GLS1 Promotes The Malignant Progression of Osteosarcoma

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Abstract

Background: Osteosarcoma (OS) is the most frequent and high-grade young malignant bone tumor. The prognosis is still poor despite the use of combined therapy involving maximal surgical resection, radiotherapy and chemotherapy. Metabolic reprogramming currently is recognized as one of the hallmarks of cancer. Glutaminase 1 (GLS1) has been associated with progression of tumor cell through CDK4 signaling pathway.

Methods: In the study, Western blot was used to detect the expression of GLS1 protein in tumor and adjacent normal tissues of osteosarcoma patients, and Western blot was used to detect the expression of GLS1 protein in osteosarcoma cell lines. GLS1 siRNA was transfected into osteosarcoma U2OS cells. Western blot was used to detect the expression of GLS1 protein. MTT and clone formation assay were used to detect cell proliferation. Transwell chamber assay was used to detect migration and invasion. Western blot was used to detect the expression of CDK4 protein in GLS1 knockdown U2OS cells. CB-839 was used to treat U2OS cells. The IC₅₀ value of CB-839 was detected by MTT. The proliferation and migration of CB-839 were detected by clone formation, scratch test, RNA seq sequencing, q-PCR and Western blot.

Results: (1) GLS1 was highly expressed in osteosarcoma tissues and cell lines; (2) After transfection, compared with the control group, GLS1 protein expression and CDK4 protein expression of U2OS cells in the knockdown group were significantly down regulated. In vitro experiments showed that the proliferation, migration and invasion of U2OS cells were significantly down regulated; (3) CB-839 promoted apoptosis and inhibited the proliferation and migration of osteosarcoma cells by acting on upstream transcription factors EGR1 and FOXO1.

Conclusion: GLS1 can promote the proliferation, migration and invasion of osteosarcoma cells by affecting the cell cycle of CDK4 signaling pathway, and can be used as a potential prognostic indicator and therapeutic target for osteosarcoma patients.

Background

Osteosarcoma (OS) is a type of primary malignant tumor with high malignant origin which originates from mesenchymal tissue. It can occur at any age. The incidence rate of osteosarcoma has two peaks. The 10–20-year youth group is the first peak of the disease. The age of 75 years old is the second peak of the disease. The incidence rate is about 4/100 million.^[1–3] The common sites of osteosarcoma are metaphysis, distal femur, proximal tibia and distal humerus. Patients with osteosarcoma in spine and pelvis usually have poor prognosis^[4–5]. Before the 1970s, there was no effective treatment for osteosarcoma. The main treatment method was simple amputation, and the overall survival rate of patients was about 20%. After the 1970s, with the emergence of adjuvant chemotherapy and neoadjuvant chemotherapy technology, high-dose methotrexate and neoadjuvant chemotherapy were used. Vincristine and doxorubicin are used as chemotherapy drugs in the treatment of osteosarcoma, and the overall

survival rate of patients is increased to about 60%. At present, the standard scheme of osteosarcoma treatment is: on the basis of preoperative neoadjuvant chemotherapy, combined with surgery to remove the tumor focus and postoperative adjuvant chemotherapy to treat osteosarcoma. However, with the development of new treatment, the survival rate of patients with metastatic and recurrent osteosarcoma has not changed significantly, and the overall survival rate is about 20%^[6-7]. Therefore, it is urgent to explore the causes of malignant progression of osteosarcoma, find new targets for the treatment of osteosarcoma, and provide new solutions for the treatment of osteosarcoma.

Glutaminase 1 (GLS1) is a key enzyme in tumor metabolism. In tumor metabolism, glutamine can transport glutamine into cells through cell membrane through transporter SLC1A5 (ASCT2). Through glutamine metabolism, GLS1 is used to decompose glutamic acid and ammonia, and its metabolites further provide nitrogen and carbon sources for tumor cells, Glutaminase is an important energy source of tumor biosynthesis, so glutaminase plays an important role in the occurrence and development of tumor^[8-11]. At present, there are two subtypes of glutaminase, one is renal glutaminase (GLS1), the other is hepatic glutaminase (GLS2)^[12]. There are two transcriptional splicing mutants in gls1: Renal glutaminase (KGA) and glutaminase C (GAC). Inhibition of KGA can clear aging cells and reduce the progression of age-related diseases^[13]. The expression of GLS1 was significantly correlated with the malignant progression of tumor. GLS1 was found to pass through Akt / GSK3 in HCC β / CyclinD1 pathway promotes the proliferation of hepatoma cells^[14]. In breast cancer, CB-839, a novel GLS1 inhibitor, can significantly inhibit the proliferation, migration and invasion of triple negative breast cancer^[15]. High expression of gls1 was found to be associated with the development of colorectal cancer. GLS1 plays an important role in tumor growth and metastasis, and its expression is closely related to hypoxia induced migration and invasion in vitro and in vivo^[16]. In non-small cell lung cancer and thyroid cancer, it is found that tumor cells are most sensitive to the changes of glutamine metabolism and inhibit the abnormal proliferation of tumor^[17-19]. In melanoma, we found that CB-839, an inhibitor of GLS1, can improve the immune function in tumor microenvironment by changing the metabolism of tumor and immune cells in varying degrees, and inhibit the activity of tumor cells by increasing the infiltration of effector T cells by combining with PD-1 and CTLA4 antibodies^[20]. CB-839, as a new inhibitor of GLS1, has a good therapeutic prospect in many preclinical studies. In triple negative breast cancer, CB-839 combined with paclitaxel can inhibit the proliferation of tumor cells by inhibiting the consumption of glutamine^[21]. In chondrosarcoma, IDH2 mutant chondrosarcoma was found to have an increased demand for glutamine, and CB-839 could significantly inhibit its proliferation^[22]. In multiple myeloma, CB-839 can enhance the stress and apoptosis of endoplasmic reticulum induced by katzomib by strongly inducing the expression of ATF4 and CHOP and the activation of cleaved caspase 3^[23]. CB-839 can increase the production of reactive oxygen species in PIK3C dependent rectal cancer, leading to nuclear translocation of NRF2, and then up regulate the expression of uridine phosphorylase 1 (UPP1) to inhibit the malignant process of rectal cancer cells^[24]. All the above studies indicate that CB-839 has good biological activity and inhibits the malignant progression of tumor cells through a variety of pathways. In 2017, two phase II clinical

studies of CB-839 have been carried out worldwide, so CB-839 may become an important tumor targeting inhibitor.

In tumor cells, transcription factors, as important upstream regulators of genes, affect important biological functions in tissues and organs, and regulate important biological processes. FOXO1 transcription factor is a member of FOX family, which is involved in a variety of biological processes, including the regulation of oxidative stress, DNA damage repair, cell cycle arrest and promoting apoptosis [25]. Studies have shown that FOXO1 is highly expressed in a variety of normal tissues, and its activity is significantly inhibited in cancer tissues [26-30]. FOXO1 is a tumor suppressor gene found in prostate cancer research. FOXO1 can inhibit the proliferation of tumor cells by acting on androgen signaling pathway and promote apoptosis through apoptosis pathway [31]. In the study of breast cancer, the expression of FOXO1 is down regulated. Estrogen E2 inhibits the expression of FOXO1 and promotes the malignant process of tumor cells [32-33]. In the study of colon cancer, FOXO1, as a tumor suppressor gene, is activated and phosphorylated by PI3K / Akt pathway, which inhibits the expression of FOXO1 and promotes the malignant process of tumor. The apoptosis rate of FOXO1 cells is higher than that of FOXO1 knockout cells [34]. FOXO1 can inhibit cell proliferation in G1/S phase of cell cycle. In conclusion, FOXO1 is closely related to the malignant process of many kinds of tumors and regulates the biological progress of tumors. EGR1 transcription factor is one of the members of the early gene family. The expression of EGR1 is closely related to the progress of a variety of human diseases, including inflammation, atherosclerosis and tumor related diseases [35-36], EGR1 affects inflammation, thrombosis and apoptosis by regulating the transcription of downstream genes. In the study of liver cancer, it was found that zidovudine can regulate the expression of EGR1, which may inhibit the proliferation of tumor cells by controlling p53 and caspase-3 pathway and promoting apoptosis [37]. In conclusion, EGR1 and FOXO1 transcription factors as tumor suppressor genes play an important role in a variety of biological processes. In the study of osteosarcoma, we found that propofol regulates Akt / GSK3 β through FOXO1 / TUSC7 axis signal pathway inhibits proliferation, migration and invasion of osteosarcoma cells [38]. EGR1 transcription factor can be inhibited by miR-191-5p and promote the malignant process of osteosarcoma cells through positive regulation of PI3K / Akt pathway [39].

We found that super enhancer plays an important role in the occurrence and development of osteosarcoma [40]. Further screening and sequencing results showed that gls1 gene associated with super enhancer may play an important role. At present, the role and mechanism of GLS1 in the malignant process of osteosarcoma have not been reported at home and abroad. Therefore, to explore the role and mechanism of GLS1 in the occurrence and development of osteosarcoma, and to explore the effect and mechanism of a new GLS1 inhibitor CB-839 on osteosarcoma will help to understand the malignant process of osteosarcoma from the molecular level. It provides a new target for targeted therapy of osteosarcoma and a new theoretical basis for CB-839 as a new strategy for the treatment of osteosarcoma.

Materials And Methods

1Reagents and specimens

DMEM medium, 2.5 g/L trypsin, fetal bovine serum, penicillin and streptomycin double antibody solution, and tetramethylazozolium salt (MTT) were purchased from sigma company. CA No.: C11995, 03-050-1A 10270-106, 03-034-1A, A056. The BCA kit was purchased from biyuntian company. LipofectamineTM 3000 was purchased from thermo, USA, and GLS siRNA was purchased from Guangzhou Ruibo biological company, vinculin, GLS1, β - Actin, CDK4, cleaved-Caspase3, FOXO1 and EGR1 antibodies were purchased from Abcam company. CB-839 was purchased from Selleck company in Shanghai. Source of specimen: the specimens of 12 cases of osteosarcoma tissue and para cancerous tissue used in the study were from amputated patients in the Department of bone oncology, the First Affiliated Hospital of Sun Yat-Sen University (approval No.: [2019] 060), and all patients were diagnosed by postoperative pathology. All patients signed informed consent.

2 Main Methods

2.1Tissue protein acquisition

After amputation, osteosarcoma and its distal normal muscle tissue far away from the reaction area were obtained. The tissue was grinded in a precooled grinder for 30 s, and the tissue was fully broken to obtain its protein, which was stored in a refrigerator at - 80 °C.

2.2Cell culture and siRNA transfection of GLS1

Osteosarcoma cell lines U2OS, U2OS / MTX300, MNNG / HOS, HOS, 143B, SJSA-1 were purchased from the American ATCC cell bank. They were cultured in DMEM medium containing 10 g/L fetal bovine serum, 1% penicillin and streptomycin at 37 °C in a 5% CO2 incubator for 2-3 days. According to the instructions of LipofectamineTM 3000, the siRNA bacterial solution of GLS1 was transfected into 50% of normal cells, namely, siGLS1-nc, siGLS1-1, and siGLS1-2. After 8 hours of transfection, the normal medium was changed. After 48 hours of culture, the cells were lysed for the detection of GLS1 protein expression and subsequent function test.

Table 1 Synthetic sequence of siRNA

group	Target sequence
siGLS1-1	5'-GCATCGATATGTTGGAAAA-3'
siGLS1-2	5'-GGTAAATGCTGGAGCAATT-3'

2.3 Western blot

After 48 hours of transfection, the cells were collected and placed in a precooled EP tube. 0.3 ml RIPA lysis buffer was added into the EP tube, and then placed in a 4 °C shaker for 30 min, followed by 15000×g. After centrifugation for 60 min, the supernatant was the total protein. According to the kit

instructions of Biyuntian, the cytoplasmic proteins were extracted. SDS electrophoresis was carried out and then transferred to PVDF membrane. 5% skimmed milk powder was sealed at room temperature for 1 h, and 1:1000 primary antibody was incubated overnight at 4 °C. The 1:5000 second antibody was incubated at room temperature for 1 h, and then the chemiluminescent agent was added for detection. The X-ray film was exposed in the dark room, and the conventional development and fixation were carried out. Image J software was used to analyze the relative expression level of target protein. The experiment was repeated three times and the average value was calculated.

2.4 MMT assay

The cells in logarithmic phase were digested and counted, and then the 96 well plate was laid, 3000 cells per well, 100ul system. For four consecutive days, 50ul MTT (1:4 diluted MTT mixture) was added at a fixed time every day. The cells were cultured in 37 °C and 5% CO₂ incubator for 4 hours. The upper liquid was absorbed, 200ul DMSO was added, and the absorbance (D) was detected at 490nm wavelength. The experiment was repeated three times, Calculate the average.

2.5Cell cloning formation assay

After the cells were digested and counted in logarithmic phase, the plates were laid in 6-well plates with 900 cells per well and 2ml DMEM. The medium was changed once every 4 days. After 2 weeks, the medium was removed. The plates were fixed with 4% paraformaldehyde for 30min and stained with 0.05% crystal violet for 30min. Then the whole plates were photographed and counted. The experiment was repeated three times and the average value was calculated.

2.6 Transwell assay

After the logarithmic phase cells were digested and counted, 8×10^4 cells were added into each upper chamber, and the culture system was 100 µL DMEM medium containing 1% FBS, adding 600 µml DMEM medium containing 10% FBS in the lower chamber was placed in 37 °C and 5% CO₂ incubator for 24 hours. A proper number of cells were observed to penetrate into the lower layer to complete the experiment. The chamber was taken out, fixed with methanol for 30 minutes, stained with crystal violet for 30 minutes, and then photographed. Five random fields were recorded under 20 times magnification for data analysis. The experiment was repeated three times and the average value was calculated.

2.7 Analysis of gene expression by quantitative real-time PCR

RNA extraction When the cell density was about 60%, CB-839 (8µM) After 48 hours, discard the medium, add 2 ml PBS, wash for 3 times, and add 700 µml PBS buffer solution, standing at 37 °C for 5 min, blowing different parts of the lysate in the dish to make it fully fused. Use the precooled centrifuge at the speed of 15000 R / min for centrifugation for 5 min, collect the liquid at the bottom, and add 700 µ L 70% ethanol, centrifuge at 10000 R / min for 1 min, then discard the liquid in 2 ml collecting tube and add 500 µml RNA wave buffer I, centrifuged at 10000 R / min for 1 min. Continue to replace the new 2 ml

collecting tube and add 500 µml. After centrifugation at 10000 R / min for 1 min, a new 2 ml collecting tube was replaced and 500 µml RNA was added. After centrifugation at 10000 R / min for 1 min, a new 2 ml collecting tube was finally replaced. Centrifugation at 15000 R / min for 2 min, the remaining liquid in the red column was dried, and the Hi bind matrix was dried. The red column was moved into a 1.5 ml collecting tube again, and 40 µml was added. The RNA solution was collected by centrifugation at the speed of 15000 R / min for 2 min. the RNA concentration in the cells was detected by microplate reader. The RNA solution was stored in an ultra-low temperature refrigerator at - 80 °C for freezing to prevent degradation.

2 mRNA Reverse transcription The genomic DNA removal reaction solution system was prepared with pre cooled ice box, and the experiment was carried out according to the operation process of TaKaRa reverse transcription reagent.

3 mRNA Real Time PCR: The experiment was carried out according to the operation procedure of TaKaRa q-PCR reagent, with 0.8 for each group µL's forward primer, 0.8 µL's reverse primer, 0.4 µL ROX reference dye II, 2 µL of cDNA template, 10 µL TB Green Premix Ex Taq II and 6 µL DEPC enzyme free water to make 20 µL. The total system was added to the q-PCR plate, covered with a film, and centrifuged at 3000 R / min for 3 min in a precooled high-speed centrifuge. In the first stage, the reaction parameters were set at 95 °C for 30 s, the second stage was set at 95 °C for denaturation for 5 s, and the next stage was annealed at 60 °C for 34 s for 40 cycles. In the third stage, the reaction parameters were set at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, about 1 h to complete the whole quantitative amplification experiment, According to the experimental data, CT value is selected for further statistics and drawing.

3 Statistical analysis

GraphPad PRISM 8.0 was used for mapping and SPSS 21.0 software (IBM Corporation, Armonk, NY) was used for statistical analysis. The experimental data were calculated by means ± Standard deviation (mean ± SD). T-test was used for comparison between the two groups, and one-way ANOVA was used for comparison between multiple groups. P < 0.05 was considered as statistically significant.

Results

1 Expression of glutaminase-1 in osteosarcoma, normal tissues and osteosarcoma cell lines

The expression of GLS1 was detected by Western blot, and vinculin (a cytoskeletal protein and focal adhesion component protein, mainly distributed in cells) was used as internal reference^[16]. The results showed that the relative amount of GLS1 protein expression in tumor tissue was significantly higher than that in normal tissue adjacent to tumor, with significant statistical difference (P < 0.05).

Immunofluorescence results showed that the expression level of GLS1 in normal tissue adjacent to osteosarcoma was lower, and the expression level of GLS1 in osteosarcoma tissue was significantly up-regulated. These results suggest that GLS1 may play an important role in the occurrence and development of osteosarcoma, as shown in Figure A and B in Figure 1. In order to further study the

specific function of glutaminase-1, the appropriate cell lines were selected for further experiments. Western blot was used to detect the protein expression of GLS1 in U2OS, U2OS / MTX300, MNNG / HOS, HOS, 143B, SJSA-1 cell lines commonly used in osteosarcoma, and the results of Western blot showed that the protein expression of gls1 in U2OS, U2OS / MTX300, MNNG / HOS, HOS, 143B, SJSA-1 cell lines commonly used in osteosarcoma was higher than that in other cell lines, the expression of GLS1 in SJSA-1 was higher than that in U2OS and MNNG / HOS cells (Figure C in Figure 1).

2 GLS1 inhibits U2OS cell proliferation, migration and invasion by acting on CDK4 signaling pathway

In order to explore the function of glutaminase-1 in osteosarcoma, U2OS and MNNG / HOS cell lines with high expression of glutaminase-1 were selected in this study. The cells were knocked down by siGLS1-nc, siGLS1-1 and siGLS1-2 respectively. The knockdown efficiency was detected by Western Blot. The results showed that the down regulated GLS1 expression cells were constructed for subsequent functional test (Figure A,B in Figure 2). The proliferation of U2OS and MNNG / HOS cells was significantly inhibited by down-regulation of GLS1 compared with the negative control siGLS1-nc group ($P < 0.001$), as shown in figures C, D in Figure 2. The results showed that down-regulation of GLS1 expression could significantly inhibit the clone formation of U2OS cells ($P < 0.001$), as shown in Figure E, F in Figure 2. Transwell chamber experiment was used to observe the effect of down-regulation of GLS1 expression on the migration and invasion of U2OS cells. It was found that the migration and invasion ability of U2OS cells decreased significantly after down-regulation of GLS1 expression ($P < 0.001$, $P < 0.001$) as shown in Figures G, H, I and J in Figure 2. Furthermore, we explored the possible mechanism of GLS1 knockdown, and detected the changes of CDK4 by Western blot. The results showed that compared with the negative control group NC, GLS1 knockdown could reduce the expression level of CDK4 ($P < 0.05$), as shown in Figure K, L in Figure 2.

3 CB-839 inhibited the proliferation and migration of U2OS and MNNG / HOS cells, and promoted apoptosis.

CB-839 0 $\mu\text{mol/L}$, 0.125 $\mu\text{mol/L}$, 0.25 $\mu\text{mol/L}$, 0.5 $\mu\text{mol/L}$, 1 $\mu\text{mol/L}$, 2 $\mu\text{mol/L}$, 4 $\mu\text{mol/L}$, 8 $\mu\text{mol/L}$, 16 $\mu\text{mol/L}$, a new GLS1 inhibitor, was used to treat 143B, HOS, MNNG / HOS, SJSA-1, U2OS, U2OS / MTX300 cells at different concentrations for 48 hours. The results showed that cb-839 could significantly inhibit the proliferation of osteosarcoma cell lines, and its IC50 values 11.5 $\mu\text{mol/L} \pm 1.3 \mu\text{mol/L}$, 10.7 $\mu\text{mol/L} \pm 1.1 \mu\text{mol/L}$, 4.1 $\mu\text{mol/L} \pm 1.2 \mu\text{mol/L}$, 12.2 $\mu\text{mol/L} \pm 1.4 \mu\text{mol/L}$, 11.0 $\mu\text{mol/L} \pm 0.4 \mu\text{mol/L}$, 10.5 $\mu\text{mol/L} \pm 0.9 \mu\text{mol/L}$ showed that CB-839 could inhibit the proliferation of osteosarcoma cell lines, as shown in figure a in Figure A in Figure 3. When CB-839 (0.1%DMSO, 4 $\mu\text{mol/L}$, 8 $\mu\text{mol/L}$) were used to treat the same cells, the results showed that the proliferation of CB-839 cells was significantly inhibited, and the number of colony formation was significantly decreased ($P < 0.05$), as shown in figures B and C in Figure 3. CB-839 (0.1%DMSO, 4 $\mu\text{mol/L}$, 8 $\mu\text{mol/L}$) were also used to treat the same cells. The effect of CB-839 on migration was observed at 0h, 24h and 48h. The results showed that the migration ability of

the experimental group was significantly inhibited at 24h and 48h ($P < 0.05$), as shown in Figure D and E in Figure 3.

4 RNA-seq sequencing to detect the mechanism of CB-839

CB-839 (0.1 %DMSO, 8 μ M) were used to treat U2OS and MNNG / HOS cells for 48 hours, then RNA seq was sent to the sequencing company with two replicates in each group. After RNA was extracted, RNA sequence was sent to detect the relevant experimental results. The results showed that compared with the control group, there were 2757 transcription factors up-regulated in cb-839 treated MNNG / HOS cells, and 1666 transcription factors up-regulated in U2OS experimental group compared with the control group

After CB-839 acted on U2OS and MNNG / HOS cells, 71 transcription factors were co expressed and up-regulated, as shown in Figure A in Figure 4. Among the signal pathways co-enriched by transcription factors, the top ten signal pathways were similar, and the first signal pathway was co-enriched in metabolic pathways, indicating that the signal pathways of drug action were consistent, as shown in Figure B, C in Figure 4.

q-PCR results showed that the control group was treated with 0.1% DMSO for 48 h; experimental group was treated with cb-839 of M for 48 h, and the relevant conclusions of sequencing were verified by q-PCR. It was found that the up-regulated transcription factors FoxO1, EGR1, Snai2, HSF4, MAFB, JUNB and FOS were expressed in the experimental group compared with the control group after CB-839 was added to the osteosarcoma U2OS and MNNG / HOS cells, and they were all statistically significant, as shown in figures D and E in Figure 4. Western blot results showed that the expression level of EGR1 and FOXO1 transcription factors in the control group and the experimental group was increased, as shown in Figure D and E in Figure 4. CB-839 (0 μ M,4 μ M,8 μ M) were used to detect the expression level of apoptosis related protein cleaved caspase 3 48 h after CB-839 treatment. It was found that the expression level of cleaved caspase 3 increased with the increase of CB-839 concentration, as shown in Figure G, H in Figure 4. These results indicate that CB-839 can activate the cleaved caspase 3 in apoptosis related pathway and inhibit the malignant process of tumor cells by acting on tumor cells and causing the rise of tumor suppressor genes FOXO1 and EGR1

Discussion

The health problems of children and adolescents are related to the happiness of every family and the future of the motherland. The high-risk population of osteosarcoma is the adolescent population in the developing stage, which is characterized by aggressive growth in the early stage, easy metastasis in the late stage, and low 5-year survival rate of patients ^[4,41]. This seriously affects the health status of young people. For osteosarcoma treatment, neoadjuvant chemotherapy, surgery, and radiotherapy are usually used in clinic, but the long-term prognosis of patients is still poor. The lack of targeting and specificity of these treatment methods also restricts the treatment effect of osteosarcoma to a certain extent ^[42].

Therefore, it is of great significance to explore more targeted treatment measures and improve the treatment strategies for osteosarcoma for the health of adolescents.

The important feature of tumor metabolism is that even in aerobic condition, the metabolism of tumor cells is still carried out by anaerobic glycolysis. It is believed that the changes of metabolism and the loss of mitochondrial function may play an important role in the occurrence and development of malignant tumors^[43]. Recent studies have shown that glutamine metabolism plays an important role in the malignant progression of tumor. GLS1 acts on glutamine metabolism as a glutaminase, and its metabolites are glutamic acid and glutamic acid α -Ketoglutarate directly or indirectly provides carbon and nitrogen sources for tumor metabolism, so GLS1 may play an important role in tumor metabolism^[44]. In recent years, articles have found that GLS1, as an oncogene, plays an important role in the occurrence and development of a variety of tumors. In breast cancer and lymphoma cell models, GLS1 can be regulated by c-Jun, a cancer promoting transcription factor, Further use of GLS1 specific inhibitor 968 can prevent the occurrence of carcinogenic transformation^[45]. In the study of liver cancer, we found that the tumor load of GLS1 negative mice was significantly less than that of GLS1 positive mice in c-MYC-induced mouse liver cancer model. Knockout of an allele of GLS1 can reduce the occurrence of liver cancer^[46]. These studies confirmed that gls1 plays an irreplaceable role in the development of tumor. GlS1 has important research value. GlS1 may be an important tumor biomarker and has potential research value. However, the role of gls1 in osteosarcoma has not been reported. In order to clarify the expression of GLS1 in osteosarcoma patients and explore the role of GLS1 in targeted therapy of osteosarcoma, we first verified our conjecture by collecting tissue samples of osteosarcoma amputated patients. Compared with the expression level of GLS1 in normal tissues adjacent to cancer, the expression level of GLS1 in osteosarcoma patients was significantly increased. The results suggest that GLS1 may be an important oncogene in osteosarcoma, which plays an important role in the development of osteosarcoma. Furthermore, the expression level of GLS1 in osteosarcoma cell line was detected by immunofluorescence assay. We found that the expression level of GLS1 was up-regulated in osteosarcoma cells. We selected osteosarcoma cell lines with high expression level of GLS1 protein and transfected siGLS1 to explore the effect of low expression of GLS1 on the function of osteosarcoma cells. We found that low expression of GLS1 could significantly inhibit the proliferation of osteosarcoma cells. These results suggest that GLS1 may be an important target gene of osteosarcoma and affect the malignant progression of osteosarcoma.

CB-839, as a novel GLS1 inhibitor, has important antitumor properties in most glutamine dependent tumors. CB-839 can block the glutamine pathway and the precursors needed to block the tricarboxylic acid cycle to inhibit the energy source of tumor and inhibit the growth of tumor. CB-839 has shown great advantages in the treatment of a variety of tumors. For glioma, triple negative breast cancer and multiple myeloma, CB-839 can significantly inhibit the proliferation of tumor cells^[47-48]. In the treatment of drug-resistant small cell lung cancer, even a low concentration of CB-839 can significantly inhibit the drug resistance of tumor cells, and induce autophagy of tumor cells to kill tumor cells, which has a good effect^[18]. CB-839 not only has a good prospect when used alone, but also shows a good synergistic effect

in combination. In the treatment of ovarian cancer and glioblastoma, the combination of CB-839 and mTOR pathway inhibitors can significantly inhibit the metabolism of glutamine and glucose, and significantly inhibit the growth of tumor^[23]. CB-839 can also be used together with EGFR, an inhibitor of tyrosine kinase, to induce the activation of oxidative stress, promote the autophagy function of tumor cells, and then lead to the occurrence of metabolic crisis. CB-839 has good anti-tumor characteristics. The study of CB-839 in normal animals at the same time shows that it has good biological safety, no drug toxicity and weight loss^[49]. In the experiment, we used cb-839 to treat osteosarcoma cells, and found that it can significantly inhibit the proliferation and migration of tumor cells, and has good biological function. Generally, the expression of FOXO1 and EGR1 transcription factors is inhibited in tumor cells, and their expression has an important impact on the occurrence and development of tumor. FOXO1 and EGR1 regulate many targets, participate in apoptosis and autophagy, antioxidant enzymes, cell cycle arrest, and are associated with immunomodulators and cell metabolism, making them a super transcription factor with a variety of complex biological activities. Therefore, its expression has a good effect on tumor inhibition. In this study, we found that CB-839 can promote the up regulation of FOXO1 and EGR1 mRNA and protein expression levels by analyzing the sequencing results. Furthermore, we detected the changes of cleaved Caspase3 protein expression level of tumor apoptosis related pathway after cb-839 treatment. In conclusion, we speculate that CB-839 may act on FOXO1 and EGR1 to activate the apoptotic pathway of tumor cells and inhibit the related functions of tumor cells.

In conclusion, through previous experimental studies, we found for the first time that the expression level of GLS1 in osteosarcoma tissues and cells is high, and found that the low expression of GLS1 can significantly inhibit the malignant process of osteosarcoma cells, including proliferation, migration and invasion. CB-839, an inhibitor of GLS1, can significantly inhibit the proliferation and migration of tumor cells. By further exploring the mechanism of CB-839, we found that CB-839 can promote the expression of FOXO1 and EGR1, activate the apoptotic pathway, and inhibit the malignant process of tumor by acting on osteosarcoma cells. In conclusion, we found that GLS1 can promote the malignant progression of osteosarcoma, CB-839 can inhibit the malignant progression of osteosarcoma, and promote the apoptosis of tumor cells by promoting the upstream transcription factors FOXO1 and EGR1 of GLS1. However, we will further explore how FOXO1 and EGR1 regulate apoptosis. Secondly, GLS1 as a valuable indicator of clinical prognosis needs more clinical data to supplement and verify. In conclusion, GLS1 may promote the proliferation, migration and invasion of OS cells by affecting CDK4 signaling pathway. CB-839 has great potential as a potential therapeutic drug. GLS1 can be used as a potential prognostic indicator and therapeutic target for OS patients.

Declarations

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Authors' contributions

Conception, design, and writing: JIN SONG, ZENG TONG and PENG LEI. Development of methodology: PENG LEI, YIN JUNQIANG, CHEN HAIBAO and LIU WEIHAI. Acquisition of data: ZHANG DI, FAN ZEPEI and SHEN JINNAN. Analysis and interpretation of data (i.e., computational analysis, statistics): PENG LEI and ZENG TONG.

Revision of the manuscript: JIN SONG and SHEN JINNAN. The authors read and approved the final manuscript.

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Ethics approval and consent to participate

The procedures were performed with informed and approved consents according to the Institutional Ethical Committee guidelines at the Hospital of Sun Yat-Sen University.

Consent for publication

Not applicable

Competing interests

No competing financial interests exist with the submitted manuscript.

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Figures

Figure 1

Expression of GLS1 in osteosarcoma tissues and osteosarcoma cell lines (Figure A represents the protein expression level of GLS1 in normal tissues adjacent to osteosarcoma and osteosarcoma tissues detected by Western blot; Figure B: immunofluorescence labeling, the left picture shows the expression level of

GLS1 in the normal tissues adjacent to osteosarcoma of amputees, the right picture shows the expression level of GLS1 in osteosarcoma of amputees, GLS1 is green fluorescence, DAPI is blue fluorescence; Figure C represents the results of statistical analysis of GLS1 protein expression level using the mapping software image J and GraphPad Prism 8, N: normal tissue, O: Osteosarcoma, *** means there is statistical significance $P<0.001$)

Figure 2

The expression level of gls1 was detected after transfection of sigls1 plasmid (Fig. A and B represent the changes of GLS1 protein expression in U2OS and MNNG / HOS cells after transfection with siGLS1 plasmid, respectively; Figures C and D represent the results of statistical analysis using image J and GraphPad Prism 8 software. The expression level of GLS1 in the experimental group was significantly decreased; Figures E and F respectively represent the effect of low gls1 expression in U2OS and MNNG / HOS cells on clonal ball formation by colony formation assay, and the following respectively represent the results obtained by using GraphPad Prism 8 software for statistical analysis; Figure G shows that the low expression of GLS1 inhibits the migration ability of U2OS cells, Figure H shows that the low expression of GLS1 inhibits the migration ability of MNNG / HOS cells, and the following shows the results of statistical analysis using GraphPad Prism 8 software; Figure I shows that the low expression of GLS1 inhibits the invasion ability of U2OS cells, figure J shows that the low expression of GLS1 inhibits

the invasion ability of MNNG / HOS cells, and the following shows the results of statistical analysis using GraphPad Prism 8 software; Figures K and I represent the inhibition of CDK4 expression in U2OS and MNNG / HOS cells by GLS1 siGLS1-NC: control group, siGLS1-1 and siGLS1-2: experimental group, * means there is statistical significance $P<0.01$, *** means there is statistical significance $P<0.001$.

Figure 3

Effect of CB-839, a new GLS1 inhibitor, on osteosarcoma cell line—Figure A shows the effect of CB-839 on the proliferation of 143B, HOS, MNNG / HOS, SJSA-1, U2OS, U2OS / MTX300 cells by MTT assay—Figures B and C represent the colony forming ability of the experimental group and the control group respectively; Figures D and E respectively represent the migration of CB-839 at different concentrations and at different times, * means there is statistical significance $P<0.01$, **/** means there is statistical significance $P<0.001$

Figure 4

RNA sequence analysis of CB-839 on U2OS and MNNG / HOS cells (Figure A represents the number of transcription factors with increased expression levels in U2OS and MNNG / HOS cells; Figures B and C represent the top ten signaling pathways in the analysis of KEGG pathway in the sequencing results of U2OS and MNNG / HOS cells, respectively.); Effect of CB-839 on expression of EGR1, FOXO1 and apoptosis related proteins (Figure a represents the changes of EGR1 and FOXO1 protein expression levels

in U2OS and MNNG / HOS cells treated with CB-839 (8 μ M) for 48 h. Figure B and C represent the changes of protein expression level of apoptosis related gene cleaved Caspase3 after CB-839 (0 μ M,4 μ M,8 μ M) treated U2OS and MNNG / HOS cells, respectively.