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Combined Use of Niraparib Enhanced the Inhibitory Effect of Anti-GD2 Antibody on Osteosarcoma Cells

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Abstract

Purpose: To study the inhibitory effect of Niraparib in combination with Anti-GD2 antibody on Osteosarcoma.

Methods: The migration ability of OS cells was detected by scratch test. Transwell experiment and CCK8 assay were used to detect the invasion and proliferation. WB was used to detect BALP and CICP protein expression. The mRNA expression of BALP and CICP was detected by QRT-PCR.

Results: Scratch test showed that the distance between cells in Niraparib+GD2 group was 1.07 ± 0.04 and 1.06 ± 0.04 at 48 h, both $p<0.05$, and the differences were statistically significant. Transwell experiment showed that the number of invasive cells was 21 ± 1.5 in Niraparib+GD2 group, $p <0.05$ and the differences were statistically significant. CCK8 assay showed that the absorbance of Niraparib+GD2 group was 0.16 ± 0.10 on day 5, $p<0.05$ and the differences were statistically significant. WB showed that compared with the Control group, the semi-quantitative results of BALP expression in Niraparib+GD2 group were 0.751 ± 0.135 and CICP expression were 1.086 ± 0.115, both $p<0.05$, and the differences were statistically significant. QRT-PCR showed that the absorbance of Niraparib+GD2 group was 0.173 ± 0.065 and 0.170 ± 0.078 on days 14, both $p<0.01$ and the differences were statistically significant.

Conclusion: Niraparib combined with Anti-GD2 antibody has a more prominent inhibitory effect on OS.

Keywords: Niraparib;Anti-GD2 antibody; Osteosarcoma cells

Introduction

Osteosarcoma (OS) is the most common primary bone malignancy, accounting for about 35% of all orthopedic tumors [1]. OS is a serious and destructive disease. In addition to being highly locally aggressive, OS is characterized by a tendency to metastasize to the lungs and distant bones. The cure rate for OS is low and is further reduced if the diagnosis is that the OS has metastasized. Despite rapid medical advances and a variety of treatment options, the 5-year survival rate for patients with OS is still low, about 60%-70% [2]. Therefore, it is necessary to develop new methods to treat OS patients. We focused on the research direction of drug combination therapy for OS in the hope of improving the survival rate of patients.

Niraparib is a Poly Adp-Ribose Polymerase (PARP) inhibitor. Niraparib was originally developed for ovarian and breast cancer. At present, Niraparib is mainly used for the treatment of recurrent epithelial ovarian cancer, fallopian tube cancer, primary peritoneal cancer and other tumor diseases. Niraparib has been found to have some effect on many tumors. In addition, monoclonal antibodies against Disialoganglioside (GD2) have been documented to inhibit tumor cell viability independently of the immune system. A recent study found that GD2 was highly expressed in OS tissues and cell lines. And more importantly, more GD2 was expressed in OS tissues at the time of disease recurrence than at the time of initial biopsy [3]. These results all suggest that GD2 plays an important role in OS progression.

Ganglioside is a sugar base part contains sialic acid sheath glycolipids, lobules of micro domains outside of plasma membrane, referred to as "synaptic sugar", engaging in biological processes such as cell proliferation [4]. Therefore, tumor-associated gangliosides would be an attractive target for immunotherapy. Although the expression of gangliosides in normal tissues is localized, it is limited to peripheral nerves and the central nervous system. But studies have found that gangliosides can be detected in sarcoma, glioma, small cell lung cancer, neuroblastoma, and many melanoma diseases [5]. Because of its distribution pattern, GD2 has been selected as a target for monoclonal antibody therapy. Early clinical trials have shown that monoclonal antibodies against tumor-associated gangliosides have certain efficacy [6]. And monoclonal antibodies may inhibit tumor cell viability through immune system such as antibody-dependent cell-mediated cytotoxicity and complement dependent cytotoxicity [7]. However, more and more researches have suggested that Anti-GD2 antibody can suppress tumor cell viability independently of the immune system [8]. In addition, studies have shown that Anti-GD2 antibody can reduce the viability of human neuroblastoma cells in a dose-dependent manner [9]. We expect Anti-GD2 antibody to act in a similar manner in the treatment of OS.

In our original research, we investigated the effect of Niraparib on OS cells alone and in combination with Anti-GD2 antibody on cell viability for the first time. The activity and invasiveness of each group of OS cells were measured by multiple detection methods to explore whether Niraparib could act on OS alone or enhance the effect of Anti-GD2 antibody on OS.

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Materials and Methods

Cells lines and reagents

SYBR Green Real-Time PCR Master Mix (Xavier Biotechnology Co., LTD.) Bone Alkaline Phosphatase (BALP), type I Collagen Carboxyl Terminal Propeptide (CICP) and GAPDH antibodies were purchased from Xavier Biotechnology Co., LTD. Experimental cell lines: MG63 (ATCC) and U2OS (ATCC). Culture conditions: Gas phase: air, 95%; Carbon dioxide, 5%. Temperature: 37°C incubator humidity, 70%-80%.

Scratch test

All sterilizable instruments should be sterilized. Ruler and marker should be irradiated by UV for 30 min before operation (in ultra-clean table). First, use markers evenly draw horizontal lines with ruler behind 6-hole plate, about every 0.5 cm~1 cm, across the hole. At least 5 wires must pass through each hole. Add about 5 × 105 cells to the hole, the exact number varies from cell to cell, and it should be fully covered overnight. The next day, compare the ruler with the spear head, try to hang as far as the back of the horizontal line scratches, the spear head should be vertical, not tilt. The cells were washed 3 times with PBS, the floating cells were removed, and serum-free medium was added. Put it into a 37°C, 5% CO2 incubator for cultivation. Take samples at 0 h, 24 h and 48 hours.

Trasnswell experiment

On the day before the experiment, a tube of Matrigel matrix glue which had been divided was put in the refrigerator at 4°C overnight from -20°C in advance, and the Matrigel glue melted from solid state to liquid state. Matrigel matrix glue was diluted at 1:8 and coated on the upper compartment surface of the bottom membrane of the Transwell chamber. Extract the residual liquid from the culture plate and add 50 μL 10 g/LBSA serum-free medium to each well at 37°C for 30 min. The cells were digested by conventional trypsin and washed 1-2 times with PBS to remove the effect of serum. The cells were re-suspended in serum-free medium, and the cell density was adjusted to 5 cells /mL × 105 cells /mL. Add 200 μL cell suspension to the upper chamber of Transwell chamber, and add 600 μL medium containing 10% FBS to the lower chamber of 24-well culture plate. The culture plate was placed in a CO2 incubator at 37°C for 24 hours. The cells were taken out, washed twice with PBS, and the cells in the upper layer of the microporous membrane of the cells were carefully wiped with cotton swabs. The cells were fixed with 4% paraformaldehyde in the 24-well plate for 20 min, and stained with crystal violet solution for 15 min. Photographs were taken under an inverted microscope, 10 fields were counted randomly for each sample, and the average value was taken for statistical analysis.

CCK8 experiment

Inoculate cell suspension in 96-well plate (100 μL/ well). The culture plate was placed in an incubator for pre-culture for a period of time (37°C, 5% CO2). Add 10 μL CCK8 solutions to each well. Incubate the culture plate in the incubator for 2 hours. The absorbance at 450 nm was measured with a microplate reader.

Western blotting

Protein (40 μg) was extracted, separated by 10% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk, followed by incubation with primary antibodies at 4°C overnight. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h. Blots were developed using the Enhanced Chemiluminescence (ECL) system. The antibodies used in our study included anti-BALP antibody and anti-CICP antibody. All experiments were repeated at least three times.

Real-time quantitative reverse transcription

Total RNA was extracted using the TRIzol method, and cDNA was synthesized with the RevertAidTM first strand cDNA synthesis kit. Real-time Polymerase Chain Reaction (PCR) was performed using the ABI PRISM 7900 HT sequence detection system. The expression of osteogenesis-related genes was normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method. The primer sequences are listed in Table 1.

Statistical analysis

Image processing was performed by Image J. Statistical analyses were performed with SPSS for Windows 10. All data values were expressed as means ± SD. Comparisons of means among multiple groups were performed with one-way ANOVA followed by post pairwise comparisons using Tukey's tests. A two-tailed p, 0.05 was considered statistically significant in this study.

Results

Scratch test

At 0 h, there was no significant difference in scratch width between the four groups of the two cell lines. In the MG63 cell line, at 48 h, the scratch width in the control group almost disappeared, with a distance of 0.34 ± 0.04. The scratch spacing in the Niraparib+GD2 group was still relatively wide, with a distance of 1.07 ± 0.04, $p<0.05$, and the difference was statistically significant. The U2OS cell line showed a similar trend. These results show that niraparib combined with Anti-GD2 antibody has a significant inhibitory effect on OS cells and can inhibit the migration of OS cells (Figure 1). Statistical data were presented in Supplementary Table 1.

Trasnswell experiment

Transwell experimental results showed that in the MG63 cell line, compared with the Control group, the invasive ability of OS cells in the Niraparib, GD2 and Niraparib+GD2 groups was inhibited. However, Niraparib combined with Anti-GD2 antibody had the most significant inhibitory effect on the invasion ability of OS cells (Figure 2). By counting, the number of cells that invaded through the basement membrane in the Control group was 87.7 ± 2.9, while the number in the Niraparib+GD2 group was 21 ± 1.5, $p<0.05$, and the difference was statistically significant. The U2OS cell line showed a similar trend. These results showed that Niraparib combined with Anti-GD2 antibody has a significant inhibitory effect on the invasive ability of OS cells. Statistical data were presented in Supplementary Table 2.

CCK8 experiment

CCK8 results showed that compared with the Control group, the Niraparib group and the GD2 group had no significant inhibitory effect on OS cell proliferation. However, Niraparib combined with Anti-GD2 antibody significantly inhibited the proliferation of OS cells (Figure 3). With the prolongation of culture time, the inhibitory effect of Niraparib combined with Anti-GD2 antibody on OS cell proliferation gradually increased. On day 5, the cell proliferation in the Control group was assessed by absorbance as 0.76 ± 0.09, while in the Niraparib+GD2 group it was 0.16 ± 0.10, $p<0.05$, and the difference was statistically significant. These results showed that Niraparib combined with Anti-GD2 antibody has a significant inhibitory effect on OS cell proliferation. Statistical data were presented in Supplementary Table 3.

Gene	Sequence(5'-3')
BALP	Forward primer：CAGAAGTGCGAGGAGGAGGT
	Reverse primer：GAAATCGTGCGGGGTCAT
CICP	Forward primer：GGTGCAGACCTAGCAGACACCA
	Reverse primer：AGGTAGCGCCGGAGTCTATTCA
GAPDH	Forward primer：GGCACAGTCAAGGCTGAGAATG
	Reverse primer：ATGGTGGTGAAGACGCCAGTA

Table 1: Primer sequences used in quantitative PCR assay.

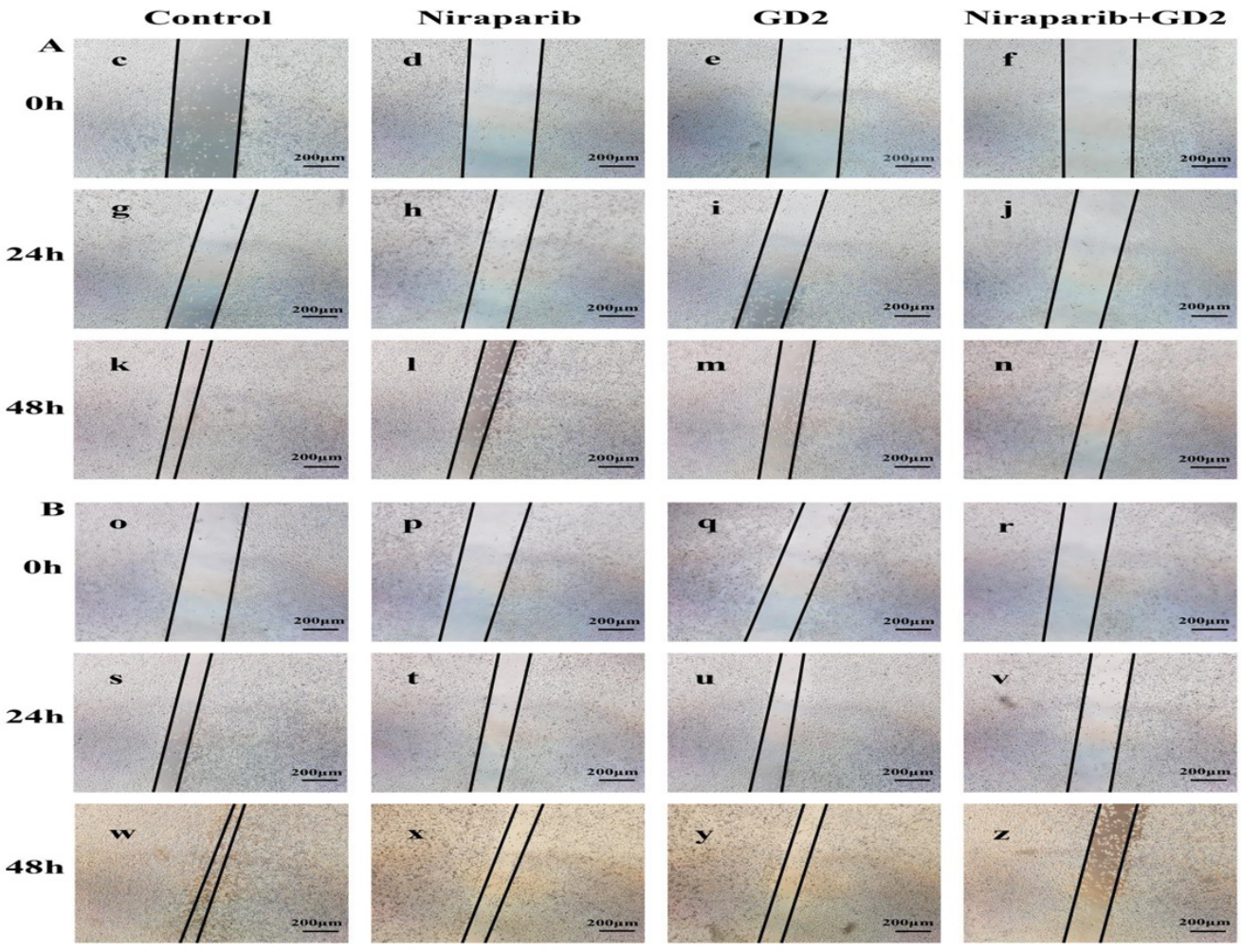

Figure 1: Two cell lines' scratch test results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group were taken at 0 h, 24 h and 48 h, respectively; (A) MG63 cell line's scratch test results; (B) U2OS cell line's scratch test results; (c,d,e,f): MG63's scratch test results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group respectively at 0 h; (g,h,i,j): MG63's scratch test results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group respectively at 24 h; (k,l,m,n): MG63's scratch test results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group respectively at 48 h; (o,p,q,r): U2OS's scratch test results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group respectively at 0 h; (s,t,u,v): U2OS's scratch test results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group respectively at 24 h; (w, x, y, z): U2OS's scratch test results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group repectively at 48 h.

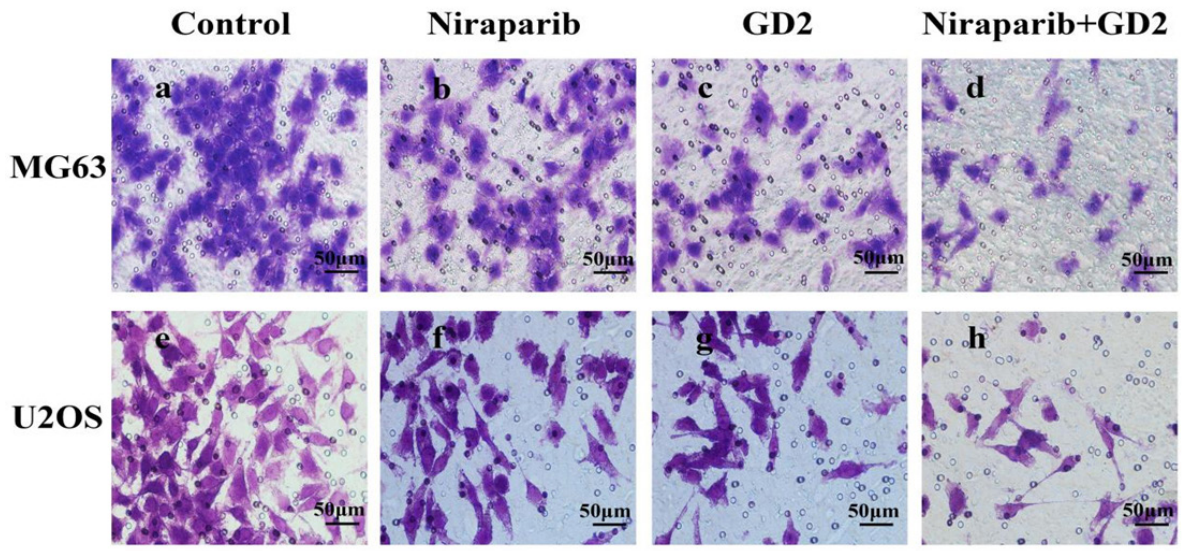

Figure 2: Two cell lines' transwell experiment results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group were recorded. (a) MG63's transwell experiment results of Control group; (b) MG63's transwell experiment results of Niraparib group; (c) MG63's transwell experiment results of GD2 group; (d) MG63's transwell experiment results of Niraparib+GD2 group; (e) U2OS's transwell experiment results of Control group; (f) U2OS's transwell experiment results of Niraparib group; (g) U2OS's transwell experiment results of GD2 group; (h) U2OS's transwell experiment results of Niraparib+GD2 group.

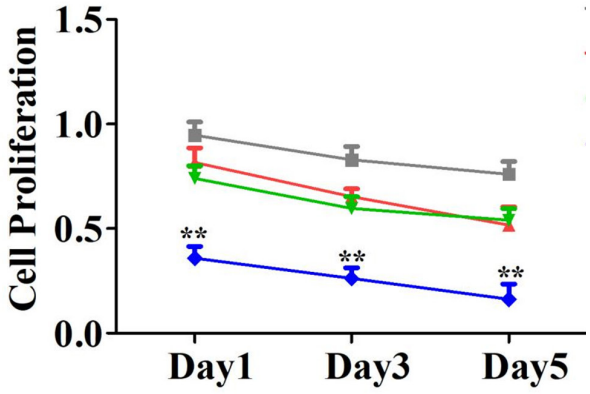

Figure 3: CCK8 assay was used to detect the proliferation level of cells. CCK8 results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group were recorded on day 1, 3 and 5 respectively. Note: (): Control; (): Niraparib; (): GD2; (): Niraparib+GD2; **: p< 0.05.

Western blotting

WB results showed that in the MG63 cell line, compared with the Control group, the protein expression levels of BALP and CICP in OS cells in the Niraparib, GD2 and Niraparib+GD2 groups were inhibited. However, Niraparib combined with Anti-GD2 antibody had the most significant inhibitory effect on the expression levels of BALP and CICP proteins in OS cells (Figure 4). Through semi-quantitative analysis, the expression levels of BALP and CICP in the Control group were 1.025 ± 0.143 and 1.216 ± 0.168, while those in the Niraparib+GD2 group were 0.751 ± 0.135 and 1.086 ± 0.115, both $p<0.05$, and the differences were statistically significant. These results showed that Niraparib combined with Anti-GD2 antibody has a significant inhibitory effect on the expression of osteogenesis-related proteins in OS cells. Statistical data were presented in Supplementary Table 4.

Real-time quantitative reverse transcription

PCR results showed that compared with the Control group, the mRNA expression levels of BALP and CICP were inhibited in the Niraparib group, GD2 group and Niraparib+GD2 group. However, Niraparib combined with Anti-GD2 antibody had the most significant inhibitory effect on the mRNA expression levels of BALP and CICP in OS cells. On day 14, the absorbance of the mRNA expression of BALP and CICP was measured. The Niraparib+GD2 group was 0.173 ± 0.065 and 0.170 ± 0.078. Compared with the Control group, both $p<0.01$, and the difference was statistically significant. These results showed that Niraparib combined with Anti-GD2 antibody had a significant inhibitory effect on the expression of osteogenesis-related RNA in OS cells (Figure 5). Statistical data were presented in Supplementary Table 5.

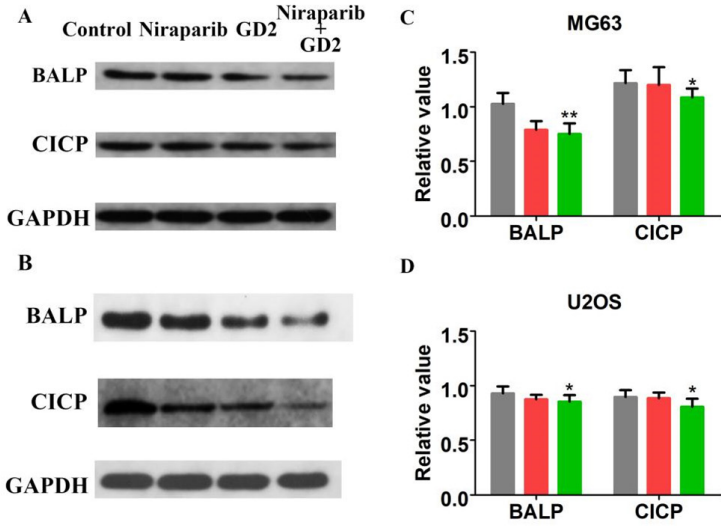

Figure 4: Two cell lines' western blotting results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group. The expression levels of BALP and CICP were detected with GAPDH as internal reference. (A) MG63's western blotting results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group; (B) U2OS's western blotting results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group; (C) MG63's target protein expression of semi-quantitative analysis; (D) U2OS's target protein expression of semi-quantitative analysis. Note: (▬): Niraparib/control; (▬): GD2/control; (▬): (Niraparib+GD2)/Control; *: p< 0.01; **: p< 0.05.

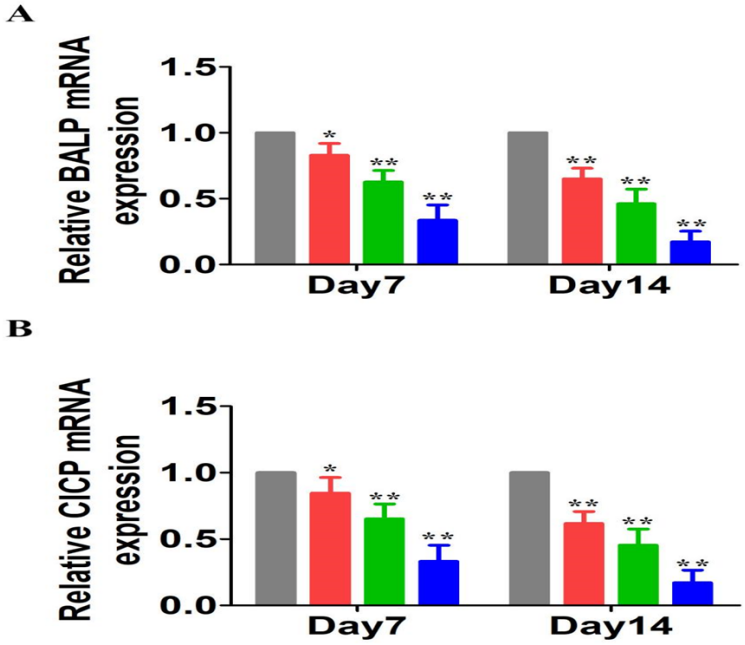

Figure 5: Real-time quantitative reverse transcription results of Control group, Niraparib group, GD2 group and Niraparib+GD2 group. (A) relative BALP mRNA expression; (B) relative CICP mRNA expression. Note: (▬): Control; (▬): Niraparib; (▬): GD2; (▬): Niraparib+GD2; *: p< 0.01; **: p< 0.05.

Discussion

The combined use of PARP inhibitors and GD antibodies has already appeared in the field of tumor treatment we made a table to summarize in Table 2. In this study, we confirmed that Anti-GD2 antibody has an effect on the invasion ability of OS cells through the results of scratch and transwell experiments. In the scratch test, cells in GD2 group crawled slower than those in control group. In Transwell, the GD2 group had less cell invasion than the Control group. Previous studies have also shown that Anti-GD2 antibody is associated with aggressive activity of tumor cells [13-16], which we confirmed in OS cells.

Author	Title	Tumor they studied	Cite
Tsao CY, et al.	Anti-proliferative and pro-apoptotic activity of GD2 ganglioside-specific monoclonal antibody 3F8 in human melanoma cells.	Melanoma	[10]
Ha SH, et al.	Exogenous and endogeneous disialosyl ganglioside GD1b induces apoptosis of MCF-7 human breast cancer cells.	Breast cancer	[11]
Durbas M, et al.	Downregulation of the PHLDA1 gene in IMR-32 neuroblastoma cells increases levels of Aurora A, TRKB and affects proteins involved in apoptosis and autophagy pathways.	Neuroblastoma	[12]
Chung TW, et al.	The ganglioside GM3 is associated with cisplatin-induced apoptosis in human colon cancer cells.	Colon cancer	[13]

Table 2: Combined use of PARP inhibitors and GD antibodies on tumors.

According to preclinical studies, PARP inhibitors have potential therapeutic value for OS [17-19], and by screening for genetic determinants of drug activity, PARP inhibitors are shown to be potential targets for OS [20-24]. The researchers also show that PARP inhibitors can enhance the activity of cytotoxic drugs [25-30]. In our study, it was confirmed by CCK8 experiment that Niraparib had a certain effect on cell activity, and the cell activity of Niraparib+GD2 group was significantly reduced, indicating that Niraparib enhanced the effect of Anti-GD2 antibody.

The preclinical studies indicate that OS cells are sensitive to PARP inhibitors in vitro [31-33], but the efficacy of PARP inhibitors as monotherapy in OS is limited, highlighting the need for combination therapy. PARP inhibitors enhance DNA damage-mediated cytotoxicity caused by topoisomerase I poisons, DNA methylators, or radiation, which is relevant to the role of PARP in repairing DNA damage caused by these cytotoxic therapies [34-37]. In our study, the osteogenesis was observed by detecting the expression of BALP and CICP. The results also confirmed that the effect of Niraparib monotherapy was limited. But Niraparib could enhance the effect of Anti-GD2 antibody, and the expression of protein associated with osteogenesis was significantly reduced.

Our study highlights the need for PARP inhibitors to be used in combination with other drugs when monotherapy is not effective in treating tumors. The study of PARP inhibitor combined with Anti-GD2 antibody in the treatment of OS showed that Niraparib combined with Anti-GD2 antibody has a certain effect in the treatment of OS.

It's very important for us to state this point here. We agree that resulting treatments need to be supplemented by preclinical studies and even clinical studies. There are many potential targets for OS, but we have only limited research into them. We therefore state that it is premature to jump from cell experiment to clinical trials based on this study alone. The construction of this study is still very imperfect, and we will gradually improve our conclusions in subsequent research.

Conclusion

By studying the inhibitory effect of Niraparib alone or in combination with Anti-GD2 antibody on OS, we found that the inhibitory effect of Niraparib combined with Anti-GD2 antibody on OS was more prominent than Niraparib alone. This provides a new idea for the treatment of OS. Subsequent clinical trials will be carried out to evaluate the inhibitory effect of Niraparib combined with Anti-GD2 antibody on OS, expecting to make a certain contribution to the clinical treatment of OS.

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Availability of Data and Materials

The data either reside within the article itself or can be obtained from the authors upon making a reasonable request.

Authors' Contributions

Conceptualization, Chen Wenyao and Que Xiangyong; methodology, Chen Wenyao and Que Xiangyong; software, Ma Shuai; validation, Li Xinzhi, Ma Shuai, Fan Yifeng, Chen Wenyao and Que Xiangyong; formal analysis, Chen Wenyao and Que Xiangyong; investigation, Ma Shuai, Fan Yifeng; data curation, Li Xinzhi; writing-original draft preparation, Chen Wenyao and Que Xiangyong; project administration, Que Xiangyong. All authors have read and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

The study was approved by the Affiliateo Renhe Hospital of China Three Gorges University, Hubei Province, China.

Patient Consent for Publication

The study was approved by the Affiliateo Renhe Hospital of China Three Gorges University, not included experiments on patient.

Conflicts of Interest

The authors declare no conflict of interest.

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