

Significance of neoadjuvant chemotherapy (NACT) in limb salvage treatment of osteosarcoma and its effect on GLS1 expression

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Abstract. – **OBJECTIVE:** We examined the clinical significance of neoadjuvant chemotherapy (NACT) in limb salvage treatment of osteosarcoma and its effect on the Glutaminase 1 gene (GLS1) expression.

PATIENTS AND METHODS: 278 patients admitted to Qianfoshan Hospital Affiliated to Shandong University with osteosarcoma were randomly divided into the study group and the control group. Patients in the study group had 3-4 courses of cisplatin, ifosfamide, and adriamycin (DIA) chemotherapy before surgical excision, while no chemotherapy was used in the control group before the surgery.

RESULTS: GLS1 expression in the study group decreased, the difference showed statistical significance compared with that of the control group ($p < 0.05$). The median survival time of the study group was 48.4 ± 19.7 months while in the control group was 42.4 ± 11.2 . The overall survival time of study group was 58.5 ± 15.2 months, which was significantly higher than control 49.4 ± 10.7 ($p < 0.05$). The higher GLS1 expression in osteosarcoma was, the shorter the patients' survival time would be.

CONCLUSIONS: Neoadjuvant chemotherapy performed before surgery could significantly decrease the GLS1 expression in osteosarcoma, effectively improving limb salvage treatment of osteosarcoma. The higher the GLS1 expression was, the shorter the patients' survival time would be.

Key Words:

Osteosarcoma, Neoadjuvant chemotherapy, Glutaminase 1 gene (GLS1).

cal symptoms with small pulmonary metastasis at diagnosis, and poor prognosis¹⁻⁴. The median survival time and five-year survival rate of patients with osteosarcoma are relatively low. The five-year survival rate of osteosarcoma has gradually increased to 60% from 20%, and the limb salvage rate has significantly increased⁵. With the development of neoadjuvant chemotherapy (NACT), the therapeutic effect of osteosarcoma gradually improves, and the prognosis could be increased. NACT refers to a course of chemotherapy before excising pathological tumor tissues by surgery, which could control the local tumor tissues to limit tumor proliferation, and improve the survival rate of patients⁶. As one of the genes regulating tumor cells metabolism, the GLS1 function has drawn increased attention^{7,8}. In tumor cells, GLS1 produces glutaminase which transforms glucose to lactose without involving oxygen, and plays a vital role in the formation of the tumor microenvironment as well as tumor migration and metastasis⁹. In this study, for limb salvage operation in patients with osteosarcoma, we used immunohistochemical methods to test the GLS1 expression in tumor tissue and peri-tumoral normal tissues before and after neoadjuvant chemotherapy, to provide a theoretical foundation for clinical medicine and osteosarcoma treatment.

Patients and Methods

Patients

278 patients with osteosarcoma admitted to Qianfoshan Hospital Affiliated to Shandong University from March 2015 to March 2017 were selected. 42 males and 36 females, with an average

Introduction

Osteosarcoma, or osteogenic sarcoma has a high degree of malignancy and less early clinical

age of 38.5±12.7 years (range 10-54 years old) were included.

Tumor diameter ranged from 5-32 cm (12.9±5.9 cm in average). Pathological sections were classified by histology: 7 cases of I level, 23 cases of II level, 18 cases of III level; Enneking surgical stage: 2 cases of I a period, 3 cases of Ib period, 4 cases of IIa period, 32 cases of IIb period, 4 cases of IIIa period, 3 cases of IIIb period. All patients had no other tumors and no histories of radiotherapy and chemotherapy. Patients were randomly divided into study group (139 cases) and control group (139 cases). DIA (cisplatin, ifosfamide, and adriamycin) project was carried out in study group for chemotherapy, performing operation following 3-4 periods; MMIA (high dose methotrexate-adriamycin-ifosfamide-cisplatin project was carried out in control group before operation. This study was approved by the Ethics Committee of Qianfoshan Hospital Affiliated to Shandong University. The written informed consents were obtained from all patients involved in this work.

Equipment and Reagents

Reverse Transcription System A3500 (Promega, Madison, WI, USA), G-BOX gel imaging system (Syngene, Frederick, MD, USA); mouse anti-human GLS1 McAb, goat anti-rabbit GADPH (Bioscience, San Jose, CA, USA), immunohistochemical EnVision™ system kit (Syngene, Frederick, MD, USA).

Chemotherapy Regimen

Chemotherapy regimens in study group (DIA regimen)⁽¹⁰⁾: consists of cis-platinum (DDP), ifosfamide (IFO) and adriamycin (ADM). Medicine order: DDP chemotherapy was firstly performed and then IFO, ADM after 1 week, which is a course, the second-course chemotherapy was given after 2 weeks. Dosage: DDP: 120 mg/m², 4-6 h dropping, all medicines were given through a vein. Operation was performed 9 weeks after diagnostic biopsy. No neoadjuvant chemotherapy was carried out in control group and all medicines were given through a vein. Operation was performed 11 weeks after diagnostic biopsy.

Detection on GLS1 Protein Expression

Tumor tissue paraffin specimens before and after neoadjuvant chemotherapy were sliced up and then mounted on glass slide processed by polylysine. EnVision system was used in immunohistochemical staining, specific staining steps refers to kit instructions.

Surgical Methods

Surgical methods were as described previously^{11, 12}. Briefly, aspiration biopsy was performed to detect tumor tissue before operation. Limb salvage operation was performed following neoadjuvant chemotherapy. Operative time was 1 month after chemotherapy. Incising primary tumor using operation could thoroughly cure osteosarcoma, and X-ray, CT, and MRI inspection were carried out before operation to determine the scope of surrounding soft tissue and bone tissue invaded by tumor. According to Enneking stage, tumor tissue should be incised thoroughly and totally in the operation, avoiding damaging tumor and causing proliferation, and the whole incision should not only include tumor itself, but also reaction zones caused by peritumoral tissues and normal tissues invaded by surrounding tissues. Normal physiological structures (bones, muscles, and so on) 5 cm from tumor should be incised completely after separating tumor from normal tissues, which means osteotomy surface should be more than 5 cm from tumor. Pathological examination should be carried out in osteotomy tissues after incising tumor to determine whether tumor transferred or not, so as to provide reference for postoperative effectiveness. Operator and assistants should change sterile gloves, and then re-build bone limb defects.

Result Detection

Results were classified into four levels according to positive cell numbers. Negative (-) no brown reaction, staining were negative in cell background and positive cell numbers less than 5%; weakly positive (+): 5%-25% of tumor cell cytoplasm dyes faint yellow or light brown; median positive (++) : 25%-50% of tumor cell cytoplasm dyes brown; strong positive (+++) : over 50% of tumor cell cytoplasm dyes brown. All staining sections were inspected by two experienced pathologists respectively, and divergences which were not determined should be discussed with another pathologist. Median and strong positive samples were recorded as GLS1 protein expression positive in final result to calculate positive rate.

Statistical Analysis

SPSS18.0 0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis; measurement data were shown as mean ± standard deviation; count data were shown as percentage; chi-square test was used in measurement data

Table I. Comparison of general clinical indexes in enrolled patients.

Groups	Cases	Age	Sex (male/female)	BMI (kg/m ²)	Course (year)
Study group	139	139.2±12.7	22/17	21.5±0.3	1.6±2.8
Control group	139	38.5±10.9	20/19	20.8±0.4	1.5±1.9
T/X ² -value	-	0.33	0.28	0.29	0.45
p-value	-	0.71	0.79	0.87	0.58

in single-factor analysis; variance analysis was carried out in measurement data when obeying normal distribution, and non-parameter analysis (rank sum test) was used when not obeying normal distribution. Single factor analysis of variance and multiple comparison between the groups was performed using S-N-K method. Multiple regression analysis was used in correlation, and difference had statistical significance when $p < 0.05$.

Results

Comparison of General Clinical Indexes in Enrolled Patients

There were no differences in age, sex, BMI, osteosarcoma courses, and other baseline data in the study group and control group (Table I).

Effect of Neoadjuvant Chemotherapy on GLS1 Expression in Osteosarcoma Tissue

Aspiration biopsy was carried out in tumor tissues to extract tissue total protein, and GLS1 protein expression was detected. The result showed that GLS1 mRNA and protein expression had no statistical difference ($p > 0.05$) in osteosarcoma tissue before chemotherapy in the two groups (Figure 1A). GLS1 levels were analyzed in tumor tissue extracted after surgery, and the results showed that GLS1 expression in study group, after receiving neoadjuvant chemotherapy, was significantly lower than that of control group ($p < 0.05$). Results indicated that DIA regimen could significantly control GLS1 expression in osteosarcoma tissue (Figure 1).

Immunohistochemical Detection on Effect of GLS1 Expression in Osteosarcoma Tissue

The immunohistochemical SP method was carried out to make immunohistochemical staining on postoperative tumor tissue. Results showed that GLS1 positive zone percentage in the study group decreased significantly compared with the control group ($p < 0.05$) (Figure 2).

Survival Time

The disease-free survival was followed and compared in the two groups of patients. PFS in study groups was significantly higher than that of control group ($p < 0.05$); median survival time in study group was 48.4±19.7 months, and in control group was 42.4±11.2 months, while overall survival time in study group was 58.5±15.2 months, and in control group was 49.4±10.7 months ($p < 0.05$). The higher the GLS1 expression in osteosarcoma tissue was, the shorter the survival time of patients would be (Log-Rank test, $X^2 = 7.98$, $p = 0.004$).

Discussion

Osteosarcoma refers to a kind of malignant tumor common in bones of teenagers and children that mostly happens in the second ten years (10-20 years old). Poor clinical prognosis due to high malignance and high occurrence of early metastasis may bring heavy burden and serious consequence to patients and family¹³⁻¹⁵. The only way to cure osteosarcoma was amputation of disease limb before adjuvant chemotherapy¹⁶. However, osteosarcoma has always potential metastasis, while cure rate and postoperative survival time of patients were always low even after amputation. Most patients may get pulmonary metastasis inevitably, and their 5-year survival time is less than 20%. Osteosarcoma is one of the diseases with the highest mortality to teenagers¹⁷.

The pathogenesis of osteosarcoma is unclear. In the late 1970s, Lehrke et al¹⁸ proposed the concept of neoadjuvant chemotherapy before surgery in certain cancer patients. They further revised the concept of neoadjuvant chemotherapy, in clinical observation, imaging, laboratory analysis, and pathologic histology, so as to determine, observe, and analyze whether preoperative chemotherapy assists in further regulation of chemotherapy after operation and in choosing operative treatment.

Preoperative chemotherapy plus surgery plus postoperative chemotherapy were gradually performed clinically with continuously improving

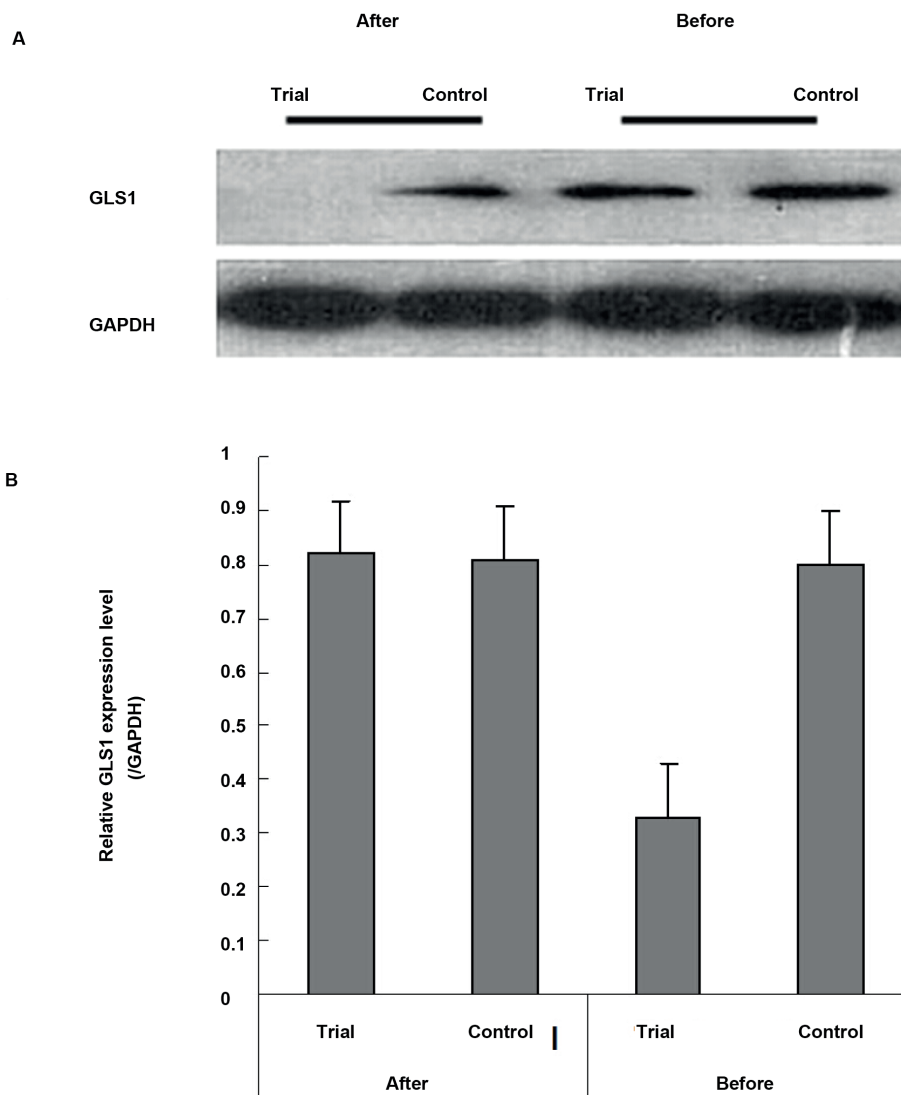


Figure 1. *A*, Western blot was performed to detect the GLS1 expression in osteosarcoma tissue of the two groups, and no statistical difference was found in the GLS1 expression of the two groups before neoadjuvant chemotherapy. After comparing GLS1 expression in osteosarcoma tissue of the two groups, it was found that GLS1 expression in study group (DIA) increased significantly compared with that of control group, and the difference showed statistical significance ($*p < 0.05$). *B*, Western blot result showed that the GLS1 protein expression in tumor tissue significantly decreased using DIA as neoadjuvant chemotherapy, which indicated neoadjuvant chemotherapy could decrease GLS1 expression in tumor tissue of patients. The difference had statistical significance ($*p < 0.05$).

neoadjuvant chemotherapy, which could significantly improve the clinical treatment of osteosarcoma. Its mechanism is mainly through limiting the distant metastasis of malignant tumor, to limit tumor cell spread and reducing operative scope and avoiding limbs' amputation¹⁹⁻²¹.

GLS1 (Glutaminase 1 gene) was separated from blood platelets by Sahai et al²² in 1983. An important biological activity of the expression product from the GLS1 gene is to influence the neurotransmitter activity of glutaminase due to

its character as a neurotransmitter. The product of GLS1 could degrade the effect of glutaminase in histocytes of organism²³. Some studies have shown that GLS1 is closely related with occurrence of some tumors. GLS1 plays a vital role in hepatocellular carcinoma and could be regulated through miRNA. Furthermore, the effect of glutaminase to regulate the glucose metabolism in tumor cells, called the Warburg effect, which means that tumor cells, with the action of glutaminase, tend to transfer glucose into lactose without

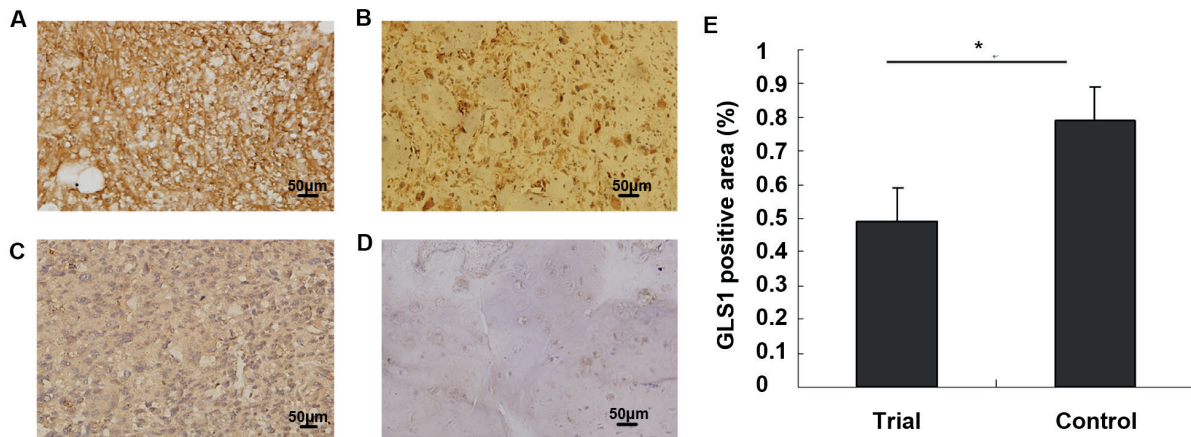


Figure 2. Immunohistochemical staining. **A**, GLS1 expresses (+++) in osteosarcoma tissue (100×). **B**, GLS1 expresses (++) in osteosarcoma tissue (100×). **C**, GLS1 expresses (+) in osteosarcoma tissue (100×). **D**, GLS1 expresses (-) in osteosarcoma tissue (100×). **E**, We showed that GLS1 expression has no statistical difference in control group and study group ($p < 0.05$).

involving oxygen. As the downstream target of GLS1, c-Myc could regulate oncogenic transcription factor through miRNA. GLS1 could be found in tumors of various organs, tissues, and diseases such as lung cancer, liver cancer, ovarian cancer, bladder cancer, leukemia, etc.²⁴.

The result of our work shows that GLS1 and its protein all express in tumor tissue of patients with osteosarcoma, which is in line with the biological character of malignant tumor. GLS1 expression significantly decreased in patients of study group receiving DIA chemotherapy compared with control group, and the difference was statistically significant ($p < 0.05$).

The result shows that combined chemotherapy of DDP, IFO, ADM has great effect in controlling osteosarcoma proliferation. Our results indicate that higher GLS1 expression may mean stronger tumor cell metabolism, and tumor cells may be resistant to chemotherapeutics, which may cause the distant metastasis of local tumor, and then make worse survival prognosis of patients with osteosarcoma.

GLS1 expression showed no change in tumor tissue before and after neoadjuvant chemotherapy. GLS1 expression showed no difference in tumor tissue following different

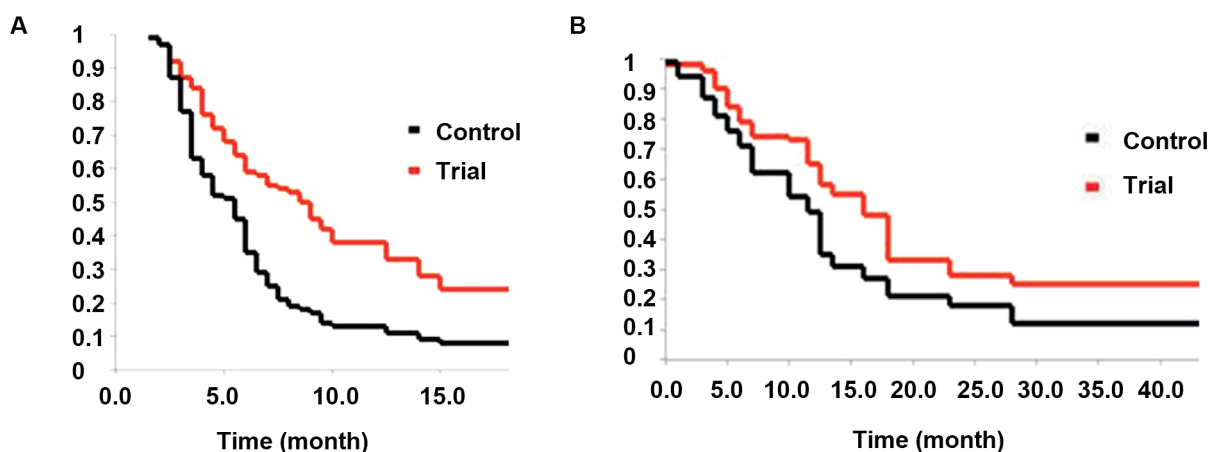


Figure 3. **A**, Disease-free survival rate was followed and compared in the two groups of patients. PFS in study groups was markedly higher than that of control group, and the difference was statistically significant ($p < 0.05$); median survival time in study group was 48.4 ± 19.7 months and in control group was 42.4 ± 11.2 months. **B**, Overall survival time in study group was 58.5 ± 15.2 months, and in control group was 49.4 ± 10.7 months, and the difference was statistically significant ($p < 0.05$). The higher the GLS1 expression in osteosarcoma tissue was, the shorter the survival time of patients had. Log-Rank Test, $X^2 = 7.98$, $p = 0.004$. The difference was statistically significant ($p < 0.05$).

neoadjuvant chemotherapies²⁵. This indicates that GLS1 expression in osteosarcoma has no relation with chemotherapy performing or choice of chemotherapy plan, but mainly relates to histologic characteristics of tumor itself. Therefore, if GLS1 expression could be known before choosing chemotherapy, it may predict the sensitivity of tumor to corresponding chemotherapy, and improve therapeutic effect of chemotherapy.

Our results were limited by a relatively small sample size. Therefore, the correlation between GLS1 expression in osteosarcoma and chemotherapy performing and chemotherapy plan needs further large-samples with a high-accuracy investigation.

Conclusions

We found that neoadjuvant chemotherapies could significantly reduce the expression and multidrug resistance of osteosarcoma. Higher GLS1 expression in osteosarcoma corresponds with shorter survival time. GLS1 could be a potential target of osteosarcoma therapy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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