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Antisense oligonucleotide targeting survivin inhibits growth by inducing apoptosis in human osteosarcoma cells MG-63

Y. F. WU[1,4], X. J. LIANG[1,4], Y. Y. LIU[1], W. GONG[2], J. X. LIU[3], X. P. WANG[1], Z. Q. ZHUANG[1], Y. GUO[1], H. Y. SHEN[1]

[1] Department of Orthopaedics, Sun-Yat-Sen Memorial Hospital, Sun-Yat-Sen University, 107 West Yanjiang Road, Guangzhou 510120, P.R., China.e-mail: doctor_shenhy@yahoo.com.cn; [2]Department of Oncology, Xiangfan Hospital (Xiangfan central Hospital), Tongji Medical College, Huazhong University of Science and Technology, Xiangfan 441021, P.R., China; [3]Department of General Surgery, Guangci Hospital of Wuhan University, Wuhan 430050, P.R., China; [4]These authors contributed equally to this manuscript.

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Survivin may play an important role in the development of osteosarcoma. In this study, we chose osteosarcoma cell line MG-63, which highly expressed survivin, to observe the effects of antisense oligonucleotide targeting survivin on the apoptosis induction and proliferation inhibition. It was shown in our results that the apoptosis rate and the proliferation inhibition rate increased significantly in survivin-positive cells MG-63 by using MTT and flow cytometry methods. We found that the growth inhibition rate and apoptosis rate were changed in a dose-dependent way. When the concentration of antisurvivin oligonucleotide was 600 nM, the effects reached the peak. RT-PCR and western-blot methods were used to detect the mRNA and protein expression of survivin in MG-63. We observed that the mRNA and protein expression of survivin reduced after transfected with antisurvivin oligonucleotides at the concentration of 200 nM, 400 nM and 600 nM. At the same time, we found that the mRNA and protein expression of Fas were up-regulated with the concentration of antisurvivin oligonucleotides from 200 nM to 600 nM. It was negative associated with the expression change of survivin. These data suggested that survivin should play an important role in the development of osteosarcoma and the survivin blockaded by using antisurvivin oligonucleotide could inhibit the proliferation and induce apoptosis of osteosarcoma by decreasing the expression of survivin and activate the Fas-mediated apoptosis. Down-regulation of survivin by antisense oligonucleotide might be an effective strategy to the treatment of osteosarcoma and might improve the therapeutic effect.

Key words: osteosarcoma, Survivin, apoptosis, Fas

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents [1]. It is described as having an extremely destructive malignancy that occurs frequently in the long bones [2]. Standard cytotoxic chemotherapy and radiation therapy is of limited effectiveness in the treatment of osteosarcoma [3]. During the past 3 decades, the 5-year survival rate of osteosarcoma however has improved significantly to 60%-70% as the result of the successful use of neoadjuvant chemotherapy [4]. However, there are more than 30% of patients with a poor prognosis due to the early pulmonary metastasis and chemotherapy resistance [5]. Gene therapy as a new method to treat tumors has been introduced to osteosracoma for a few years and seems to be very successful [6–8]. Therapeutic strategies using antisense oligonucleotides have been found to be an effective way to regulate the expression of some oncogene and factors related to the development of tumors and increase the chemosensitivity in tumor cells [9–12].

Suvivin, which is located at chromosome 17q25, is a novel member of the inhibitors of apoptosis protein (IAP) gene family. It is known that Survivin is over expressed in several tumors, including breast cancer, hepatocellular carcinoma, colon cancer, ovarian cancer, lung cancer, and some embryonic tissues but it is scarcely expressed in differentiated normal tissues [13–16]. Furthermore, the overexpression of Survivin is associated with their biologically aggressive characteristics and represents a poor prognosis. Several studies have shown that survivin is also up-regulated in osteosarcoma which suggested that survivin may be involved in the development of osteosarcoma and may be a useful target for the therapy [17, 18].

Although a few experiments have been made to observe the effects of antisurvivin oligonucleotides on several cancer cells in vitro, there are no relatived reports about the use of antisurvivin oligonucleotides on osteosarcoma cells [19, 20]. The presented study, is focused on survivin for its potential role

in the development of osteosarcoma. A human osteosarcoma cell line MG-63 was selected for Lip-ASODN transfection to evaluate the possible therapeutic effects. This may provide a new method in the treatment of osteosarcoma.

Materials and methods

Cell culture. Human osteosarcoma cells line MG-63, obtained from American Type Culture Collection, was cultured in the medium supplemented with 100 u/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in complete RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS) at 37°C in humidified air with 5% CO_2. For subculture, the cell monolayer was washed twice with phosphate buffered solution (PBS) and then incubated with trypsin solution (0.25%) for 5 min at 37 °C to detach the cells. The effect of trypsin was inhibited by adding the FBS at 37°C. Cells were resuspended in RPMI 1640 complete medium for reseeding.

Antisurvivin oligonucleotides. Antisurvivin 20-mer phosphorothioate antisense oligonucleotide targeting nucleotides 232-251 of survivin mRNA (accession number NM_001168) was used. The control oligonucleotide was the reverse of the antisense sequence. Oligonucleotides were provided by Bosia Company of ShangHai in China in the form of phosphorothioate oligonucleotides. The antisurvivin oligonucleotide sequence was 5'-CCC AGC CTT CCA GCT CCT TG-3', and the control primer was 5'-GTT CCT CGA CCT TCC GAC CC-3'.

*Transfection***.** One day before transfection, MG-63 cells were collected and rinsed with 2 ml PBS. Then, 2×10^5 cells per well were planted into 6-well tissue culture plates. Oligonucleotides were delivered in the form of complexes with Lipofectamine 2000 (Invitrogen, USA) in accordance with the follows: antisense oligonucleotides (200 nM, 400 nM and 600 nM) and control oligonucleotides mixed with 20 μl liposome in 2 ml serum-free RPMI-1640 were added to the MG-63 cells. Six hours later, the medium was replaced by complete RPMI-1640 and MG-63 cells were cultured in 5% CO_2 at 37°C for an additional 18 h or longer.

MTT assays. The growth inhibition rate of MG-63 cells was determined by Methyl thiazolyl tetrazolium (MTT) assay. Cells were plated in 96-well tissue culture plates with the cells' density of 5×10^4/ml and divided into different groups as follows: cells transfected with antisense oligonucleotides (200 nM, 400 nM and 600 nM), cells treated with liposome alone, and cells treated with control oligonucleotides. After transfected by antisense oligonucleotides for 6 h, the serum-free medium was replaced by complete medium. The cells were further incubated in 5% CO_2 at 37°C for another 48 h. Subsequently, 20 μl of MTT reagent (Sigma, 5 mg/ml) and 180 μl RPMI 1640 were changed and allowed to react for 4 h at 37°C. Then 150 ul Dimethyl Sulphoxide (DMSO) was added to each well. The plate was shaken on a rotary platform for 10 min, and then the absorbance at wavelength 490 nm was measured by use of microplate reader. The ratio of the absorbance of treated cells relative to that of the control cells was calculated and expressed the percentage of cell death.

Apoptosis assay. MG-63 cells were planted in 6-well tissue culture plates and collected after pre-treatment (cells underwent different treatment in the different groups as described in MTT assays) for 48 h. Being washed twice in PBS and resuspended in 200 μl of PBS, cell suspension was transferred to the addition of 1 ml 70% ice-cold ethanol for 30 min at 4°C. The cells were centrifuged again and resuspended in 200 μl PBS and kept at 4°C overnight. 100 μl of RNase I (1 mg/ml) and 100 μl of propidium iodide (PI) (100 mg/ml) were added to the cell suspension and then the cell suspension was incubated at 37°C for 30 min and analyzed by flow cytometry. Data were pooled from three independent experiments in each group.

Reverse transcription PCR. After pre-treatment (cells underwent different treatment in the different groups as described in MTT assays) for 48 h, MG-63 cells were washed twice in PBS, centrifuged and collected. Total RNA was isolated by Trizol reagent (Invitrogen, USA). Then RT-PCR was performed by using a one-step RT-PCR kit (Life Technologies, USA) according to the manufacturer's instructions. The primer pairs were designed according to the sequences in the GenBank as follows. Survivin(374 bp): forward primer 5'- AGC TGG CTG CCA TGG ATT GA-3'; and reverse primer 5'- GCT CTG CCC ACG CGA ACA AA--3'; Fas(316 bp): forward primer 5'- ATC ACC ACT ATT GCT GGA GTC-3'; and reverse primer 5'- CAC TCT AGA CCA AGA TTT GGA-3'; and internal control GAPDH (230 bp): forward primer 5'- ACG GAT TTG GTC GTA TTG GG -3; reverse primer 5'- TGA TTT TGG AGG GAT GTC GC -3'. The cDNA synthesis and predenaturation were performed as follows: 1 cycle of 94°C for 5 min. PCR amplification was continued for 35 cycles of 94°C for 15 s, (60°C, Survivin; 58°, Fas; and 58°, GAPDH) for 30 s, and 72°C for 1 min. A final extension was performed for 10 min at 72°C. PCR products were identified by 1.5% agarose gels electrophoresis containing 0.5% ethidium bromide (EB). All experiments were repeated at least three times.

Western blotting. About 1×10^7 MG-63 cells were gathered after transfected with antisense oligonucleotides (600 nM) for 24 h and 48 h as described previously. After medium was removed, cells were rinsed twice with PBS solution at room temperature. Plasmosin was extracted. Subsequently, cell lysates were centrifuged at 12,000 rpm for 10 min at 4 °C. Supernantant was collected and protein concentrations were determined. Mouse anti-human monoclonal antibodies directed against Survivin, Fas and rabbit anti-**β**-actin polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, USA). Horseradish peroxidase conjugated goat anti-rabbit was used as secondary antibodies. Proteins were visualized with chemiluminescence's luminal reagents (Santa Cruz Biotechnology, USA).

Statistical analysis. All experiments were repeated at least three times. Statistical analysis was performed with *t* test. $P < 0.05$ was considered to be statistically significant.

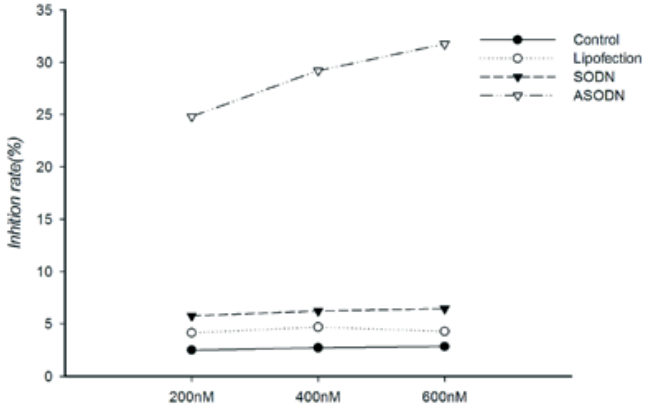

Fig.1 Effect of antisense oligonucleotide on cell proliferation. The effect of antisurvivin oligonucleotides on the proliferation of human osteosarcoma cells line, MG-63 was examined by MTT assay. Shown in the value of absorbance at 490 nm. Untreated cells, cells treated with liposome alone, and cells treated with the control oligonucleotide were used as comparison.

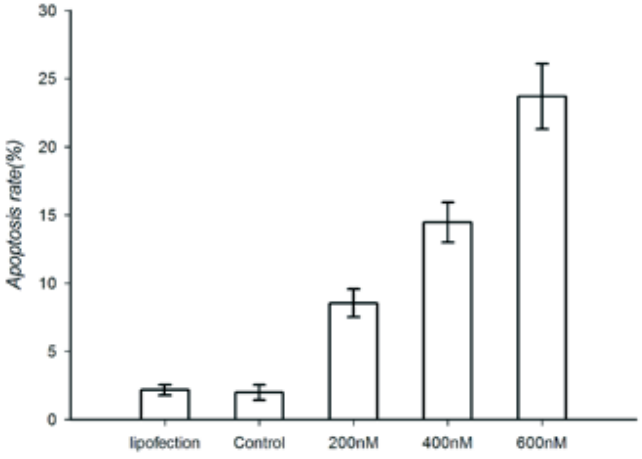

Fig.2 Apoptosis of osteosarcoma cells line MG-63 induced by antisurvivin oligonucleotides was evaluated by flow cytometry. Cells were divided into four groups as described before. After transfected for 48 h, cells were collected and the apoptosis rate was evaluated by flow cytometry.

Results

*Effects of antisurvivin oligonucleotides on MG -63 cells' proliferation***.** Previous studies have confirmed that survivin was highly expressed in osteosarcoma and involved in cell proliferation and apoptosis. To observe the effects of antisurvivin oligonucleotides on MG-63 cells' proliferation, methyl thiazolyl tetrazolium (MTT) was used after the treatment as described before. As shown in Fig.1, Transfected with antisurvivin oligonucleotide caused the growth inhibition rate rising conspicuously in MG-63 cells 48 h later in a dose dependent manner ($P<0.05$). When the concentration of antisurvivin oligonucleotide was 600 nM, the growth inhibition rate reached the peak.

Inhibition of the expression of survivin by antisurvivin oligonucleotides enhances the apoptosis in osteosarcoma cells. After the treatment as described before for 48 hours, flow cytometry analysis was used to detect the apoptosis rate of human osteosarcoma cells MG-63. As shown in Fig.2, the apoptosis rates of cells in the experimental groups treated with antisurvivin oligonuceotides (200 nM, 400 nM and 600 nM) were significantly higher than that in control groups which suggested that survivin blockaded by oligonuceotides could induce apoptosis in vitro. The dose-dependent effects could also be observed in our experiment. A strong effect was achieved with a high antisurvivin oligonucleotide concentration (600 nM). (Fig.2)

Down-regulating effects of antisurvivin oligonucleotides on the mRNA and protein expression of survivin. As we had described in the MTT and flow cytometry assay, great changes had been observed 48 h after transfected. Thus we chose 48 h as the time point to survey the expression of survivin mRNA and protein by RT-PCR and western blot. In our results, the expression of survivin mRNA and protein in the cells treated with different concentration of antisense oligonucleotides were decreased when comparing with the control groups. The most significant effect happened in the cells transfected by antisense oligonucleotide with the concentration of 600 nM. (Fig.3)

Effects of antisurvivin oligonucleotides on the mRNA and protein expression of Fas. For further evaluating the effect on the expression of Fas, we detected the mRNA and protein expression of Fas by RT-PCR and western blot after transfected with different concentration of antisense oligonucleotides. As shown in Fig. 3, we found that the mRNA and protein expression of Fas in MG-63 cells rose after transfected with antisurvivin oligonuceotides which suggested that the expression of Fas can be regulated by survivin blockaded.

Discussion

Osteosarcoma is the most common primary malignant bone tumor which occurs mainly in children and young adults [21]. It is an extremely aggressive disease and occurs mainly in long bones with a high degree of lung metastases [22]. The five years survival rate of osteosarcoma patients is only 20% when treated with traditional methods such as surgery, radiotherapy and chemotherapy [23]. Some patients have shown a good response to neoadjuvant chemotherapy and they were eventually cured by the subsequent surgery, however many osteosarcoma patients remained resistant to treatment due to rapid progression, metastasis, and tendency to chemotherapy resistance [24]. Thus, several strategies have been made to identify new prognostic factors of the patients with osteosarcoma and to improve the curative effects. Gene therapy as a new method has been found to be an effective way in the therapy of cancer

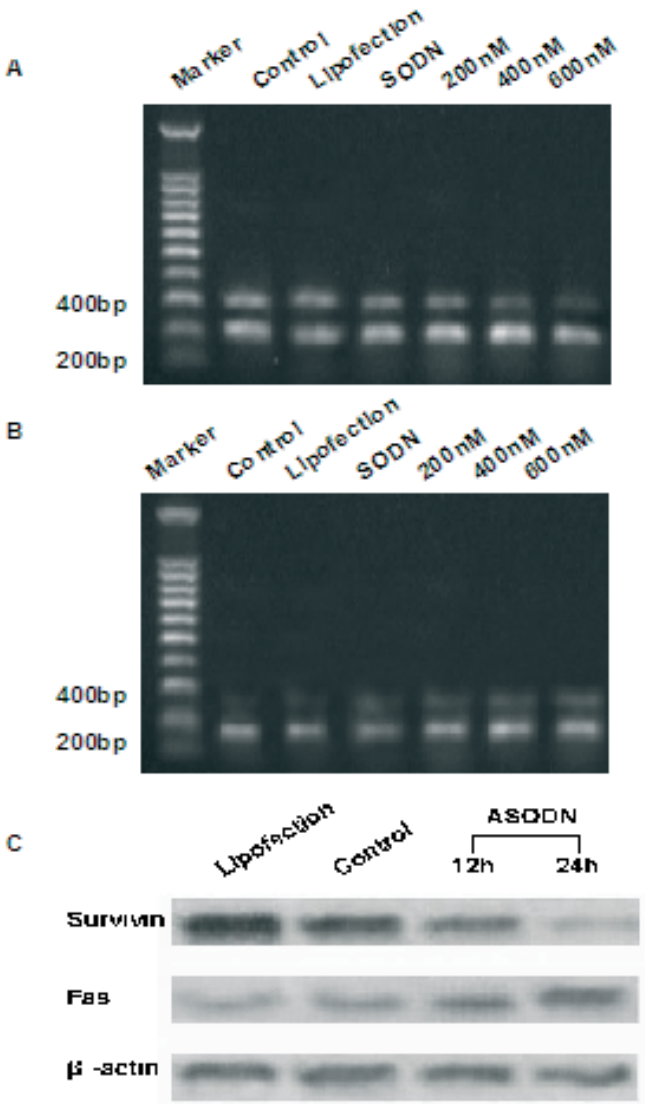

Fig.3 The mRNA and protein expression of Survivin and Fas in human osteosarcoma cells line MG-63 after transfected with antisurvivin oligonucleotides. A. The mRNA expression of Survivin in MG-63 cells. PCR products were identified by 1.5% agarose gels electrophoresis containing 0.5% ethidium bromide. Relative sizes of RT-PCR products are as follows: Survivin, 374 bp; and GAPDH, 230 bp. Molecular size markers are the 100 bp ladders. B. The mRNA expression of Fas in MG-63 cells. RT-PCR products are as follows: Fas, 316 bp; and GAPDH, 230 bp. C. Immunochemical detection of the expression of Survivin and Fas protein in MG-63 cells by mouse anti-human monoclonal antibodies. The expression of β-actin protein was used as control.

by regulating the expression of some oncogenes and factors related to the development of cancer [25].

Survivin is a new member of inhibitors of apoptosis (IAPs) family and is expressed during fetal development and in tumor tissues, but it lacks expression in the terminally differentiated adult tissues [26]. Several studies have discovered that survivin was overexpressed in a variety of human cancers. It was reported that the expression of survivin was up-regulated in non-small cell lung cancer, colorectal cancer, ovarian cancer, breast cancer, and even other sites [27–30]. Overexpression of Survivin correlates with the resistance to chemotherapy and the poor prognosis. Recent studies have found that Survivin was overexpressed in osteosarcoma and was associated with the development, progression, and resistance to chemotherapy of this tumor. It is suggested that survivin may be a useful target for the therapy of osteosarcoma [31].

Several studies have reported that antisense oligonucleotides could be used for their ability to block gene expression in tumor cells and induce apoptosis [32]. Therapeutic strategies using antisense oligonucleotides have been found to be an effective way to down-regulate survivin and reduce the apoptotic threshold in tumor cells. After survivin has been considered as a potential target for cancer therapy, a number of antisurvivin oligonucleotides have been described to be used for their ability to block survivin expression in tumor cells [33–35]. Leech [36] used survivin antisense oligonucleotide to induce the apoptosis of lung cancer cells and got a great achievement.

In our study, we used the antisurvivin oligonucleotides 4003, which had been confirmed to be the most effective method in inducing growth inhibition and apoptosis in a lung carcinoma cell line A549 by Olie [37], to down-regulate the Survivin expression in survivin-positive cells line, MG-63, and demonstrate its ability to induce apoptosis and proliferation inhibition. We found that the cell viability was significantly decreased after transfected with antisurvivin oligonucleotide 48 h later as compared with controls by MTT assay. As shown in Fig.1, both low and high concentrations (200 nM, 400 nM and 600 nM, respectively) of antisurvivin oligonucleotides could inhibit the growth of MG-63 cells. When the concentration of antisurvivin oligonucleotide was 600 nM, the growth inhibition rate reached the peak. It could be suggested that antisurvivin oligonucleotide decrease the proliferation of osteosarcoma cells in vitro. Furthermore, we investigated the apoptosis rate of MG-63 cells after transfected with antisurvivin oligonucleotide by flow cytometry analysis. We observed that different concentrations of antisurvivin oligonucleotides could induce different amount of apoptosis. Compared with control, 200, 400 and 600 nM antisurvivin oligonucleotide caused 21.3%, 27.9%, 33.6% apoptosis in MG-63 cells, respectively, which revealed a dose-dependment increase of apoptotic cells in MG-63. All these data discovered that notable decreased cell growth and increased cell apoptosis were observed in the cells transfected by antisurvivin oligonucleotides.

It was reported that high expression of survivin represented the ability of migration and invasion of tumor cells which is associated with clinical resistance to chemotherapy and progression of osteosarcoma. We have confirmed that transfection with antisurvivin oligonucleotide could induce apoptosis and inhibit the proliferation of MG-63 cells. To detect the mRNA and protein expression of survivin, we used RT-PCR and western-blot methods. In our results, we found that the

mRNA and protein expression of survivin degraded significantly after transfected with antisurvivin oligonucleotide. The mRNA and protein expression of survivin reduced in MG-63 cells were observed with the concentration of ASODN from 200 nM to 600 nM. When the concentration of ASODN was 600 nM, the reduction effect reached the peak. Some authors have demonstrated that survivin could inhibit Fas-mediated apoptosis induced by immune cells [38]. Survivin might block Fas-mediated apoptosis through direct inhibition of caspase-3 and -7, which act as terminal effectors in the apoptotic protease cascade [39]. This indicated that cancer cells could protect themselves from apoptosis by survivin expression. In our study, we down-regulated the expression of survivin by use of different concentration of antisurvivin oligonucleotide and observed the expression of Fas in osteosarcoma cells by RT-PCR and western-blot. We found that the mRNA and protein expression of Fas were up-regulated with the increased concentration of ASODN from 200 nM to 600 nM, which was negative associated with the expression change of survivin. All this suggested that antisurvivin oligonucleotide could decrease the expression of survivin and activate the Fas-mediated apoptosis.

In conclusion, we have shown that survivin played an important role in the development of osteosarcoma and antisense oligonucleotide targeting survivin could significantly down-regulate the expression of survivin and reactivate the Fas-mediated apoptosis resulting in proliferation inhibition and apoptosis induction of osteosarcoma cells in vitro. Targeting survivin by using antisurvivin oligonucleotide might be an effective therapeutic strategy of osteosarcoma. Therefore, it is expected that survivin inhibition in combination with chemotherapy might be a useful therapeutic approach to improve the clinical effectiveness in the treatment of osteosarcoma.

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