -actin were analyzed by image Master VDS 2.0 image acquisition and analysis system, and results are shown in Table 2. Compared to Saos-2/MTX2.2 and Saos-2/MTX4.4 cells, expression of RFC mRNA in Saos-2 cells was significantly higher (Figure 2).

### $^3\text{H}$ -MTX concentrations in Saos-2 and Saos-2/MTX4.4 cells

The concentrations of  $^3\text{H}$ -MTX expressed in DPM1 (decayed atomic number per minute) in Saos-2 and Saos-2/MTX4.4 cells at the different times are shown in Table 3. Results showed that the intracellular  $^3\text{H}$ -MTX concentration reached a peak at 50 min. After 70 min, it was maintained at a plateau phase. During this phase, the  $^3\text{H}$ -MTX concentration in Saos-2 cells was 2.15 times that of the Saos-2/MTX4.4 cells.

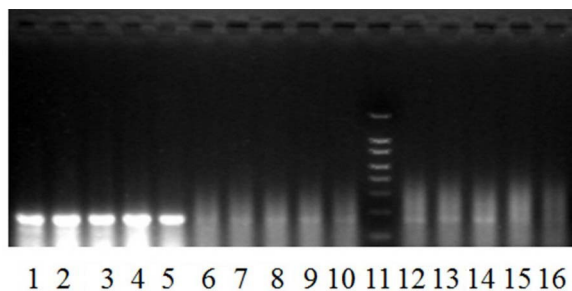


**Figure 1.** Expression of RFC mRNA in Saos-2, Saos-2/MTX2.2 and Saos-2/MTX4.4 cells. *Lane 1* = molecular marker (100, 250, 500, 750, 1000); *lane 2* = Saos-2; *lane 3* = Saos-2/MTX2.2; *lane 4* = Saos-2/MTX4.4.

**Table 2.** Expressions of RFC mRNA in Saos-2, Saos-2/MTX2.2 and Saos-2/MTX4.4 cells.

Cells	RFC/ $\beta$ -actin
Saos-2	8.56 $\pm$ 2.35
Saos-2/MTX2.2	0.35 $\pm$ 1.15*
Saos-2/MTX4.4	0.27 $\pm$ 2.24*

RFC = reduced folate carrier. \*P < 0.05 compared with Saos-2.



**Figure 2.** Expression of  $\beta$ -actin in Saos-2, Saos-2/MTX2.2 and Saos-2/MTX4.4 cells. *Lane 11* = molecular marker (100, 250, 500, 750, 1000); *lanes 1-5* = Saos-2; *lanes 6-10* = Saos-2/MTX4.4; *lanes 12-16* = Saos-2/MTX2.2.

**Table 3.** Intracellular  $^3\text{H}$ -MTX concentrations (DPM1) in Saos-2 and Saos-2/MTX4.4 cells.

Culture time (min)	Saos-2	Saos-2/MTX4.4
5	2220 $\pm$ 343.23	2604.66 $\pm$ 493.00
10	2055 $\pm$ 953.97	2612.33 $\pm$ 457.83
30	2411.66 $\pm$ 422.58	3775 $\pm$ 298.60
50	3820 $\pm$ 530.36	7397 $\pm$ 1167.80
70	2908 $\pm$ 915.75	6154.66 $\pm$ 423.42
100	2878 $\pm$ 867.66	6179.66 $\pm$ 649.11

DPM1 = decayed atomic number per minute.

## DISCUSSION

The mechanisms of multidrug resistance in osteosarcoma are not very unclear, which may be related with: 1) reduced endocytosis of MTX caused by RFC expression decrease or dysfunction; 2) amplification and enhanced transcription of dihydrofolate reductase (DHFR) gene, with alteration of TS activity, 3) decreased ratio of folypolyglutamate synthetase (FPGS)/folypolyglutamate hydrolase (FPGH), with reduced MTX-PG content, 4) binding of multidrug resistance protein to ATP to pump MTX out of cell. At present, the research shows that the number and function of RFC are important factors affecting the endocytosis of MTX. The decreased number and reduced ability of membrane transport proteins expressed by RFC may be the main contributors to resistance.

Normal body cells require folic acid, which is involved in the differentiation and proliferation of cells. Since cells cannot synthesize folic acid, they need to take it from their external environment. MTX, an anticancer drug, is transported following a pathway similar to that of folic acid. Research has confirmed (Horne, 1993; Moscow, 1998; Sirotiak and Tolner, 1999; Matherly and Goldman, 2003) that 2 classic transport pathways of folic acid are mediated by the RFC and folate receptor (FR). The structure, function, environment, and substrate of RFC and FR are different, and therefore, their affinities to folic acid and MTX differ. It has been suggested that there is a strong affinity between FR and folic acid, and that this plays a key role in the endocytosis of folic acid. Compared to the FR, RFC has greater affinities to MTX and leukovorin (LV). Thus, RFC-mediated MTX transmembrane transport is the main pathway in the endocytosis of MTX.

As an encoding gene, iRFC (RFC-1, FOLT, RFT-1, or SLC19A1) is also known as RFC1. As early as 1995, Moscow et al. cloned the human RFC gene (hRFC1) successfully. It is located on chromosome 21 q22, q22. 3 and contains 7 exons, in which 2 selectively noncoding exons are exons 1 and 2. It is an integral membrane protein with 591 amino acids, 12 typical transmembrane transport proteins, a short N end, and a C terminal of about 65 kDa. The hRFC protein in adult tissue is often expressed in the jejunum, ileum, colonic, duodenal brush-border membrane, renal tubular epithelial basement membrane, liver, choroid plexus, and other tissues (Matherly and Goldman, 2003). There are differences in the RFC gene structure in different species. The selective shear of the human RFC gene can lead to the removal of approximately 100 amino acids, and can result in extensive glycosylation. In contrast, there is no glycosylation in rodents. The change in RFC gene structure and the difference in post-translational modification can lead to a change in affinity between RFC and the folic acid antagonist.

We carried out an impact method by gradually increasing the doses of drugs to induce drug resistance in the primary human osteosarcoma cell line. This method was consistent with the characteristics of treatment of clinical osteosarcoma. The large doses of MTX in osteosarcoma treatment are effective, but this therapy needs an LV rescue. In our experiment, there was no LV rescue, and therefore, we chose a moderate dose and gradually increased the concentrations to induce resistance. This method not only ensured a smooth experimental process but it also conformed to the clinical characteristics of drug therapy. According to the theory and past reports about resistance mechanism (Godwin et al., 1992; Zhao et al., 1997; Vandier et al., 2000), we can identify the primary drug resistance cell line through the impact method, and the secondary drug resistance can be determined by gradually increasing the drug concentration.

*In vitro*, the resistance indexes of Saos-2/MTX2.2 and Saos-2/MTX4.4 cell lines to MTX are 4.87 and 12.73 times than that of the Saos-2 cell line. With elevated drug concentration and time, the resistance index improved gradually. There are varying degrees of resistance in Saos-2/MTX2.2 and Saos-2/MTX4.4 cell lines for IFO, ADM, EPI, THP, and PTX because of the different structures and mechanisms of these anticancer drugs. There may be a mechanism underlying multidrug resistance; but in the DDP group, there was no secondary resistance. This mechanism requires further study. With regard to the level of resistance of Saos-2/MTX4.4 cells to different drugs, the resistance to MTX is the strongest. Clinically, first several treatment courses have a good effect. However, after killing the most sensitive cells, the residual cells present obvious resistance characteristic, so the same therapy has a weakened effect.

The result of RT-PCR was a significant decrease in the RFC mRNA in drug-induced cell lines compared with that in the primary cell line Saos-2. The expression of RFC mRNA in drug-induced cell lines is 24.45 and 31.70 times higher. MTX transmembrane transport plays an important role in high-dose MTX chemotherapy, which is mediated by RFC. The increased protein and improved activity of RFC can increase the concentration of MTX in tumor cells, and enhance the antitumor effect of MTX. Low expression and changes in molecular structure, function, and activity can affect the capacity of MTX transmembrane transport, leading to drug resistance in tumor cells. Zhao et al. (1997) transfected RFC1 into an L1210 leukocyte with a defective carrier to investigate the characteristics of the RFC protein. The results showed that the increased RFC1 activity can lead to increased endocytosis and exocytosis of MTX, suggesting that the resistance of MTX depends on the mutation of the RFC gene. Matherly and Goldman (2003) and Sirotnak and Tolner (1999) summarized that the low expression of RFC is one of the contributors to the resistance of tumors to MTX chemotherapy. Guo et al. (1999) believed that the reduction of RFC in osteosarcoma is the main defense mechanism for primary resistance. Hattinger et al. (2003) found a significantly reduced expression of RFC in Saos-2 MTX-resistant variant cell lines, and its degree is associated with the resistance of MTX. Ifergan et al. (2003) detected 11 bone sarcoma specimens and 9 osteosarcoma recurrence specimens that had received high-dose MTX chemotherapies through Western blot. The data showed a positive correlation between the expression of RFC and sensitivity of tumors to chemotherapy ( $P = 0.0016$ ).

Conversely, Kaufman et al. (2004) found that in a child with acute lymphoblastic tumor, the mutations of genes are not a potential cause of MTX resistance. Guo et al. (1999) analyzed RFC and DHFR mRNA in 42 cases (primary and metastatic osteosarcoma specimens) by semi-quantitative RT-PCR and found the mechanism of osteosarcoma chemotherapy resistance. The main reason may attribute to reduced MTX volume transported by folate vector and increased expression of DHFR.

Serra et al. (2004) studied a series of cell lines (sensitive/insensitive to MTX) and found that in the U-20S osteosarcoma-resistant cell line, there was an elevated expression of DHFR with slightly elevated expression of RFC. However, in the Saos-2 cell line and the tumor suppressor gene RBI-negative cells, there was a negative correlation between drug resistance and RFC expression. According to the discussed research, in osteosarcoma, there is a significant correlation between RFC and MTX resistance. Low expression levels and dysfunction of RFC are the main factors contributing to primary drug resistance of osteosarcoma in MTX chemotherapy. Our results showed a decreased expression of RFC mRNA in drug-induced cells. In primary cells, RFC mRNA is significantly higher than that of the inducible cell



line, and this is consistent with the literature (Guo et al., 1999; Hattinger et al., 2003, Ifergan et al., 2003; Kaufman et al., 2004).

L5178Y (a cell line with deficient active transport) and normal transport cell lines were cultured in medium with 10  $\mu$ M MTX L5178Y cell line by Chabner and Young (1973). There was a mutation of the RFC gene, causing the expression and function of the RFC gene to decline. This affected the intracellular concentration of MTX, causing the intracellular concentration of MTX in normal transport cells to be 6.3 times higher. Our experiment found that in different cell lines cultured with  $^3$ H-MTX, the time to reach peak concentration of  $^3$ H-MTX in the cells is 30 min. After 50 min, the intracellular drug concentration reaches the plateau. In plateau, the intracellular drug concentration of the resistant cell line is 2.15 times higher than that of the primary cell line, suggesting that the declined expression of RFC downregulates the intracellular drug concentration of the drug-resistant cell line. This is caused by disorders associated with cell transport, which results in the reduction in MTX antitumor ability. Drug resistance is the final outcome.

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