MICRORNA-218 INHIBITS THE GROWTH AND INVASION OF OSTEOSARCOMA CELLS BY TARGETING SURVIVOR

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ABSTRACT

Objective: *The purpose of this study was to explore the relationship between microRNA-218, survival hormone and its mechanism in osteosarcoma.*

Methods: *The transcription levels of microRNA-218 and survivin in osteosarcoma cells MG63 and U2OS and human osteoblast cell line hFOB1.19 were measured by quantitative RT-PCR, and the protein expression of survivin and related apoptotic proteins Caspase-3 and Caspase-9 were detected by western blotting. The effect of microRNA-218 on cell proliferation was evaluated by a cell counting kit, and the effect of microRNA-218 on cell migration and invasion was investigated by Transwell assay. The interaction and mechanism between microRNA-218 and the survivor were studied by qRT-PCR, western blotting and luciferase assay.*

Results: *RT-qPCR results showed that microRNA-218 was downregulated in osteosarcoma cell lines MG63 and U2OS compared with human osteoblast cell line hFOB1.19. Western blotting and PCR results showed that compared with hFOB1.19 cells, the expression of survivin in MG63 and U2OS cells was significantly increased. They were negatively correlated with each other, while the expression of apoptosis proteins Caspase-3 and Caspase-9 was significantly decreased. The binding sequence of survivor to microRNA-218 was predicted by Targetscan, and the results of double luciferase assay showed that the survivor was the direct target of microRNA-218. The overexpression of microRNA-218 significantly decreased the proliferation, migration and invasion of osteosarcoma cells. The expression of the survivor decreased, while the overexpression of microRNA-218 blocked the inhibitory effect of the upregulation of microRNA-218 on the proliferation and invasion of osteosarcoma cells.*

Conclusion: *Our data show that microRNA-218 affects osteosarcoma cells by regulating the expression of survivin, which may provide a theoretical basis for the treatment of osteosarcoma with microRNA-218.*

Keywords: *MicroRNA-218, survivor, osteosarcoma.*

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Introduction

Osteosarcoma (OS) is an invasive malignant tumour, which mainly occurs in children and adolescents and is caused by primitive bone-forming mesenchymal cells[1]. The main common sites of osteosarcoma are the distal femur, proximal humerus and proximal humerus. More than half of osteosarcoma occurs around the knee joint, and it is characterised by high mortality and a low cure rate[2, 3]. Neoadjuvant chemotherapy, surgical resection and limb salvage surgery are the main treatment strategies for osteosarcoma[4]. For high-grade osteosarcoma, the five-year survival rate of osteosarcoma patients without tumour metastasis was less than 60-70%[5, 6], while the survival rate of osteosarcoma patients with low-grade osteosarcoma was significantly higher than that of low-grade osteosarcoma[7, 8]. Unfortunately, most osteosarcoma patients are diagnosed at an advanced stage, and the prognosis of these patients is still poor[9]. Although more and more new evaluation methods have been used in the diagnosis and treatment of osteosarcoma, the overall survival of osteosarcoma has not improved since the 1990s, and molecular targeted therapy has been seen as a potential strategy for the treatment of osteosar-

coma[10]. Therefore, it is very important to identify new targets and the potential molecular mechanisms driving the pathogenesis of osteosarcoma. MicroRNAs (miRNAs/mirs) is a short non-coding RNA that is composed of 18-25 nucleotides. It can regulate gene expression by binding to the three-terminal UTR of target mRNA, thus inhibiting the translation of mRNA or inducing its degradation[11]. Previous studies have shown that miRNA exhibits disorder in human cancer. MiRNA regulate gene expression and thus regulate the growth and differentiation of human cancer cells [8≤12].

More and more studies have proved that miRNA plays an important regulatory role in the occurrence and in the development of osteosarcoma[12, 13]. For example, miR-449a is downregulated in osteosarcoma and promotes apoptosis of osteosarcoma cells by targeting B lymphocytes[14]. In addition, miR-20a was overexpressed in osteosarcoma and induced the proliferation of osteosarcoma cells[15]. Studies have shown that miR-218 is significantly downregulated in human cancer tissues compared with adjacent non-cancer tissues, so miR-218 plays an important role in inhibiting miRNA in human cancer[16]. Studies have also shown that miR-218 can inhibit the growth and invasion of cancer cells by regulating carcinogenic genes[17-21], and miR-218 increases the chemical sensitivity of many types of human cancer cells, including oesophageal cancer, colorectal cancer, breast cancer and gastric cancer[22-25]. In this study, we evaluated the expression of miR-218 in osteosarcoma cells and identified the downstream target of miR-218.

The results showed that miR-185 was significantly downregulated in osteosarcoma tissues and cells. The ectopic overexpression of miR-185 inhibited the growth and migration of osteosarcoma cells. These findings suggest that miR-185 has a potential tumour inhibitory effect in osteosarcoma.

Method

Cell culture

Human osteosarcoma cell line MG63, U2OS, and human osteoblastic cell line hFOB1.19 were all from Shanghai cell institute of the Chinese Academy of Sciences.

Cells were cultured in DMEM (Life Technologies, CA, USA) supplemented with 10% FBS, 1% penicillin and 1% streptomycin (Invitrogen, Carlsbad, CA, USA) in a constant temperature incubator of 5% CO2 at 37°C.

Transfection

MiR-NC and miR-218 mimics were purchased from Ambion (Austin, TX, USA). MiR-218 mimics, inhibitors or controls were diluted for 15 minutes in opti-mem medium (Life Technologies, CA, USA) at room temperature (RT), then human osteosarcoma cells were transfected with miR-218 mimics or inhibitors and cultured for 48 hours. The expression of mir-218 was detected by qRT-PCR.

Lipofectamine RNAiMAX reagent (Invitrogen, CA, USA) was used to transfect siRNA according to the manufacturer's instructions. The expression of survivin was examined by qRT-PCR and western blotting.

RNA extraction and RT-qPCR

Total RNA was isolated from control or osteosarcoma cells were transfected using TRIzol reagent (Invitrogen). MiRNA complementary DNA (cDNA) was transformed from total RNA using the PrimeScript miRNA cDNA synthesis Kit (TaKaRa, Tokyo, Japan), and survivin cDNA was synthesised from total RNA using the PrimeScript RT Master Mix Kit (TaKaRa).

TaqMan MicroRNA Assays (Applied Biosystems, CA, USA) were used to quantify mir-218 with specific primers and probes, and RNA U6 was used as an internal control. QPCR assay (SYBR Green; Bio-Rad, USA) was used to measure the mRNA expression of viable protein. GAPDH was used as internal control and relative mRNA expression was calculated.

The sequence of specific primer for mRNA amplification is as follows: U6 forward primer: 5'-TGCGGGTGCTCGCTTCGGC-3', reverse primer: 5'-CCAGTGCAGGGTCCGAGGT-3'; Positive primer of survivin: 5'-GGACCACCGCATCTCTACA T-3', reverse primer: 5'-GACAGAAAGGAAAGCCAAC-3'; GAPDH forward primer: 5'-TCGAGTCAGCCGCATC-TTCTTT-3', reverse primer: 5'-ACCAAATCCGTTG-ACTCCGACCT T-3 '.

Cell viability detection

Cell activity was measured by CCK-8 (Promega) according to the manufacturer's protocol. In short, transfected human OS cells were inoculated into 96-well plates and left to adhere overnight. Freshly prepared simulants, inhibitors or siRNA were then added to the Wells as planned and placed in an incubator for 72 hours.

Finally, CCK-8 solution was added to the 96-well plate and incubated at 37°C for another two

hours. Finally, the absorbance at 450nm was measured using the microplate reader.

Luciferase reporter gene assay

A fragment of the 3'UTR from the viable protein gene was amplified by PCR from the genomic DNA, which contained the predicted binding site for mir-218. The amplified fragment was cloned into the UTR downstream of luciferase gene in pMIR-reported luciferase vector (Ambion, USA). Corresponding mutant constructs were used as controls. Human osteosarcoma cells were co-transfected with luciferase reporter plasmid and Renilla luciferase plasmid. Then, cells were harvested, and the Dual-Glo Luciferase Assay System was used to measure Dual Luciferase activity, and Renilla was used as a standardised internal control.

Western blotting

Whole cell extracts were prepared by lysis of cells in a cold lysate buffer. The insoluble substances were separated by high speed centrifugation and the protein concentration of the supernatant was determined using the BCA protein assay kit. Each group was sampled with 10% SDS-PAGE gel, and the transformed strips were incubated overnight with the corresponding primary antibody at 4°C. After washing, the protein strip on PVDF membrane was incubated with the corresponding secondary antibody, the colour was developed by an ECL reagent box test, and the strip was analysed after exposure by the gel imager. The protein content was expressed as the relative value of the corresponding internal parameter strip.

Results

Negative correlation was found between miR-218 and survivin expression in osteosarcoma cells

We measured the transcription levels of miR-218 in osteosarcoma cells MG63 and U2OS and human osteoblastic cell line hFOB1.19 by RT-qPCR. The results showed that the transcription levels of miR-218 in osteosarcoma cells were significantly decreased.

At the same time, the RT-qPCR and western blotting respectively tested the osteosarcoma MG63 cells and U2OS hFOB1.19 ossification cell line with people in the transcription and expression level of survivin and related apoptosis protein Caspase-3, and the expression of Caspase-9, according to the results of survivin in osteosarcoma MG63 cells and U2OS transcription and expression level increased significantly.

At the same time, related apoptosis protein Caspase-3 and the expression of Caspase-9 significantly reduced. The above results showed that mir-218 was negatively correlated with the expression of survivin in osteosarcoma cells.

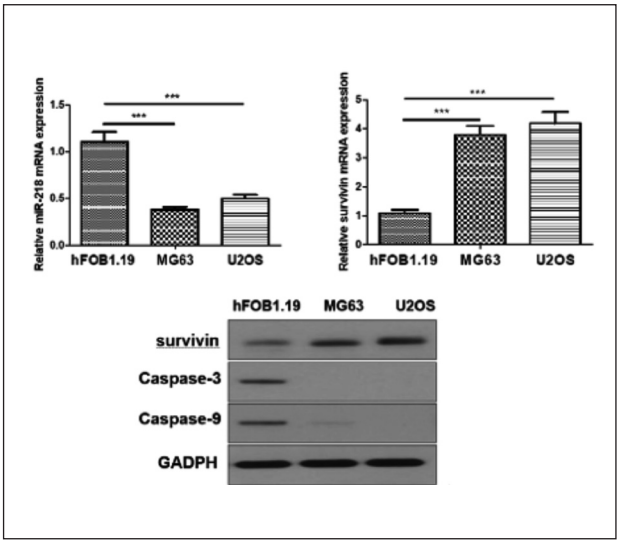

Figure 1: miR-218 and survivin are negatively correlated in osteosarcoma cells.
Transcription levels of microRNA-218 and survivin in osteosarcoma cells MG63 and U2OS and human osteoblast cell line hFOB1.19 were determined by quantitative RT-qPCR. The expression of survivin and Caspase-3 and Caspase-9 in osteosarcoma cells MG63 and U2OS and human osteoblasts was detected by western blotting. Data are shown as mean ± SD. $^{***}P<0.001$.

Survivin is a direct target of miR-218 in osteosarcoma cells

In order to further determine whether the miR-218 in osteosarcoma play a role in molecular basis, we used the mirbase database (http://microrna.sanger.ac.uk/cgi-bin/) to predict the target genes of miR-218. By using prediction, we found that survive protein is a potential target of miR-218. At the same time, in order to determine whether survivin OS cell is the direct target of miR-218, we will have survivin wild type (WT) or mutant (Mut) 3 'UTR of miR-218 plasmid and luciferase report plasmid transfection to OS cells, and then we will measure the value of each of the luciferase.

The results showed that mir-218 significantly reduced luciferase activity in MG63 and U2OS cells in WT group, while miR-218 did not affect luciferase activity in Mut group. These results indicated that survivin was a direct target of mir-218 in OS cells.

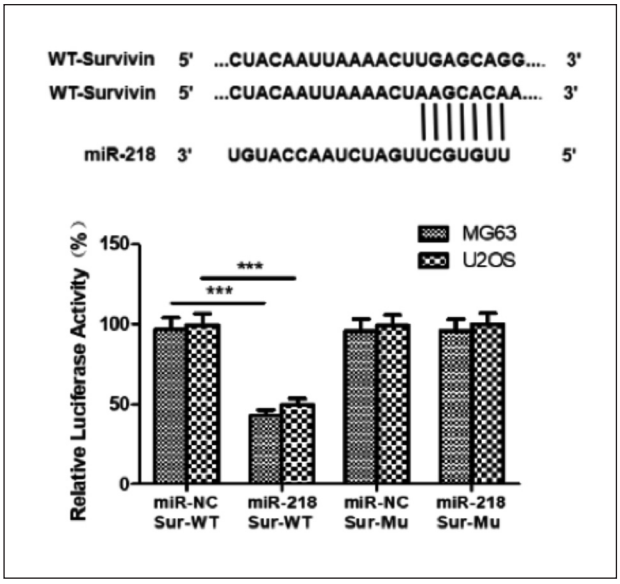

Figure 2: Survivin is a direct target of miR-218 in osteosarcoma cells.
Wild type and mutant miR-218 targeting sequences and miR-218 sequences in survivin mRNA. Luciferase activity in OS cells. ***p<0.001.

Overexpression of miR-218 inhibits the growth and invasion of osteosarcoma cells by inhibiting survivin

In order to further confirm the role of miR-218 in osteosarcoma cells by regulating the survival factor, we constructed osteosarcoma cells overexpressing miR-218 by transfection of mir-218 plasmid into MG63 and U2OS cells.

First, western blotting was used to detect the expression levels of survivins and related apoptotic proteins caspase-3 and caspase-9 in miR-218 overexpressed MG63 and U2OS cells as well as those in the control group. The results showed that the expressions of survivins in mir-218 overexpressed MG63 and U2OS cells were significantly decreased, while the expressions of related apoptotic proteins caspase-3 and caspase-9 were increased. Then we tested the proliferation levels of MG63 and U2OS cells overexpressed by miR-218 over time and those of the control group through CCK-8 experiment. The results showed that the proliferation ability of miR-218 overexpressed MG63 and U2OS cells was significantly reduced.

Simultaneously, we conducted Transwell experiments in each group of cells, and the results showed that the invasion ability of miR-218 overexpressed MG63 and U2OS cells was significantly reduced compared with the control group.

It can be seen from the above results that the overexpression of miR-218 inhibits the proliferation and invasion ability of osteosarcoma cells by inducing apoptosis, and it may be realised by regulating the survival factor.

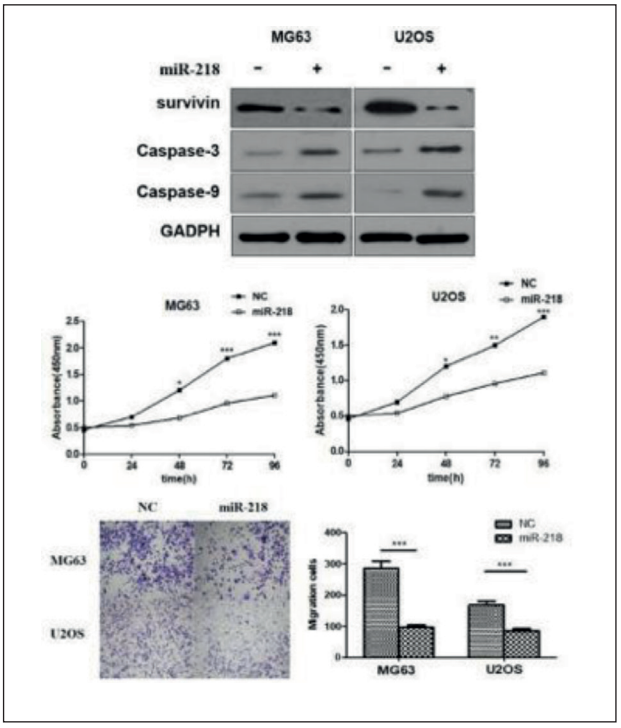

Figure 3: Overexpression of miR-218 inhibits the growth and invasion of osteosarcoma cells by inhibiting Survivin. Survivin, Caspase-3 and Caspase-9 expression of control and miR-218 overexpressing osteosarcoma cells MG63 and U2OS was detected by western blotting. The proliferation activity of the control and miR-218 overexpressing osteosarcoma cells MG63 and U2OS was examined by CCK-8 assay. The invasion and migration ability of the control and miR-218 overexpressing osteosarcoma cells MG63 and U2OS were examined by Transwell assay. *, p<0.05; **, p<0.01; ***, p<0.001.

The overexpression of induced survivin blocked the inhibition of upregulated microRNA-218 on the proliferation and invasion of osteosarcoma cells

We spent the miR-218, expressed MG63 and further transfected the survival quality in the grain of the U2OS cells in the survivin expression. Through the expression of survivin through miR-218 expression MG63 and in contrast to the U2OS cell through the western blotting test related to the apoptosis protein Caspase-3 and the expression of Caspase-9 levels, the results showed that survivin expression significantly reduced the miR-218 expression MG63 and U2OS cell apoptosis protein expression. Then we conducted cck-8 experiments on the overexpression of survivin and the control miR-218 overexpressed MG63 and U2OS cells,

and the results showed that the overexpression of survivin led to the decreased proliferation ability of miR-218 overexpressed MG63 and U2OS cells. At the same time, the Transwell experiment was carried out on each group of cells, and the results showed that overexpression of survivin resulted in decreased invasion and migration ability of miR-218 overexpressed MG63 and U2OS cells. These results showed that the overexpression of survivin reversed the inhibitory effect of miR-218 overexpression on the proliferation and invasion of osteosarcoma cells.

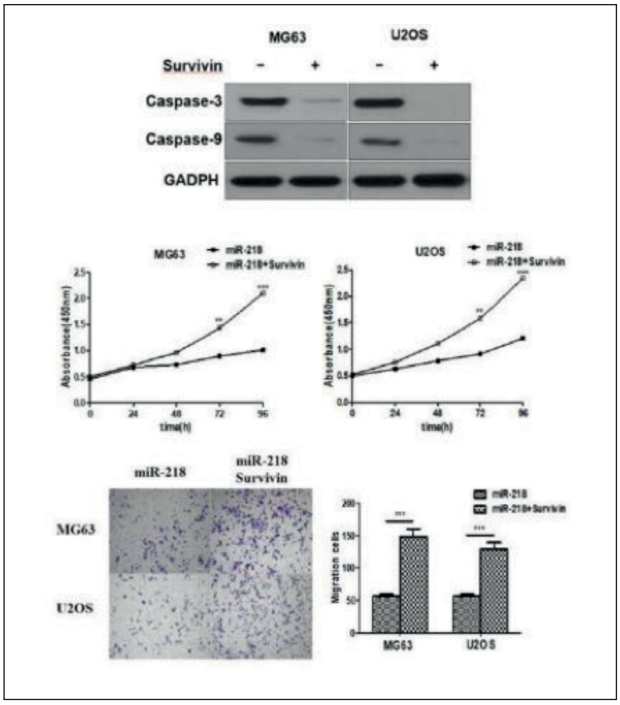

Figure 4: Induction of survivin overexpression inhibits the inhibitory effect of upregulation of microRNA-218 on proliferation and invasion of osteosarcoma cells.
This demonstrates the detection of related expression levels of caspase-3 and caspase-9 by western blotting. The proliferation activity of survivin overexpression and control miR-218 overexpressing osteosarcoma cells MG63 and U2OS was detected by CCK-8 assay. Transwell assay was used to detect the invasion and migration of survivin overexpression and control miR-218 overexpressing osteosarcoma cells MG63 and U2OS. **, $p<0.01$; ***, $p<0.001$.

Discussion

MiRNA is a non-coding single-stranded RNA, which is widely found in eukaryotes (26). Previous studies have shown that several miRNAs are abnormally expressed in a variety of tumours, and they regulate many biological processes in cancer cells, including cell proliferation, apoptosis and the stress response[27-28]. MiR-218 is an intron miRNA that encodes in the intron sequences of tumour suppressor genes SLIT2 and SLIT3[29]. MiR-218 has been reported to be downregulated in a variety of human cancers, including non-small cell lung cancer, oral cancer and cervical cancer[30-34]. Studies have also shown that miR-218 is associated with chemical resistance of cancer cells[35-37], but the biological role of miR-218 in osteosarcoma cells is not fully understood. In order to study the role of miR-218 in the occurrence and development of osteosarcoma, we conducted various assays and comparisons between osteosarcoma cells MG63 and U2OS and the human osteoblastic cell line hFOB1.19. The results showed that mir-218 was significantly downregulated in osteosarcoma cells MG63 and U2OS compared with human osteoblastic cell line hFOB1.19, and it significantly enhanced the apoptosis degree of osteosarcoma cells through overexpression of miR-218, while reducing their growth and invasion ability. Then, by using the miRNA target tool for prediction, we screened out the target gene of survival as miR-218 and confirmed that survival was the direct target gene of miR-218 by double luciferase assay. Therefore, the regulation of miR-218 on the growth and invasion of osteosarcoma cells may be carried out by regulating survival.

Survivin, a member of the apoptosis inhibitor family, is encoded by a baculovirus inhibitor that repeats apoptosis[38]. Studies have found that the imbalance of survivin has been observed in many types of human cancers, including breast cancer, lung cancer, prostate cancer, gastric cancer and colon cancer[39]. Studies have shown that survivin can inhibit the activity of caspase-9 to demonstrate the anti-apoptotic ability[40]. Studies have also shown that survivin plays an important biological role in the chemical resistance of cancer cells, and that survivin will become a biomarker in cancer treatment[41]. In this study, we reported that the expression of survivin in osteosarcoma cells MG63 and U2OS cells was significantly upregulated, and the overexpression of survivin enhanced the proliferation and invasion ability of osteosarcoma cells. We also reported that the downregulation of mir-218 increased the expression of survivin, while the overexpression of miR-218 decreased the expression of survivin. At the same time, the results showed that miR-218 could inhibit cell apoptosis, so we believed that miR-218 regulated the proliferation and invasion of osteosarcoma cells by regulating survivin.

In conclusion, in this study, we reported that survivin is an important mediator for miR-218 to

inhibit the growth and invasion of osteosarcoma cells. MiR-218 regulates the development of osteosarcoma by affecting the proliferation and invasion of osteosarcoma cells, which may be regulated by survivin in osteosarcoma cells. These results suggest that regulation of miR-218 may be beneficial to the clinical treatment of osteosarcoma in the future.

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