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MicroRNA-1247 inhibits the viability and metastasis of osteosarcoma cells via targeting NRP1 and mediating Wnt/β-catenin pathway

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Abstract. – OBJECTIVE: Osteosarcoma (OS) is a common malignant bone tumor that poses a serious threat to the health of adolescents or children. A large number of studies have proposed the role of microRNAs (miRNAs) in OS, except for miR-1247. Therefore, this research was designed to explore the molecular mechanism of miR-1247 in OS.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) or Western blot analysis was used to measure the expressions of miR-1247 and genes. The function of miR-1247 was investigated using Cell Counting Kit-8 (CCK-8) and transwell assays. The Dual-Luciferase reporter assay was used to explore the relationship between miR-1247 and neuropilin-1 (NRP1).

RESULTS: MiR-1247 was downregulated in OS, which was related to the aggressive behavior of OS patients. Moreover, miR-1247 inhibited cell viability and metastasis in OS. At the same time, miR-1247 promoted apoptosis and inactivated the Wnt/β-catenin pathway in OS. Furthermore, it was confirmed that NRP1 was a direct target of miR-1247. Upregulation of NRP1 attenuated the inhibitory effect of miR-1247 in OS.

CONCLUSIONS: MiR-1247 played a suppressive role in OS by suppressing cell viability and metastasis.

Key Words:
MiR-1247, NRP1, Osteosarcoma, Viability, Metastasis.

Introduction

Osteosarcoma (OS) is a primary malignant or soft tissue tumor that accounts for approximately 34% of malignant bone swelling[1]. In addition, OS can occur at any age, but most OS patients are between the ages of 10 and 25[2]. Now, patients with low-grade malignant OS mainly relies on surgery, who have a good prognosis. However, most of OS tumors are highly malignant. For patients with a highly malignant OS, a combination of chemotherapy and surgery is needed[3]. Surgery is also an extremely important part of the entire treatment process. Whether the tumor can be completely removed is the key to successful or failed treatment. Incomplete resection will inevitably lead to recurrence, and recurrence will greatly increase the risk of distant metastases[4]. The prognosis of patients with malignant OS is extremely poor, and lung metastases can occur within a few months. Furthermore, the survival rate after amputation is only 5% to 20% in 3 to 5 years[5]. Therefore, early detection and timely treatment are the keys to improving the survival rate of OS.

In recent years, microRNAs (miRNAs) have become important regulators in human cancer, and their roles can be found in various processes of tumorigenesis and development. Moreover, miRNAs act as oncogenes or tumor suppressors in human cancer by regulating the expression of related target genes[6]. In OS, more and more miRNAs have been reported to be involved in different processes of malignancy. For example, miR-488 inhibited proliferation, invasion and epithelial-mesenchymal transition (EMT) in OS by targeting AQP3[7]. However, Jiao et al[8] found that miR-645 promoted OS metastasis *via* targeting NME2. In addition, miR-1270 was found to be associated with poor prognosis in OS patients, and its inhibitory effect produced an anti-cancer mechanism in OS[9]. Among many miRNAs, miR-1247 has attracted our attention. Moreover, abnormal expression of miR-1247 had been identified in several cancers, including gastric cancer, colon cancer and prostate cancer[10-12]. Besides that, it had been found that downregulation of miR-1247 was associated with poor prognosis and facilitated tumor cell growth in breast cancer[13]. However, upregulation of miR-1247 was detected

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in resistant prostate cancer[14]. These results indicated that expression changes and abnormal functions of miR-1247 depend on the type of cancer. However, there are few reports on the function of miR-1247 in OS.

Neuropilin-1 (NRP1) was originally identified based on its pivotal role in the development of the nervous system[15]. Moreover, upregulation of NRP1 was observed in breast cancer, which was associated with poor prognosis[16]. Furthermore, high NRP1 expression was associated with lymph node metastasis in breast cancer patients[17]. Consistently, upregulation of NRP1 was also detected in uveal melanoma and non-small-cell lung cancer (NSCLC)[18,19]. In addition, the apoptotic rate was decreased by upregulation of NRP1 in glioma[20]. Hong et al[21] found that targeting NRP1 can be used as an anti-tumor strategy for lung cancer. Previous studies have shown that NRP1 plays a carcinogenic role in human cancer and can be targeted as a functional tumor suppressor.

In this work, the alternation of miR-1247 and NRP1 expression was assessed in OS. Their relationship and interaction were also observed in OS. In addition, we investigated the effect of miR-1247 on the Wnt/β-catenin pathway. These data will be beneficial to understand the pathogenesis of OS.

Patients and Methods

Tumor Sample and Cell Line

Tumor and normal tissues used in this experiment were obtained from 73 patients with OS in Caoxian People's Hospital. Patients with OS did not receive radiotherapy or chemotherapy prior to surgery. All of the participants provided informed consents before the research, and the experiment was approved by the Human Ethics Committee of Caoxian People's Hospital.

The normal human osteoblastic cell line hFOB 1.19 and U2OS, MG63 OS cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were then seeded in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). Next, they were incubated in an atmosphere with 5% CO_2 at 37°C.

Cell Transfection

According to the experimental design, miR-1247 mimics, miR-1247 inhibitors or NRP1 plasmid (GenePharma Co., Shanghai, China) were transferred to U2OS cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively. Untreated U2OS cells were set as controls.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

We extracted total RNA from U2OS cells using the TRIzol reagent (TaKaRa Bio, Dalian, China). The miScript Reverse Transcription kit (Qiagen, Beijing, China) was added to obtain complementary deoxyribose nucleic acid (cDNA). The qRT-PCR standard reaction system mixture was added to the QuantiTect SYBR Green RT-PCR kit (Qiagen, Beijing, China). Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis was performed on the ABI 7500 fast Real Time-PCR system (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for miR-1247 and NRP1, respectively. Their expressions were quantified using the $2^{-\Delta\Delta ct}$ method. The primers used in our work were as follows: miR-1247, forward primer: 5'-ACCCGTCCCGTTCGTC-3', reverse primer: 5'-TGCAGGGTCCGAGGTATT C-3'; U6, forward primer: 5'-CTCGCTTCGGCAGCACA-3', reverse primer: 5'-AACGCTTCACGAATTTGCGT-3'; NRP1 forward primer: 5'-CTCCAACGGGGAAGACTGGA-3', reverse primer: 5'-GTTGCAGGCTTGATTCGGAC-3';GAPDH forward, 5'-ACATCGCTCAGACACCATG-3', reverse, 5'-TGTAGTTGAGGTCAATGAAGGG-3'.

Cell Viability Assay

The prepared U2OS cells were incubated in 96-well plates for 24 hours (37°C, 5% CO_2). Next, U2OS (3×10^4/well) cells were incubated for 24, 48, 72, and 96 h. After that, the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) solution was added, and the cells were further incubated for 4 hours. The absorbance at 450 nm was observed with a microplate reader (Molecular Devices, Eugene, OR, USA).

Cell Migration and Invasion Assay

The upper chamber surface of the transwell chamber bottom membrane (8-μm pore size membranes) was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), and Matrigel was polymerized into a gel at 37°C for 30 min. The U2OS cell suspension (5×10^4/ well) was added to the transwell chamber, and the medium containing 20% FBS was added to the lower chamber.

After 24 hours of routine incubation, the transwell chamber was fixed and stained. The number of invading cells was observed under a microscope. The transwell cell migration assay performed without Matrigel, and other procedures were essentially the same as the invasion assay.

The Luciferase Reporter Assay

A pGL3 Luciferase vector (Invitrogen, Carlsbad, CA, USA) containing the 3'-untranslated region (3'-UTR) of wild or mutant type NRP1 gene was prepared. The Luciferase vector and miR-1247 mimics were co-transfected into U2OS cells. After 48 hours, the Luciferase activity was examined using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Western Blot Analysis

Protein samples were lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Next, 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protein loading buffer was added to collect the protein samples. After denaturation of the protein, the protein samples were loaded directly into the SDS-PAGE gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The PVDF membrane was incubated overnight at 4°C with the corresponding primary antibodies, including Bcl-2, Bax, NRP1 and GAPDH. The washing solution was added for 5-10 minutes, and the diluted secondary antibody was added and incubated for 1 hour at room temperature. Finally, proteins were detected using electrochemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Data were analyzed using Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (La Jolla, CA, USA). The difference between the groups was calculated by the Chi-squared Test. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference). Kaplan-Meier analysis with the log-rank test was used to compare survival differences. Data were shown as mean ± SD (Standard Deviation). A significant difference was defined as $p<0.05$.

Results

The Expression of MiR-1247 Was Reduced in OS

Primarily mRNA expression of miR-1247 was examined by qRT-PCR assay in OS tissues and cell lines. We found that the expression of miR-1247 was decreased in OS tissues compared to normal tissues (Figure 1A). At the same time, downregulation of miR-1247 was found in U2OS and MG63 cells compared to hFOB 1.19 cells (Figure 1B). Next, OS samples were divided into two groups based on miR-1247 expression levels. The correlation between miR-1247 expression and clinical characteristics of OS patients was then analyzed. The dysregulation of miR-1247 was found to be associated with clinical stage and distant metastasis (Table I). Based on these results, we concluded that miR-1247 might be involved in the pathogenesis of OS.

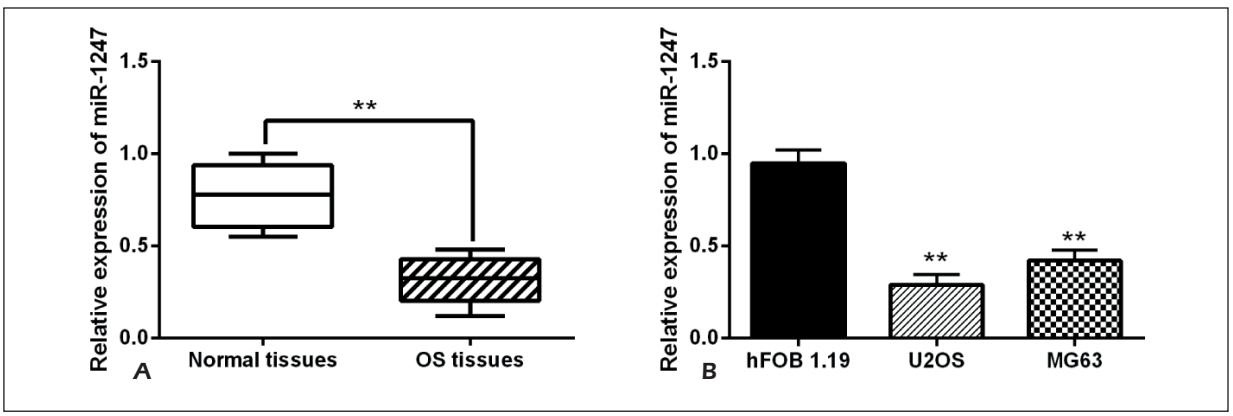

Figure 1. The expression of miR-1247 was reduced in OS. ***A,*** The mRNA expression of miR-1247 was measured in OS tissues. ***B,*** The expression of miR-1247 was determined in U2OS, MG63 and hFOB 1.19 cells. ***p*<0.01.

Table I. Relationship between miR-1247 expression and their clinic-pathological characteristics of OS patients.

Characteristics	Cases	miR-1247		*p*-value
		High	Low	
Age (years)				0.201
≥ 20	30	12	18	
< 20	43	15	28	
Gender				0.254
Male	42	17	25	
Female	31	10	21	
Tumor size (cm)				0.303
≤ 8	45	14	31	
> 8	28	13	15	
Distant metastasis				0.021*
Absent	48	20	28	
Present	25	7	18	
Clinical stage				0.033*
I-II	47	18	29	
III	26	9	17	

Statistical analyses were performed by the χ^2-test.
*$p<0.05$ was considered significant.

MiR-1247 Inhibited OS Cell Viability and Metastasis

Next, miR-1247 mimic or inhibitor was transfected into U2OS cells for gain-loss experiment. The qRT-PCR assay showed that miR-1247 mimics significantly promoted its expression, while miR-1247 inhibitor reduced its expression (Figure 2A). Then, the CCK-8 assay indicated that overexpression of miR-1247 inhibited U2OS cell proliferation. However, when miR-1247 was silenced, cell proliferation was promoted (Figure 2B). After that, how miR-1247 regulates cell metastasis was investigated in U2OS cells using the transwell assay. MiR-1271 mimics were found to suppress cell migration, while miR-1271 inhibitor promoted U2OS cell migration (Figure 2C). Similarly, cell invasion was inhibited by overexpression of miR-1247, but was promoted by knockdown of miR-1271 (Figure 2D). Briefly, miR-1247 inhibited OS cell viability and metastasis from regulating OS progression.

NRP1 Was a Direct Target of MiR-1247

To further disclose how miR-1247 suppresses OS progression, the target genes of miR-1247 were searched in TargetScan (http://www.targetscan.org/) databases. As shown in Figure 3A, miR-1247 has sites that bind to the 3'-UTR of NRP1. The Luciferase reporter assay was then designed to testify the prediction. As we predicted, overexpression of miR-1247 inhibited Luciferase activity of Wt-NRP1, but had no effect on Mut-NRP1 Luciferase activity (Figure 3B). Next, NRP1 expression regulated by miR-1247 mimics or inhibitor was assessed in U2OS cells. We found that mRNA and protein expression of NRP1 was inhibited by miR-1247 mimics, but was promoted by the miR-1247 inhibitor (Figure 3C, 3D). Therefore, it was confirmed that miR-1247 directly targeted NRP1 and negatively regulated NRP1 expression in OS.

Upregulation of NRP1 Weakened the Inhibitory Effect of MiR-1247 in OS

Next, abnormal expression of NRP1 was detected in OS. The expression of NRP1 was dramatically increased in OS tissues compared to normal tissues (Figure 4A). Furthermore, a negative correlation between miR-1247 and NRP1 expression was identified in OS tissues (Figure 4B). It indicated that their interaction might exist in OS development. To verify the above conjecture, miR-1247 mimics and NRP1 vector were co-transfected into U2OS cells. As we suspected, the reduction of NRP1 expression mediated by miR-1247 was recovered by the NRP1 vector in U2OS cells (Figure 4C). Functionally, upregulation of NRP1 impaired the inhibitory effect of miR-1247 on cell proliferation (Figure 4D). For cell migration and invasion, the suppressive effect of miR-1247 was also abolished by the NRP1 vector (Figure 4E). Collectively, upregulation of NRP1 attenuated the inhibitory effect of miR-1247 on the progression of OS.

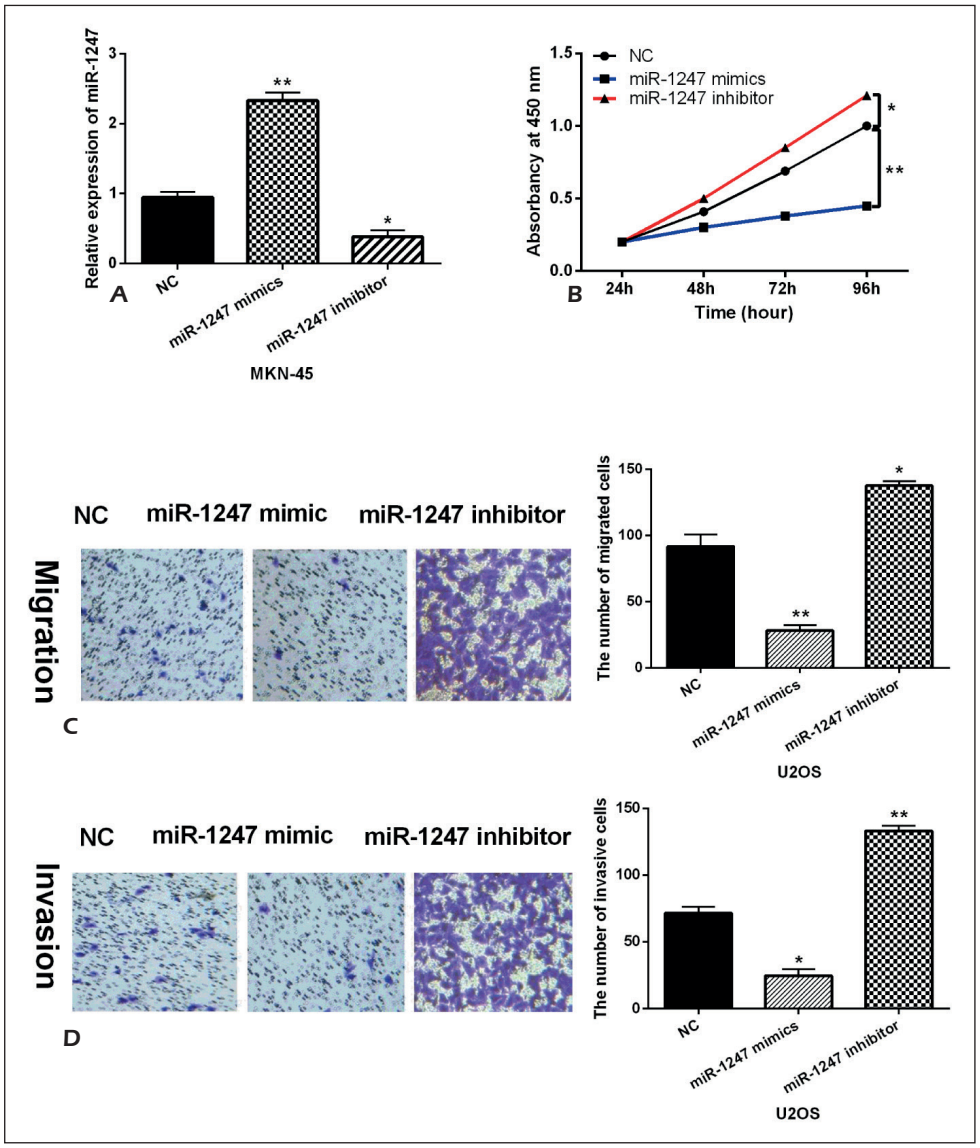

Figure 2. MiR-1247 inhibited cell viability and metastasis in OS. ***A,*** The mRNA expression of miR-1247 was determined in U2OS cells with miR-1247 mimics or inhibitor. ***B,*** Cell proliferation was regulated by miR-1247 mimics or inhibitor in U2OS cells. ***C-D,*** Cell migration and invasion were regulated by miR-1247 mimic or inhibitor in U2OS cells (magnification: 40×). *p<0.05, **p<0.01.

MiR-1247 Promoted Apoptosis and Inactivated the Wnt/β-Catenin Pathway in OS

Finally, we investigated whether miR-1247 regulates apoptosis and Wnt/β-catenin pathway in OS. The expression of the apoptosis-associated protein (Bcl-2/Bax) was assessed in U2OS cells with miR-1247 mimics or inhibitor. The expression of survival gene Bcl-2 was inhibited by overexpression of miR-1247, but was promoted by miR-1247 silencing (Figure 5). In contrast, overexpression of miR-1247 promoted Bax expression, while knockdown of miR-1247 decreased Bax expression levels (Figure 5). This indicated that miR-1247 could induce apoptosis in OS. In addition, it is well known that the Wnt/β-catenin pathway is involved in the pathogenesis of malignancies. In this work, the effect of miR-1247 on the expression of β-catenin was investigated in OS. The expression of p-β-catenin was suppressed by miR-1247 mimic and was promoted by the miR-1247 inhibitor. However, miR-1247 had no effect on the expression of β-catenin (Figure 5). MiR-1247 promoted apoptosis and inactivated the Wnt/β-catenin pathway to inhibit OS development.

Discussion

Recently, more and more scholars have recognized the role of miRNAs in tumor progression[22]. Furthermore, the same phenomenon was also found in OS. For example, miR-567 was downregulated in OS, and overexpression of miR-567

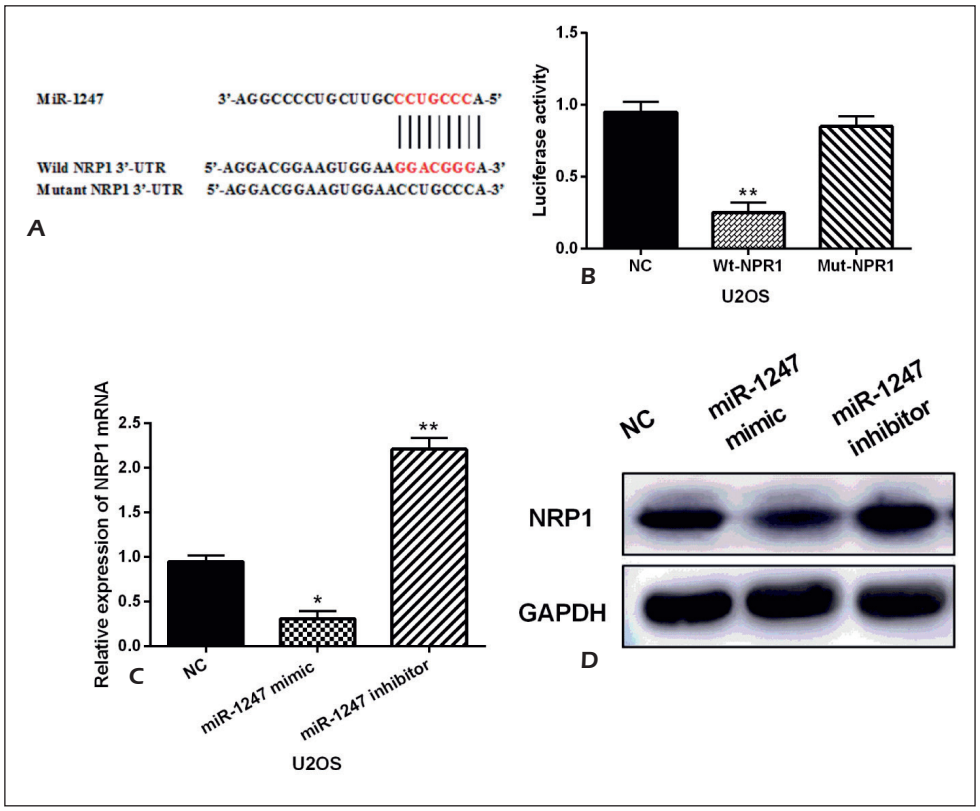

Figure 3. NRP1 was a direct target of miR-1247. ***A,*** There are binding sites between NRP1 and miR-1247. ***B,*** Luciferase reporter assay. ***C-D***, The expression of NRP1 regulated by miR-1247 mimics or inhibitor was observed in U2OS cells. *$p<0.05$, **$p<0.01$.

inhibited cell proliferation, migration and invasion in OS by suppressing FGF5 expression[23]. Here, the downregulation of miR-1247 was measured in OS and overexpression of miR-1247 inhibited the viability and metastasis of OS cells. MiR-1247 also promoted apoptosis and inactivated the Wnt/β-catenin pathway in OS. These findings indicated that miR-1247 exerted an inhibitory effect in OS.

Previous studies have shown that miR-1247 was aberrantly expressed in different human cancers. Zhao et al[24] found that miR-1247 was significantly down-regulated in OS, which was the same as our results. In addition, the association of miR-1247 expression with clinicopathological parameters and prognosis was identified in breast cancer[25]. In the current work, abnormal expression of miR-1247 was associated with clinical stage and distant metastasis in OS patients. Functionally, it had been reported[26] that miR-1247 inhibited cell proliferation in childhood neuroblastoma. Zhang et al[27] demonstrated that knockdown of miR-1247 promoted invasion and migration of NSCLC cells. Here, miR-1247-induced inhibition of cell viability and metastasis was also observed in OS. Besides that, miR-1247 was found to promote apoptosis and inactivate the Wnt/β-catenin pathway in OS. Similarly, miR-1247 was detected to suppress tumor cell growth and facilitate apoptosis *via* the Wnt/β-catenin signaling pathway in breast cancer[13]. The results of the previous studies confirm the accuracy of our conclusions.

Furthermore, we indicated that miR-1247 directly targeted NRP1 in OS cells. Upregulation of NRP1 dramatically restored the inhibitory effect of miR-1247 in OS. Moreover, upregulation of NRP1 and a negative correlation between miR-1247 and NRP1 expression were identified in OS tissues. The same results were also showed in squamous cell carcinoma of the esophagus and oral cancer[28,29]. Moreover, NRP1 had been reported[30] to be involved in tumorigenesis, growth, metastasis and immunity. As a target gene, NRP1 was found to be regulated by several miRNAs in different cancers, such as miR-365, miR-338 and miR-9[31-33]. Furthermore, miR-338 was found to suppress the growth and metastasis of OSCC cells by targeting NRP1[34]. In particular, miR-1247 had been reported to be correlated with the prognosis of pancreatic cancer and to inhibit cell proliferation by targeting neuropilins[35]. Consistently, we also found that miR-1247 inhibited the viability and metastasis of OS cells *via* targeting NRP1.

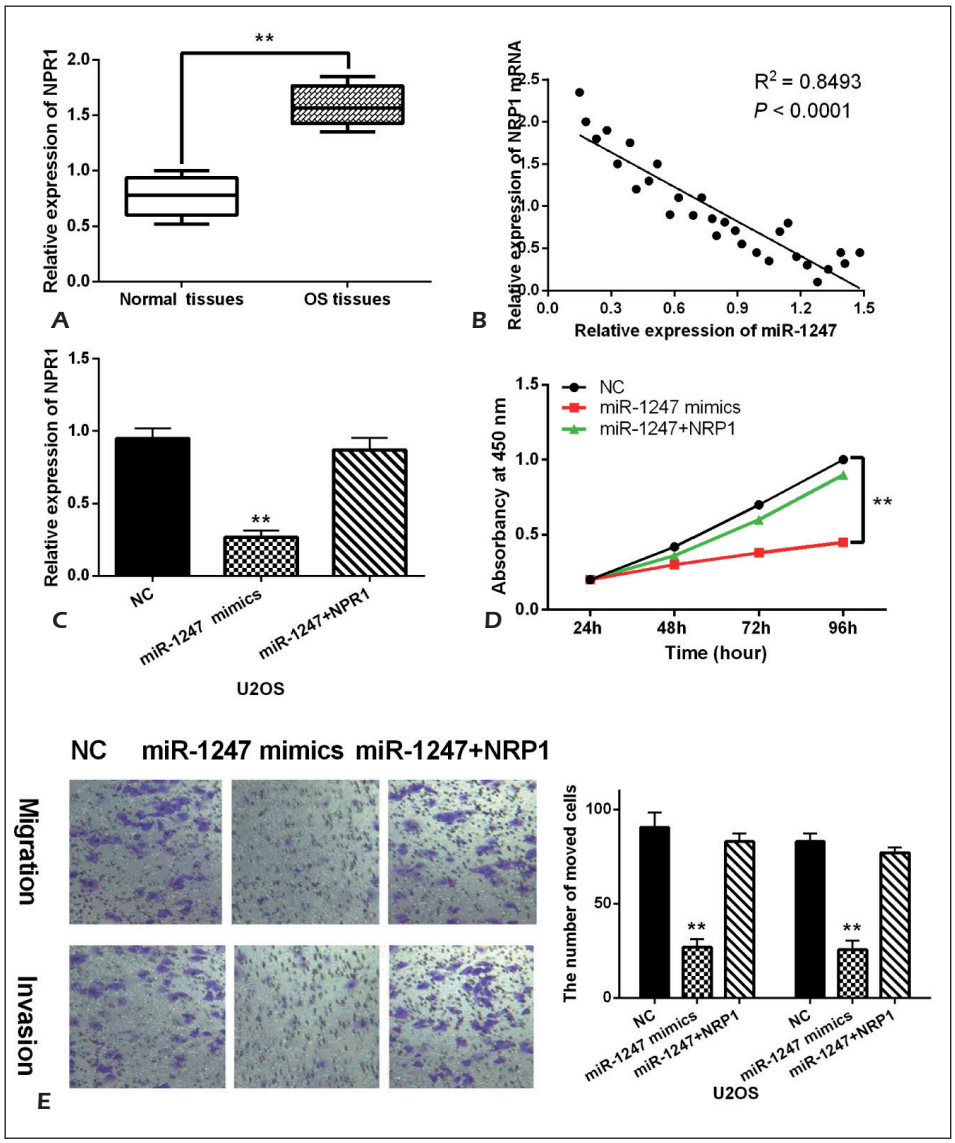

Figure 4. Upregulation of NRP1 weakened the inhibitory effect of miR-1247 in OS. ***A,*** The expression of NRP1 was detected in OS tissues. ***B,*** MiR-1247 was negatively correlated with NRP1 expression in OS tissues. ***C,*** The mRNA expression of NRP1 was detected in U2OS cells with NRP1 vector and miR-1247 mimics. ***D,*** Cell proliferation was identified in U2OS cells with NRP1 vector and miR-1247 mimics. E, NRP1+miR-1247 mimics regulated cell migration and invasion in U2OS cells (magnification: 40×). $^{**}p<0.01$.

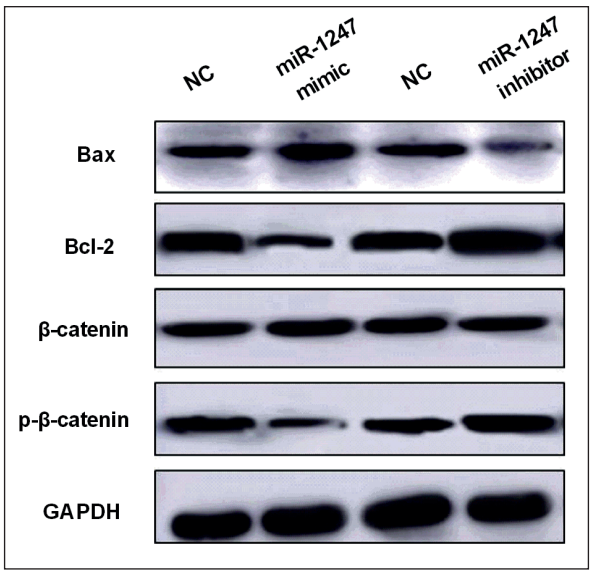

Figure 5. MiR-1247 promoted apoptosis and inactivated the Wnt/β-catenin pathway in OS. The protein expressions of Bax, Bcl-2, β-catenin and p-β-catenin were regulated by miR-1247 mimics or inhibitor in U2OS cells.

Conclusions

MiR-1247 was downregulated in OS, which was related to the aggressive behavior of OS patients. Functionally, miR-1247 inhibited cell viability and metastasis by suppressing NRP1 expression in OS. Moreover, miR-1247 was also found to induce apoptosis and inactivate the Wnt/β-catenin pathway in OS. These results fully indicate the conclusion that miR-1247 acts as a tumor suppressor in OS.

Conflict of Interests

The authors declare that they have no conflict of interest.

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