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MiR-361 inhibits osteosarcoma cell lines invasion and proliferation by targeting FKBP14

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Abstract. – OBJECTIVE: MicroRNAs have been reported to contribute to the development of osteosarcoma via negatively regulating the target genes. Nevertheless, the undiscovered function of miR-361 on osteosarcoma development remains uncertain.

PATIENTS AND METHODS: MiR-361 and FKBP14 (FK506-binding protein 14) expression in osteosarcoma samples were detected by Real-time polymerase chain reaction (PCR). Cells invasive ability was examined via the transwell invasion assay. The luciferase reporter assay was used to examine the regulation mechanism. The protein level of FKBP14 was detected by Western blot. Cell Counting Kit-8 (CCK-8) was used to detect cell lines proliferation.

RESULTS: MiR-361 was reduced both in osteosarcoma samples and cell lines. Up-regulation of miR-361 significantly inhibited cells invasive and proliferative abilities, while down-regulation of miR-361 promoted cell lines invasion and proliferation. miR-361 could negatively regulate FKBP14 in osteosarcoma. Suppression of FKBP14 could reverse the function of miR-361 inhibitor.

CONCLUSIONS: MiR-361 inhibits osteosarcoma cell lines invasion and proliferation by targeting FKBP14.

Key Words:
MiR-361, FKBP14, Invasion, Proliferation, Osteosarcoma.

Introduction

Osteosarcoma is a type of malignant bone tumors in the world. A lot of strategies have been used for the diagnosis and therapy of osteosarcoma, but the average 5-year overall survival rate is still poor[1]. Many previous studies reported that the molecular and cellular mechanisms contribute to osteosarcoma development[2-4]. Thus, to improve the therapy effect of osteosarcoma, we need to investigate the molecular mechanism and confirm the therapeutic target. MicroRNAs (miRNAs), a small non-coding RNAs, have been confirmed to be able to influence the biological processes of different diseases by negatively regulating the targeting genes[5-7]. miR-130a as an oncogenic microRNA, targets PTEN to drive malignant cell survival and tumor growth[8]. The expression of miR-30a was decreased in colorectal cancer tissues, and might act as a therapeutic strategy for colorectal cancer metastasis[9]. MiRNAs were also implicated in the biological process of osteosarcoma. For instance, miR-448 inhibits cells invasion and proliferation of osteosarcoma via regulating Ephrin type-A receptor 7 (EPHA7) gene[10]. MiRNA-203 acts as a tumor-suppressor inhibiting osteosarcoma cells invasive and proliferative ability through controlling Runt-related transcription factor 2[11].

MiR-361 was reduced and inhibited HCC cells proliferation and invasion by regulating vascular endothelial growth factor A (VEGFA)[12]. Up-regulation of miR-361 could predict better prognosis for patients with breast cancer[13]. MiRNA-361 could depress HCC cells growth through controlling the target gene chemokine receptor 6 (CXCR6)[14]. The potential functions and mechanisms of miR-361 in osteosarcoma development are unclear.

Patients and Methods

Clinical Sample

A total of 50 samples were collected from the patients with osteosarcoma in our hospital. The patients had provided the written informed consent. This study was approved by the Ethics Committee of Yantaishan Hospital.

Cell Transfection

Osteosarcoma cell lines (U2OS, MG-63, Saos-2 and HOB) were cultured in Roswell Park

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Memorial Institute 1640 (RPMI-1640) Medium with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 5% CO_2 at 37°C. Through the lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA), miR-361 mimics/inhibitor reagents were purchased from GenePharma Company (Shanghai, China) for cells transfection. Moreover, cells were also transfected with siRNA FKBP14 purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with miR-361 inhibitor and siRNA/FKBP14.

Real-time PCR

Through miRNA Isolation Kit (Ambion, Austin, TX, USA), all RNA was obtained. The primers of miR-361 were:

Forward: ATAAAGTGCTGACAGTGCAGATAGTG, Reverse: TCAAGTACCCACAGTGCGGT; FKBP14: Forward: TGAAGGACAGCACCAATAG, Reverse: GCACATTT ACCACCAACTC. This assay was examined by ABI PRISM7500 system (Applied Biosystems, Foster City, CA, USA).

Cell Counting Kit-8 Assay

Based on the detail instructions, in a 96-well plate (Corning, Corning, NY, USA), cell lines were seeded 4×10^3 cells per well. Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Kumamoto, Japan) was added to each well at 0, 24, 48, and 72 h respectively. After that, cells were incubated 2 h at 37°C. Through a microplate reader (Bio-Rad, Hercules, CA, USA), OD (optical density) value was examined.

Cell Invasion Assay

With Matrigel (Biosciences, San Jose, CA, USA) and chambers (Millipore, Billerica, MA, USA), the transwell assay was conducted. In a 24-well plate, all transfected cell lines (1×10^5) were seeded in the upper chamber (8-μm) and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium without serum, incubating in 37°C and 5% CO_2. After 24 h, invaded cells were fixed and stained with crystal violet. All the images were obtained by the NIS Elements software (Nikon, Tokyo, Japan) and analyzed by ImagePro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Western Blot

Through a BCA Kit (Beyotime, Shanghai, China), all the protein collected from the cell lines was examined. The antibodies against FKBP14 and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies at the dilution of 1:2,000 in phosphate buffered saline and Tween 20 (PBST-20) (0.1%) were used for incubation.

Luciferase Reporter

Based on the protocol, we firstly cloned 3′-UTR area that is the predicted region into pGL3 vector; next, we cloned 3'-UTR region with the mutant site. The reporter activity was examined through Victor 1420 Multilabel Counter (Wallac, NY, USA).

Statistical Analysis

The expression levels of miR-361 and FKBP14 in the tissues were detected by x^2 tests. All the histograms were investigated via GraphPad 11.0 (La Jolla, CA, USA), which was used for statistical analysis. $p<0.05$ was regarded as a significant difference.

Results

MiR-361 was Decreased Both in the Osteosarcoma Tissues and Cell Lines

The expression level of miR-361 was detected in the osteosarcoma tissues and the corresponding adjacent area. As compared to the corresponding adjacent tissues, low-expression of miR-361 was detected in the tumor samples ($p<0.05$, Figure 1A). As exhibited in Table I, miR-361 was implicated in metastasis ($p=0.003$) and tumor stage ($p=0.038$). Moreover, we tested miR-361 expression in osteosarcoma cell lines ($p<0.05$). The expression of miR-361 was also downregulated in osteosarcoma cell lines as shown in Figure 1B. Collectively, low-expressed miR-361 acted as a crucial role in the development of osteosarcoma.

MiR-361 Inhibited Osteosarcoma Cell Lines Invasion

Here, to explore the underlying functions of miR-361 in osteosarcoma progression, cells were respectively transfected with negative control, mimics, inhibitor negative control and inhibitor (Figure 2A). Furthermore, by using cell transwell assay, we discovered that up-regulation of miR-361 could inhibit cell lines invasion; inversely, down-regulation of miR-361 could enhance cell lines invasion (Figure 2B). In conclusion, all the findings suggested that

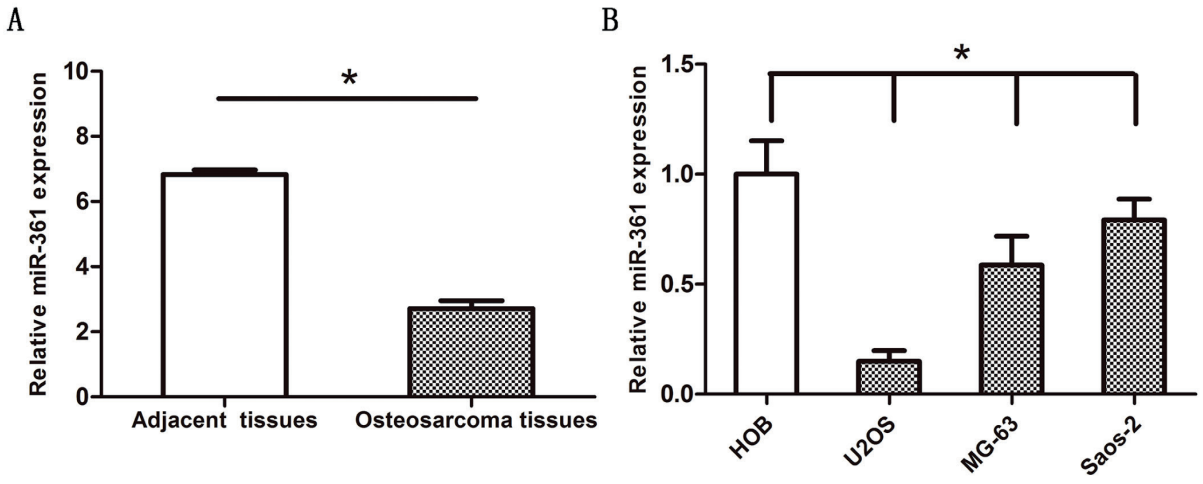

Figure 1. miR-361 expression in osteosarcoma tissues and cell lines. (A) miR-361 in 50 tumor samples and the corresponding adjacent areas by rt-PCR. (B) miR-361 in osteosarcoma cell lines (U2OS, MG-63 and Saos-2) and a normal cell line (HOB) by rt-PCR. miR-361 was normalized with GAPDH expression. *$p<0.05$.

dysregulation of miR-361 plays a regulation role in osteosarcoma metastasis.

MiR-361 Also Could suppress Cells Proliferative Ability

To explore the function of miR-361 on osteosarcoma cells proliferative ability, we performed CCK-8 assay. The results showed significant difference among cells proliferative ability at 24, 48, 72 h (Figure 2C). This data suggested that dysregulation of miR-361 could also suppress cell lines proliferation.

MiR-361 Could Regulate FKBP14 in Osteosarcoma Cells

From TargetScan (http://www.targetscan.org/), FKBP14 was regarded as a target gene; its 3'-UTR might be binding to miR-361 sequence. FKBP14, an oncogene, promotes the osteosarcoma progression[15]. In our study, the expression of FKBP14 was detected in the osteosarcoma tissues and the corresponding adjacent area by using Real-time PCR assay. As compared to the corresponding adjacent tissues, FKBP14 was upregulated in the osteosarcoma tissues ($p<0.05$, Figure 3A). Furthermore,

Table I. Expression levels of miR-361 and FKBP14 in tumor samples and corresponding adjacent tissues.

Characteristics	All patients	miR-361 low expression	miR-361 high expression	p	FKBP14 low expression	FKBP14 high expression	p
N	50	24	26		29	21	
Age (yr)				0.817			0.815
≤60	30	16	14		17	13	
>60	20	10	10		12	8	
Sex				0.982			0.340
Male	27	14	13		14	13	
Female	23	12	11		15	8	
Tumor size (cm)				0.402			0.963
≤2	26	15	11		15	11	
>2	24	11	13		14	10	
TNM stage				0.038			0.035
T1-2	30	12	18		21	9	
T3-4	20	14	6		8	12	
Metastasis				0.003			0.018
No	31	11	20		22	9	
Yes	19	15	4		7	12	

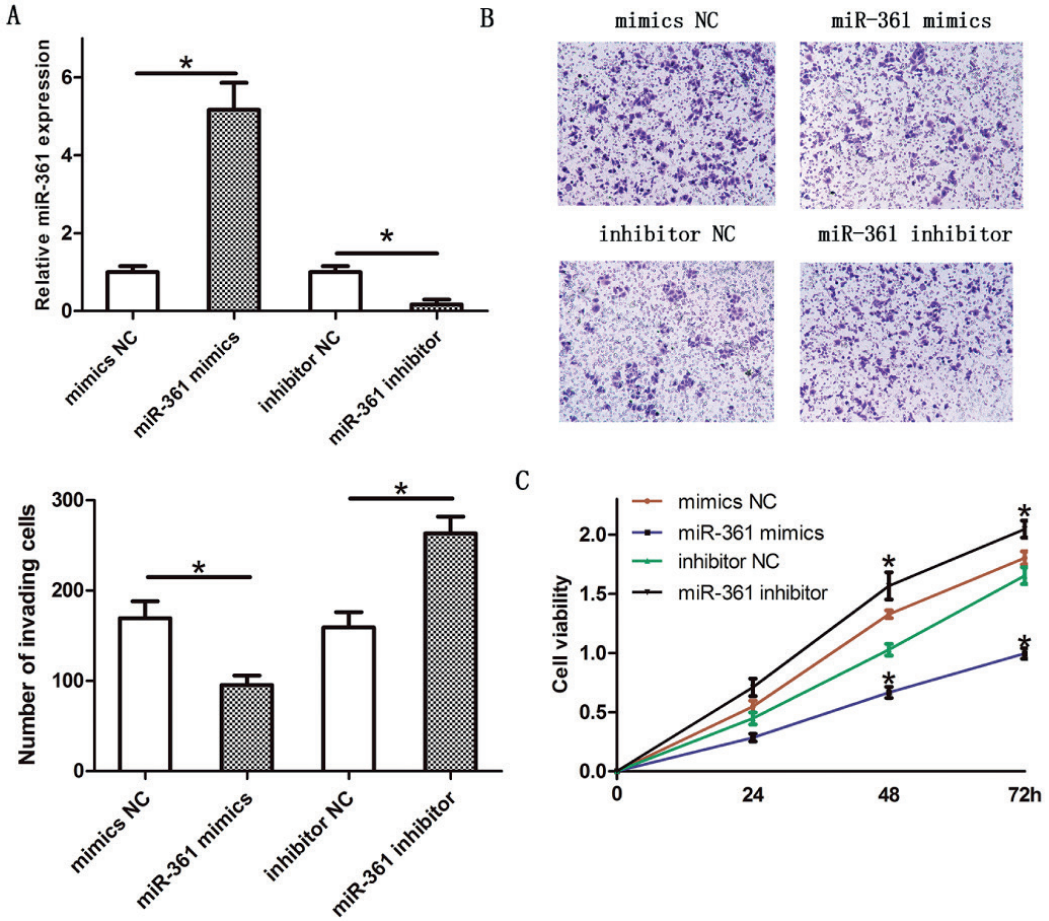

Figure 2. Abnormal expression of miR-361 could regulate cells invasive and proliferative abilities. ***(A)*** Cells were transfected with mimics NC, miR-361 mimics, inhibitor NC and miR-361 inhibitor, the transfection effect was identified via rt-PCR. ***(B)*** Transwell invasion assay was conducted to test cells invasive ability. ***(C)*** CCK-8 assay was used to detect cells proliferative ability when 24, 48, 72 h. **p*<0.05.

there was a negative correlation between miR-361 and FKBP14 (Figure 3B).

To explore whether miR-361 could bind to FKBP14 mRNA, we performed the luciferase reporter assay. The activity was significant weaken in cell lines transfection of miR-361 mimics/pGL3- FKBP14 vectors. On the contrary, there was no effect on the luciferase activity in cell lines transfection of pGL3- FKBP14-mut vector (Figure 3C). To confirm the mechanism of miR-361 and FKBP14, we examined both mRNA and protein expression of FKBP14 with up-regulation or down-regulation of miR-361 expression (Figure 3D-E). The results showed up-regulated miR-361 reduced FKBP14 gene whilst knockdown miR-361 enhanced FKBP14 gene. Based on the above findings, we illustrated that miR-361 could negatively regulate FKBP14 by binding its 3'-UTR.

Silence of FKBP14 Expression Could Reverse the Effect of miR-361 Inhibition

Finally, we down-regulated FKBP14 expression using small interference RNA (siRNA) technology. The knockdown effect was identified through Western blot (Figure 4A). Moreover, to explore whether silence of FKBP14 could influence the cell line invasion and proliferation of miR-361 inhibitor, the CCK-8 and cell invasion assays showed that the promotion effect was reversed when silence of FKBP14 in miR-361 inhibitor group (Figure 4B and C). In sum, this finding indicated that the molecular function of miR-361 in osteosarcoma development depends on regulating its target gene FKBP14.

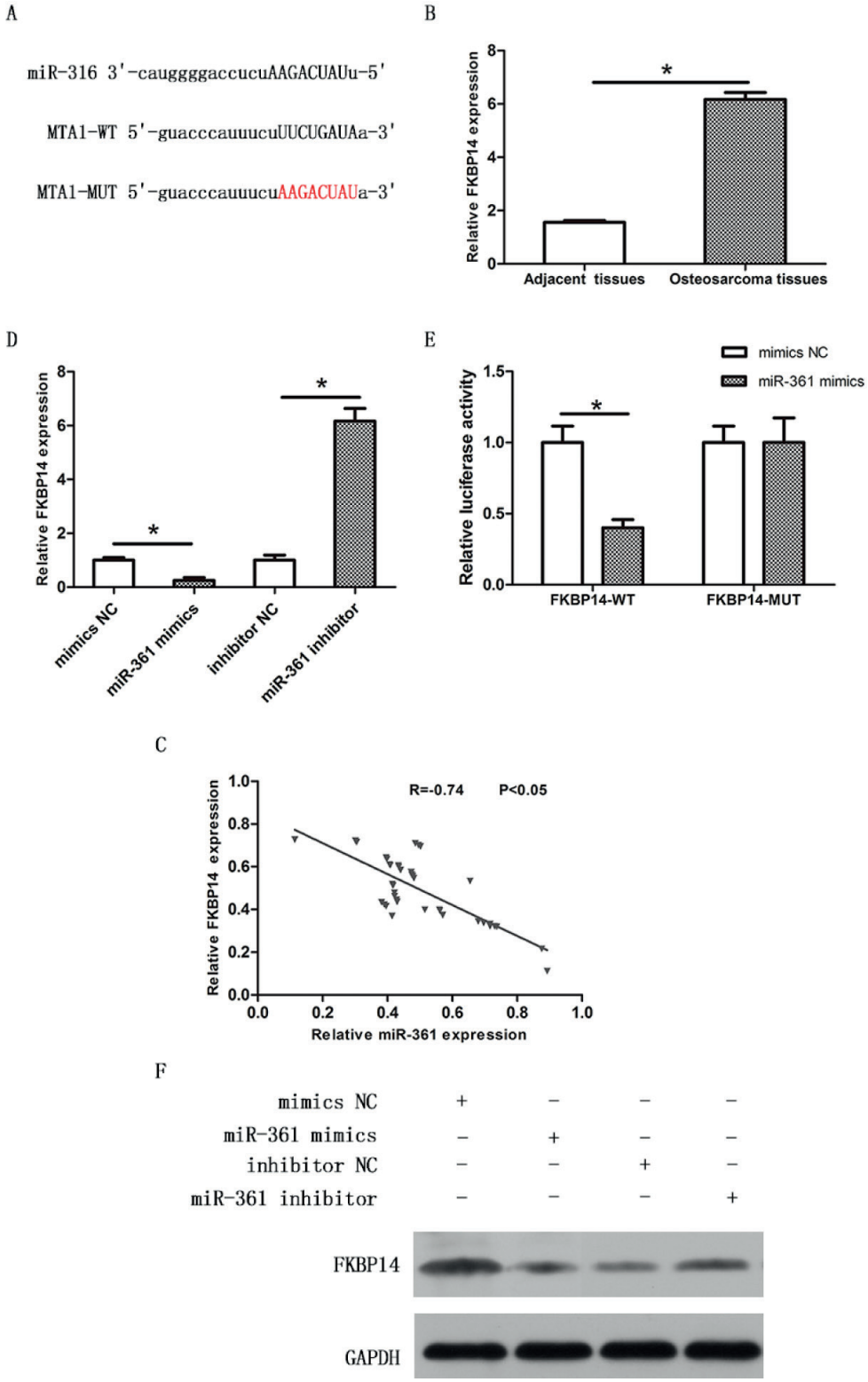

Figure 3. miR-361 negatively regulated FKBP14. ***(A)*** miR-361 in 50 tumor samples and the corresponding adjacent areas by rt-PCR. ***(B)*** An inverse relation was observed between miR-361 and FKBP14 expression R= -0.74. ***(C)*** Luciferase reporter was performed to explore miR-361 targeting FKBP14. ***(D-E)*** miR-361 negatively regulated FKBP14 by rt-PCR and Western blot. **p*<0.05.

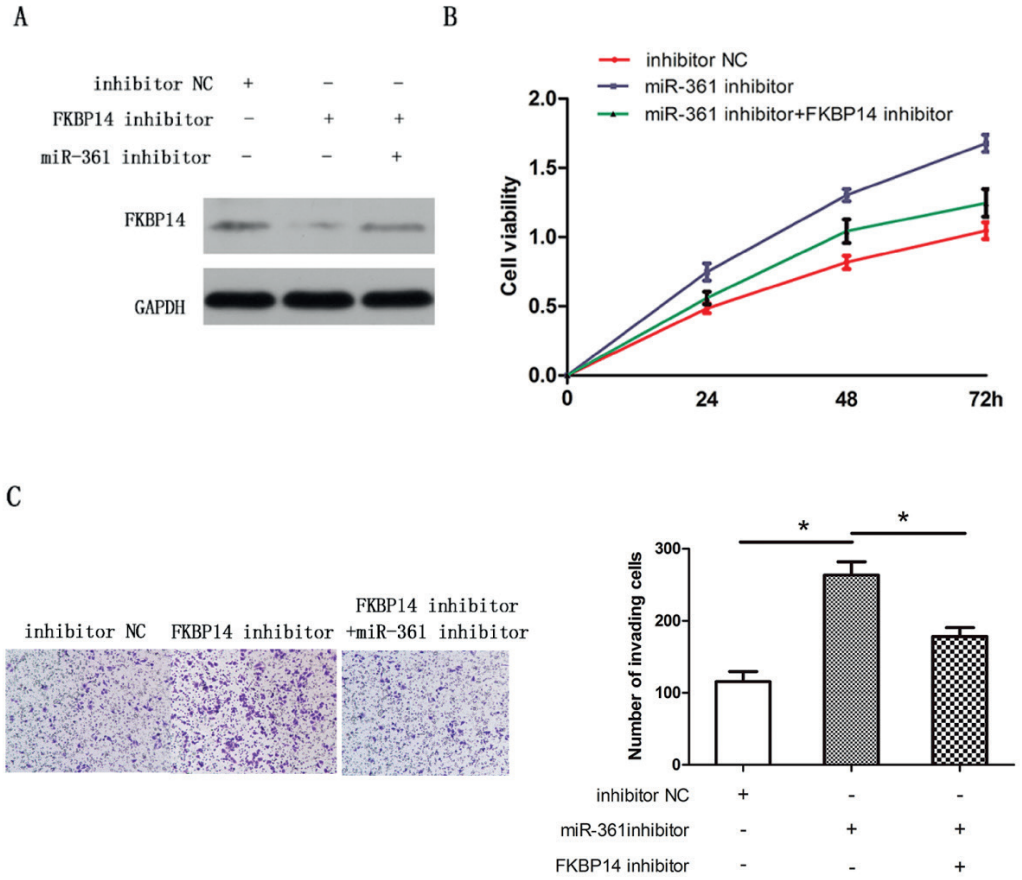

Figure 4. Silence of FKBP14 expression could reverse the effect of miR-361 inhibition. ***(A)*** Silence of FKBP14 expression via siRNA could effectively down-regulate FKBP14 expression, which was identified by Western blot. ***(B)*** A CCK-8 assay was used to detect cells proliferative ability when 24, 48, 72 h. ***(C)*** A transwell invasion assay was conducted to test cells invasive ability. *$p<0.05$.

Discussion

The previous studies had reported that miRNAs contribute to the progression of osteosarcoma[16-18]. Sun et al[19] observed that miR-19 mediated SOCS6 in osteosarcoma growth and revealed miR-19/SOCS6/JAK2/STAT3 pathway as a potential therapeutic strategy for osteosarcoma patients. miR-491-3p was identified as a tumor-inhibitor in osteosarcoma to attenuate the potential of growth and invasion by targeting Tetraspanin 1 (TSPAN1)[20]. MiR-361 has been found to influence the molecular biology of different cancers[12-14]. For example, miR-361 inhibited lung cancer cells proliferation via targeting 3'UTR of Yes-associated protein (YAP)[21]. Many studies have showed that abnormal expression of FKBP14 participated in various types of malignant tumors. For gastric cancer, knockdown FKBP14 expression could suppress cells proliferation, adhesion and invasion[22]. For ovarian cancer, silence of FKBP14 expression could inhibit cells growth[23]. Importantly, for osteosarcoma, Huang et al[15] found that FKBP14 was increased both in the tumor tissues and cell lines. Its abnormal expression level was related to tumor metastases and tumor stage. *In vitro*, down-regulation of FKBP14 could attenuate cell invasion and inhibit PCNA, CDK1 and CCNB1 expression, thus enhancing cell cycle and increasing Bax, caspase-3/7.

In our study, we determined the molecular mechanism of miR-361 in osteosarcoma cells invasion and proliferation. Firstly, from the clinic samples with osteosarcoma, miR-361 expression was downregulated in tumor tissues. By transwell invasion assay and CCK-8 assay, we discovered that up-regulation of miR-361 could inhibit cell lines invasion and proliferation; inversely, down-regulation of miR-361 could enhance cell lines invasion and proliferation. Secondly, using

bioinformatics software, we found FKBP14 was a candidate target gene. FKBP14 expression was increased in tumors, and had an inverse association with miR-361. Through a rescue experiment, our work found that miR-361 could negatively regulate FKBP14 by binding to its 3'UTR region, and the negative regulation effect of miR-361 on cells invasion and proliferation was depended on FKBP14.

Conclusions

Taken together, this study identified that miR-361 is frequently low-expressed in osteosarcoma tissues and cell lines. Moreover, we discovered a negative correlation between miR-361 and FKBP14 expression level, and they were associated with tumor metastasis and tumor stage. We concluded that miR-361 could depress osteosarcoma cells invasion via inhibiting FKBP14. MiR-361 may be a potential therapeutic target in treating osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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