European Review for Medical and Pharmacological Sciences 2018; 22: 62-69

MiR-372-3p inhibits the growth and metastasis of osteosarcoma cells by targeting FXYD6

S.-Y. XU[1], P.-F. XU[2], T.-T. GAO[1]

[1]Department of Orthopaedics, Shanghai Eighth People's Hospital, Shanghai, China
[2]Department of Radiotherapy, Affiliated Hospital of Shaanxi University of Chinese Medicine, Xianyang, China

Abstract. – OBJECTIVE: Growing evidence has suggested that dysregulation of miR-372-3p may contribute to tumor development and progression in various tumors. However, the function of miR-372-3p in osteosarcoma has not been investigated. In the present study, we aimed to study the effects of miR-372-3p on osteosarcoma cell proliferation and metastasis and its regulation on FXYD6.

MATERIALS AND METHODS: The expression levels of miR-372-3p and FXYD6 mRNA were quantified by RT-PCR in human osteosarcoma cell lines and tissues. The effects of miR-372-3p up-regulation on osteosarcoma cell proliferation and metastasis were assessed by MTT, wound healing assay and transwell assay. Finally, the potential regulatory effect of miR-372-3p on FXYD6 expression was confirmed.

RESULTS: Our data showed that miR-372-3p was downregulated in osteosarcoma tissues compared with matched normal tissues, and the expression level of miR-372-3p was significantly lower in osteosarcoma cell lines in comparison with the normal human osteoblastic cell line. Transfection with the miR-372-3p mimic enhanced the osteosarcoma proliferation and metastasis. In vivo assay indicated that forced expression of miR-372-3p significantly suppressed tumor growth. Then, Bioinformatics prediction and experimental validation results confirmed that the function of miR-372-3p was achieved by targeting FXYD6 expression.

CONCLUSIONS: Our findings revealed that miR-372-3p served as a tumor suppressor gene by targeting FXYD6 in osteosarcoma. Thus, miR-372-3 might be a potential therapeutic method for osteosarcoma.

Key Words:
miR-372-3p, FXYD6, Osteosarcoma, Proliferation, Metastasis.

Introduction

Osteosarcoma, the most common primary bone tumor in children and adolescents, derives from primitive bone-forming mesenchymal cells[1]. The real mechanism of osteosarcoma tumorigenesis is still unclear, but it is known to us that genetic factors are involved in the development and progression of osteosarcoma[2]. Despite advanced progress obtained in diagnosis and surgery combined with multi-drug chemotherapy for osteosarcoma in recent years, the five-year survival rate of patients with osteosarcoma is among the lowest in all tumors[3-5]. Osteosarcoma is correlated with poor outcome due to its high incidence of metastasis and chemoresistance[6]. In addition, recent years, scientists identified several molecular markers, but these lack sensitivity and specificity for evaluation of the prognosis and diagnosis of osteosarcoma patients[7,8]. Therefore, the molecular mechanisms of osteosarcoma dissemination from the primary tumor are important to develop novel therapeutic approach.

MicroRNAs (miRNAs) are small noncoding endogenous RNA gene products consisting of 18-25 nucleotides and regulate gene expression through binding to the 3' untranslated region (3'UTR) of their target mRNAs[9,10]. More and more evidence indicates that miRNAs play an important role in a variety of pathogenic conditions[11]. In recent decades, various researches have shown that miRNAs are dysregulated in almost all types of human tumors. Moreover, the role of several miRNAs has been identified. For instance, Danza et al[12] reported that aberrant expressions of miR-20b, miR27a and miR-181a were involved in the regulation of chemotherapeutic response in gastric cancer by modulating HIF1A and HIPK2. Liu et al[13] found that miR-217 inhibited EMT in gastric cancer through targeting PTPN14. Wang et al[14] suggested that miR-495 inhibited gastric cancer cell migration and invasion via targeting HMGA2. Recently, miR-372-3p has been reported to serve as a tumor suppressor or oncogene, and was abnormally expressed in various tumors[15-17]. However, to our

Corresponding Author: Tian-tian Gao, MD; e-mail: gtt1924g@163.com

best knowledge, the detail function of miR-372-3p in progression of osteosarcoma has not been investigated.

In the present study, we firstly determined the expression levels of miR-372-3p in both osteosarcoma tissues and cell lines. Furthermore, we experimentally confirmed that up-regulation of miR-372-3p could suppress proliferation, migration, and invasion by targeting FXYD domain containing ion transport regulator 6 (FXYD6).

Materials and Methods

Tissue Samples

Thirty pair surgical specimens of osteosarcoma tissues and their adjacent normal tissues were obtained from patients with osteosarcoma who underwent surgery at Department of Orthopedics, Shanghai Eighth People's Hospital. All the histological diagnoses for osteosarcoma were reviewed and recognized by two pathologists independently. Osteosarcoma tissues were immediately frozen at –80°C. The study was approved by the Ethics Committee of Shanghai Eighth People's Hospital and informed consent was obtained.

Cell Culture and miR Transfection

Osteosarcoma cell lines (U2OS, SOSP-9607, Saos-2, and MG-63) and osteoblastic cell line (hFOB1.19) were purchased from Cancer Research Institute, Central South University (Changsha, Hunan, China), and maintained at 37°C, 5% CO^2 in RPMI 1640 complete medium with 10% fetal bovine serum.

The miR-372-3p mimics and negative control(NC) were synthesized by GenePharma (Pudong, Shanghai, China). For RNA transfection, the cells were seeded into each well of 24-well plates, incubated overnight. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Total RNA Extraction and qRT-PCR Analyses

Total RNA was isolated from tissues and cell cultures with Trizol reagent (TaKaRa, Otsu, Shiga, Japan) according to the standard protocol. The isolated total RNA was reversed transcribed into cDNA using SuperScript Reverse Transcriptase II (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed with a TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) on ABI7500 real-time PCR detection system. Primers for miR-372-3p and FXYD6 were synthesized by Genechem (Pudong, Shanghai, China) Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to the individual or GAPDH level.

Cell Proliferation Assay and Colony Formation Assay

The MTT assay was conducted to evaluate the rate of cell proliferation, according to manufacturer's instructions. Cells were reseeded into 96-well plates for 0, 24, 48,72 and 96. Then 15 ml of MTT solution was added into each well. The optical density (OD) values were measured at 490 nm. Each experiment was performed in triplicate and repeated three times.

For colony formation assay, cells were seeded in 96-well plates and incubated for 7 days at 37°C with 5% CO^2. Cells were cultured for another 10 days under standard conditions. Colony numbers were quantified using AlphaView software version 2.0 (ProteinSimple, San Jose, CA, USA).

Wound Healing, Migration and Invasion Assays

For wound healing, the cells were seeded in six-well plates and incubated until 90% confluence in serum-free medium before wounding. The detached cells were washed off twice gently, and the medium was then replaced with 1% FBS complete medium. Gap areas were photographed at different time points using light microscope.

For migration assay, 5×10^4 cells were seeded into the upper chamber of transwells (TaKaRa, Otsu, Shiga, Japan). For invasion assay, 1×10^5 cells were added into the upper chamber precoated with Matrigel (TaKaRa, Otsu, Shiga, Japan). After 48 h of incubation at 37°C, the cells were removed from the upper sides of the Transwell membrane filter inserts with cotton-tip swabs. The cells in the lower chambers were fixed in 4% formaldehyde, then rinsed with PBS and subjected to microscopic inspection.

In vivo Tumor Growth Assay

Osteosarcoma cells were resuspended and injected intraperitoneally (2×10^6 cells/mouse) into 4-week-old male nude mice (Tiangen Biotechnology Co., Ltd, Beijing, China). Animals were maintained in a sterile animal facility. After 7 weeks, mice were killed. Then, the mice were sacrificed under ether anesthesia and the tumors were dissected for further analysis. Tumor growth

Tumor volume was evaluated using the following formula: volume = (width + length)/2 × width × length × 0.5236.

Western Blot

Protein was extracted from tissues and cells using RIPA lysis buffer containing proteinase inhibitor (Sigma, St. Louis, MO, USA). The protein concentrations were measured. Equal amounts of protein were separated by 10% SDS-PAGE gel, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were incubated with the primary antibodies anti-FXYD6. The anti- GAPDH antibody was used to normalize the protein input. Blots were detected using an ECL detection system.

Statistical Analysis

SPSS 12.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the results. Student's *t*-test was used to assess significant differences between groups. $p < 0.05$ was considered statistically significant.

Results

miR-372-3p is Downregulated in Osteosarcoma Tissues and Cell Lines

We first investigated whether miR-372-3p expression was linked to osteosarcoma. miR-372-3p expression in osteosarcoma tissues and cell lines was measured by qRT-PCR. Figure 1A showed that miR-372-3p expression was significantly lower in osteosarcoma tissues than in the corresponding noncancerous bone tissues ($p < 0.01$). As expected, Figure 1B showed that miR-372-3p was downregulated in osteosarcoma cell lines (SOSP-9607, Saos-2, MG-63, U2OS,) compared to Hfob ($p < 0.05$, respectively).

miR-372-3p Inhibit the Growth of MG-63 Cells in vitro and in vivo

After the miR-372-3p expression in osteosarcoma tissues and cell lines was investigated, we further analyzed the role of miR-372-3p in the MG-63 proliferation. As shown in Figure 2A, the results of RT-PCR indicated that miR-372-3p was markedly increased by its mimics treatment. MTT assay showed that in miR-372-3p up-regulated MG-63 cells; proliferation was inhibited (Figure 2B). Subsequently, The growth suppressing effect of miR-372-3p was also demonstrated by colony formation assays (Figure 2C). Next, we investigated whether miR-372-3p could inhibit tumor growth *in vivo*. The results indicated that the size and weight of xenograft tumors in nude mice was significantly decreased after mimics treatment of miR-372-3p (Figure 2D and 2E).

Over-Expression of miR-372-3p Inhibited the Migration and Invasion of MG-63 Cells

Next, we further assessed the effect of miR-372-3p on tumor migration and invasion. The

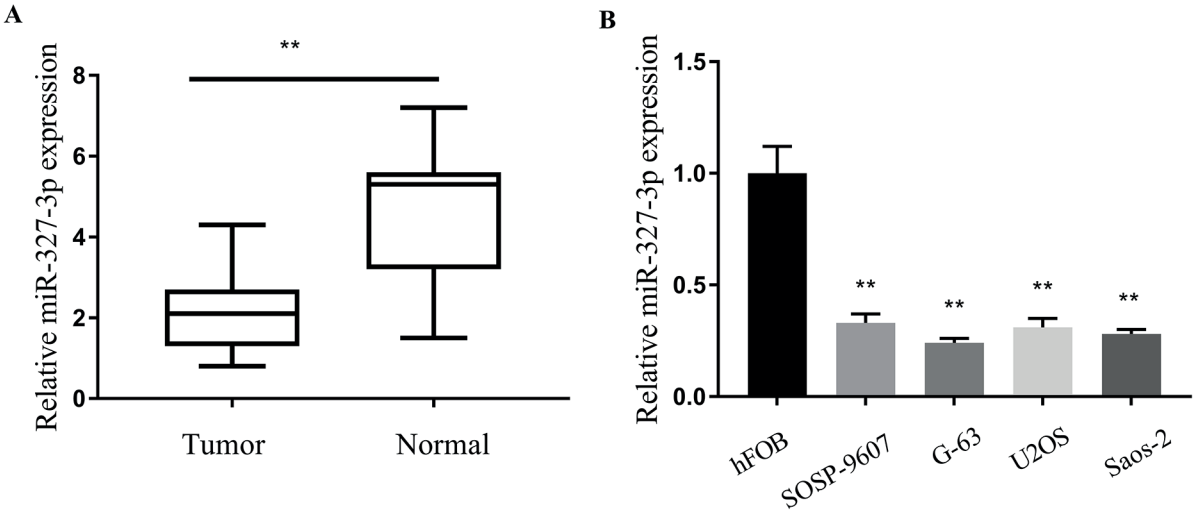

Figure 1. miR-372-3p expression is decreased in osteosarcoma tissues and cell lines. ***(A)*** Expression of miR-372-3p was measured in osteosarcoma samples and normal tissues via RT-qPCR. ***(B)*** The expression levels of miR-372-3p were tested in osteosarcoma cell lines and osteoblastic cell line by RT-qPCR. $^{**}p < 0.01$, $^{*}p < 0.05$.

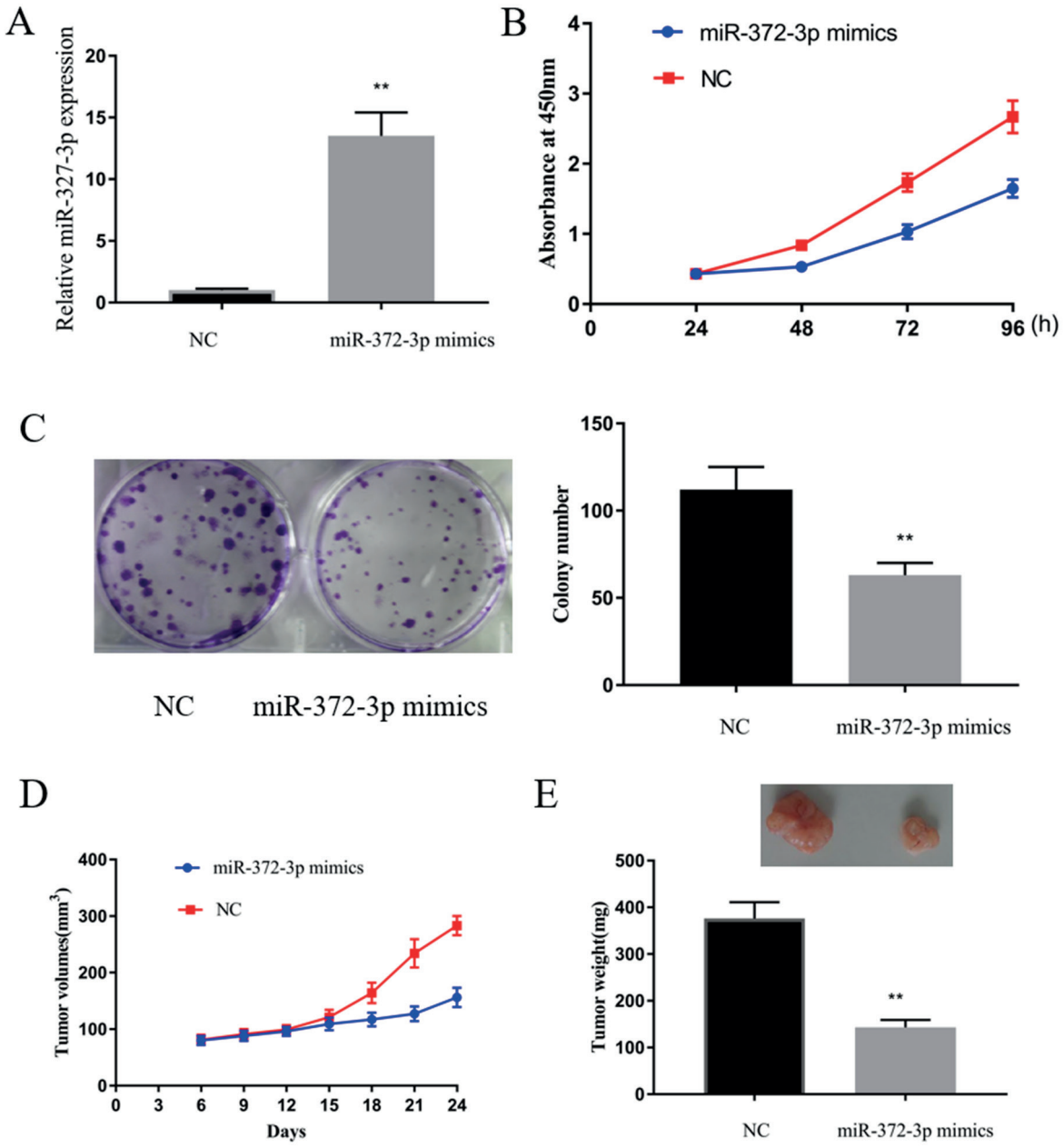

Figure 2. miR-372-3p upregulation suppressed osteosarcoma cell proliferation *in vitro* and *in vivo*. ***(A)*** miR-372-3p levels in MG-63 cells transfected with miR-211 mimics or si-NC. (B) MTT assays were performed to determine the proliferation of MG-63 cells. ***(C)*** The duplicated cells were subjected to colony formation assay. The graph is summarized data of the colony formation assay. ***(D)*** Tumor volumes were measured on the indicated days. ***(E)*** Representative photographs and average weights of the tumors from each group. $^{**}p < 0.01$, $^{*}p < 0.05$.

wound-healing assay revealed that MG-63 cells with miR-372-3p overexpression exerted a slower closing of scratch wound, compared with NC (Figure 3A). Moreover, the results of transwell showed that the migratory and invasive capacity of MG-63 cells overexpressing miR-372-3p was significantly reduced compared with the NC (Figure 3B). These data suggested that miR-372-3p suppressed osteosarcoma migration and invasion.

FXYD6 is a Direct Target of miR-372-3p

To explore the molecular mechanism of miR-372-3p function in osteosarcoma cells, we used Targetscan to predict the targets of miR-372-3p,

and found FXYD6 might be a target for miR-372-3p (Figure 4A). Then, we generated the luciferase vectors containing WT or MUT of FXYD63'-UTR (Figure 3C and D). The detection of a luciferase activity revealed that miR-372-3p significantly inhibited the activity of luciferase combined with wild-type FXYD6 3'-UTR in MG-63 cells (Figure 4B). To confirm the effect of miR-372-3p in regulation of FXYD6, we further performed RT-PCR and the results suggested that overexpression miR-372-3p significantly suppressed FXYD6 mRNA (Figure 4C). Subsequent western blot also indicated that overexpression miR-372-3p significantly suppressed FXYD6 proteins (Figure 4D). These findings indicated that FXYD6 is a direct target of miR-372-3p.

Discussion

MiR-372-3p which is located in 19q13.42 belongs to miR-371~373 cluster which has been confirmed to be involved in various tumor progression[18]. Previous studies indicated that miR-372-3p served as a tumor suppressor or oncogene in different tumors. For instance, Cho et al[19] reported that down-expression of miR-372 suppressed gastric cancer proliferation by regulation of LATS2. Yamashita et al[20] found that up-regulation of miR-372 was remarkable associated with poor prognosis of patients with colorectal cancer. Another study by Li et al[21] also revealed that miR-372 may act as an oncogenic miRNA in gliomas and an independent progno-

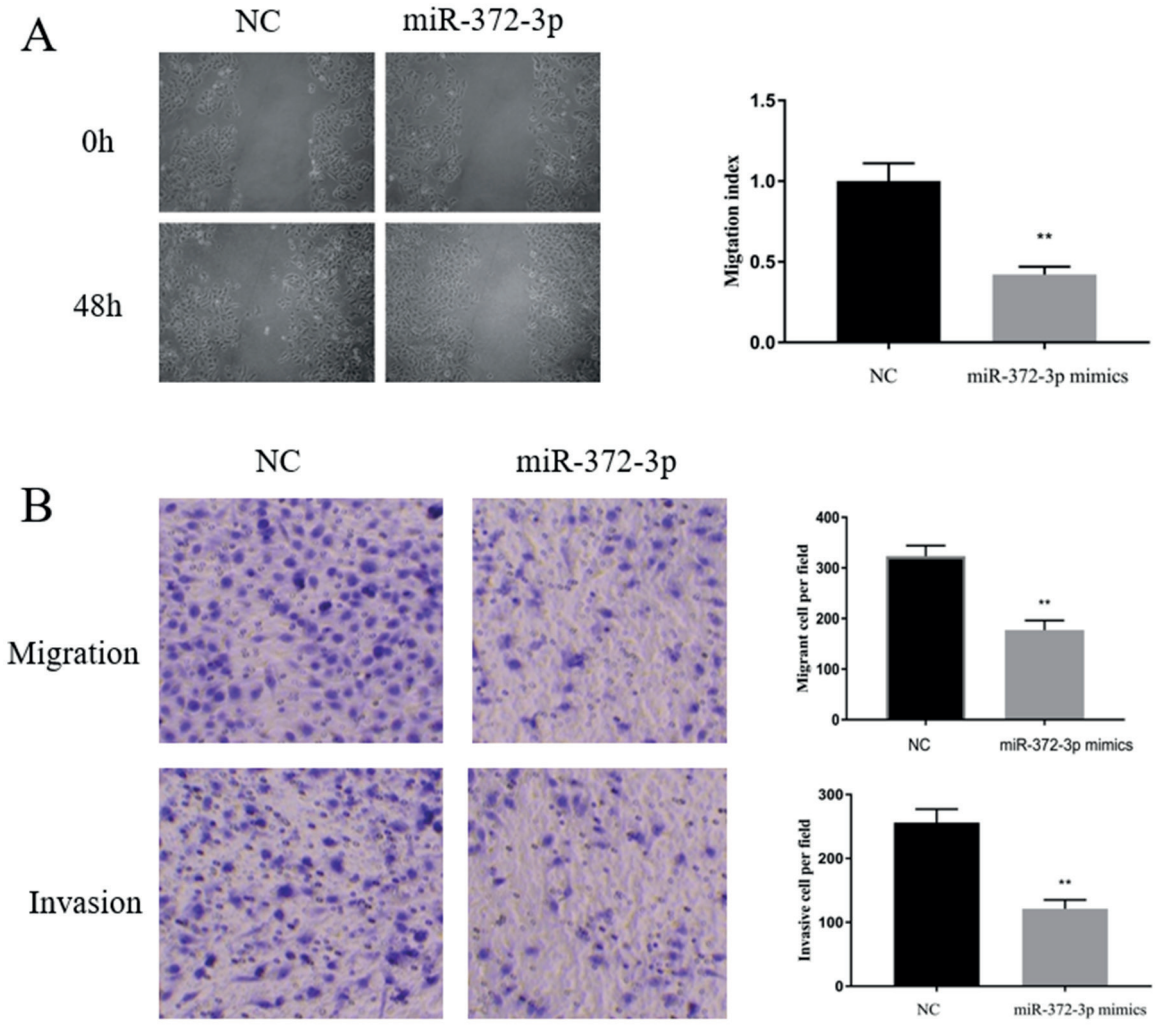

Figure 3. MiR-372-3p regulates cell migration and invasion in osteosarcoma cells. (A) *In vitro* wound healing after miRNA transfection. (B) Transwell assays were used to investigate the changes in the migratory and invasive abilities of osteosarcoma cells. $^{**}p < 0.01$, $^{*}p < 0.05$.

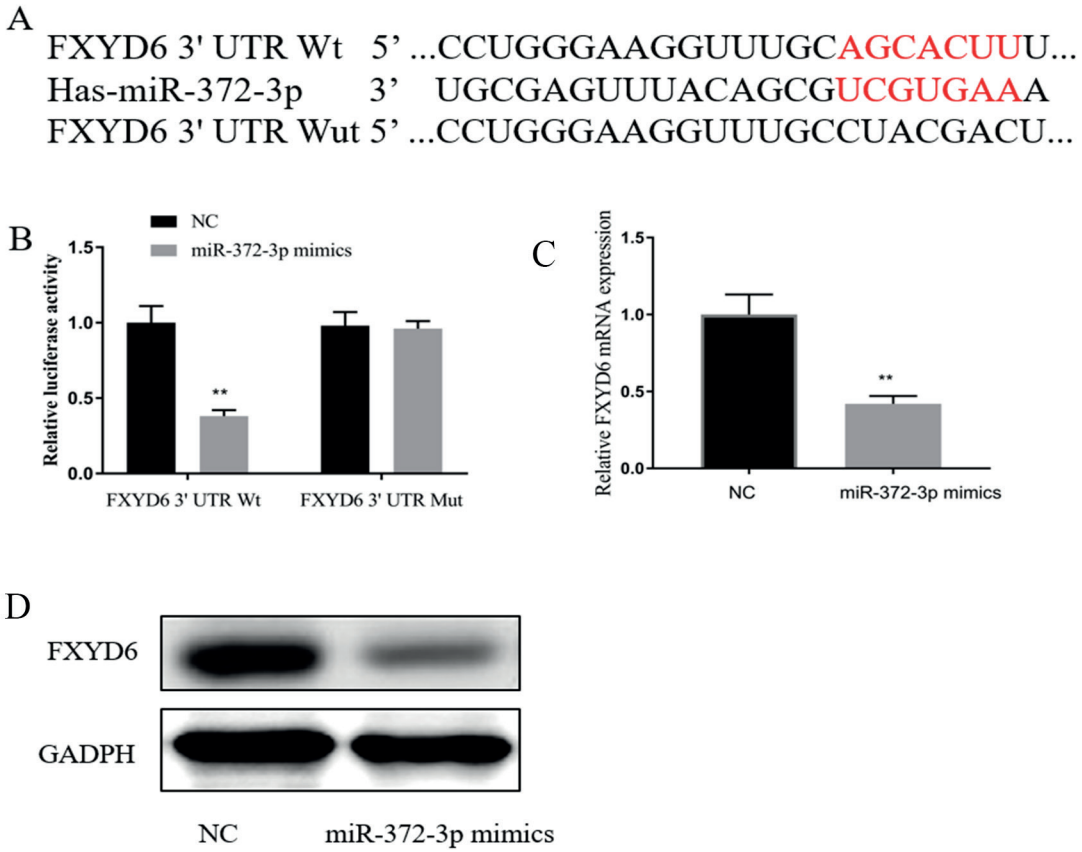

Figure 4. FXYD6 is a direct target of miR-372-3p. ***(A)*** Diagram of the mature miR-372-3p sequence and miR-372-3p target site in the 3'-UTR of FXYD6 mRNA. ***(B)*** Luciferase reporter assay of MG-63 cells transfected with the wide-type or mutant-type FXYD6 reporter plasmid and miR-372-3p mimic or miR-NC. ***(C)*** Western blot analysis of FXYD6 expression in MG-63 cells transfected with miR-372-3p mimics or negative control (NC). $^{**}p < 0.01$, $^{*}p < 0.05$.

stic biomarker in gliomas. By contrast, the role of tumor suppressor in some tumors was also reported. For example, Tian et al[22] found that ectopic expression of miR-372 suppressed cell growth by targeting CDK2 Cyclin A1 in cervical cancer. Kong et al[23] also found that miR-372 inhibited the proliferation activity, migration, and invasion of prostate cancer cells by targeting p65. These findings miR-372 exerted contrary function depending on its targeting genes. Although the role of miR-372 has been reported in several tumors, the expression and function of miR-372-3p in osteosarcoma remains unknown.

In the present study, we firstly assessed the expression levels of miR-372-3p in osteosarcoma tissues and cell lines. We found that miR-372-3p was significantly lowly expressed in both osteosarcoma tissues and cell lines. Then, we proved that forced expression of miR-372-3p inhibits the proliferation, migration, and invasion of osteosarcoma cells *in vivo* and *in vitro*. These data suggested that miR-372-3p played important roles in osteosarcoma procession. To further explore the underlying mechanisms participating in the miR-372-3p-mediated inhibitory effects on cell proliferation and metastasis in osteosarcoma cells, we focused on FXYD6 which played a complex role in progression of several tumors[24,25].

FXYD6 is a new member of the FXYD protein family and a regulator of Na, K-ATPase[26]. FXYD6 has been demonstrated to play a positive regulator in development of tumors. For instance, Gao et al[27] found that FXYD6 protein was significantly up-regulated in hepatocellular carcinoma tissues, and forced expression of FXYD6 promotes tumor growth *in vivo* and *in vitro*. Chen et al[28] reported that the positive expression rate of FXYD6 was significantly associated poorer cholangiocarcinoma stages. Notably, FXYD6 mRNA was upregulated in osteosarcoma cells[29]. Furthermore, Li et al[30] revealed that knockdown of FXYD6 significantly suppressed prolifera-

tion and metastasis in osteosarcoma. Given the important role of FXYD6 in tumor progression, we identified FXYD6 may be a putative target of miR-372-3p by bioinformatics databases. Subsequently, we confirmed that miR-372-3p could directly target the 3'UTR of FXYD6 mRNA by dual-luciferase reporter gene assay. Furthermore, the results of Western blot and RT-PCR indicated that over-expression of miR-372-3p significantly suppressed the expression levels of FXYD6 protein and mRNA. Taken together, our findings suggested that the tumor-suppressive function of miR-372-3p in osteosarcoma was correlated with FXYD6 modulation.

Conclusions

We firstly demonstrated that miR-372-3p expression was downregulated in osteosarcoma tissues. Moreover, forced miR-372-3p expression suppressed the osteosarcoma proliferation by targeting FXYD6. Our findings suggested that miR-372-3p and FXYD6 may become promising candidates for the treatment of osteosarcoma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Geller DS, Gorlick R. Osteosarcoma: a review of diagnosis, management, and treatment strategies. Clin Adv Hematol Oncol 2010; 8: 705-718.
2) Puls F, Niblett AJ, Mangham DC. Molecular pathology of bone tumours: diagnostic implications. Histopathology 2014; 64: 461-476.
3) Pang PC, Shi XY, Huang WL, Sun K. miR-497 as a potential serum biomarker for the diagnosis and prognosis of osteosarcoma. Eur Rev Med Pharmacol Sci 2016; 20: 3765-3769.
4) Marina N, Gebhardt M, Teot L, Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist 2004; 9: 422-441.
5) Longhi A, Fabbri N, Donati D, Capanna R, Briccoli A, Biagini R, Bernini G, Ferrari S, Versari M, Bacci G. Neoadjuvant chemotherapy for patients with synchronous multifocal osteosarcoma: results in eleven cases. J Chemother 2001; 13: 324-330.
6) Rainusso N, Wang LL, Yustein JT. The adolescent and young adult with cancer: state of the art -- bone tumors. Curr Oncol Rep 2013; 15: 296-307.
7) Xu B, Wu DP, Xie RT, Liu LG, Yan XB. Elevated NDC80 expression is associated with poor prognosis in osteosarcoma patients. Eur Rev Med Pharmacol Sci 2017; 21: 2045-2053.
8) Wu D, Chen K, Bai Y, Zhu X, Chen Z, Wang C, Zhao Y, Li M. Screening of diagnostic markers for osteosarcoma. Mol Med Rep 2014; 10: 2415-2420.
9) Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116: 281-297.
10) Liu ZF, Liang ZQ, Li L, Zhou YB, Wang ZB, Gu WF, Tu LY, Zhao J. MiR-335 functions as a tumor suppressor and regulates survivin expression in osteosarcoma. Eur Rev Med Pharmacol Sci 2016; 20: 1251-1257.
11) Hesse M, Arenz C. MicroRNA maturation and human disease. Methods Mol Biol 2014; 1095: 11-25.
12) Danza K, Silvestris N, Simone G, Signorile M, Saragoni L, Brunetti O, Monti M, Mazzotta A, De Summa S, Mangia A, Tommasi S. Role of miR-27a, miR-181a and miR-20b in gastric cancer hypoxia-induced chemoresistance. Cancer Biol Ther 2016; 17: 400-406.
13) Liu YP, Sun XH, Cao XL, Jiang WW, Wang XX, Zhang YF, Wang JL. MicroRNA-217 suppressed epithelial-to-mesenchymal transition in gastric cancer metastasis through targeting PTPN14. Eur Rev Med Pharmacol Sci 2017; 21: 1759-1767.
14) Wang H, Jiang Z, Chen H, Wu X, Xiang J, Peng J. MicroRNA-495 Inhibits Gastric Cancer Cell Migration and Invasion Possibly via Targeting High Mobility Group AT-Hook 2 (HMGA2). Med Sci Monit 2017; 23: 640-648.
15) Wang Q, Liu S, Zhao X, Wang Y, Tian D, Jiang W. MiR-372-3p promotes cell growth and metastasis by targeting FGF9 in lung squamous cell carcinoma. Cancer Med 2017; 6: 1323-1330.
16) Yeh LY, Liu CJ, Wong YK, Chang C, Lin SC, Chang KW. miR-372 inhibits p62 in head and neck squamous cell carcinoma in vitro and in vivo. Oncotarget 2015; 6: 6062-6075.
17) Wu G, Wang Y, Lu X, He H, Liu H, Meng X, Xia S, Zheng K, Liu B. Low mir-372 expression correlates with poor prognosis and tumor metastasis in hepatocellular carcinoma. BMC Cancer 2015; 15: 182.
18) Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, Drost J, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, Nojima H, Looijenga LH, Agami R. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell 2006; 124: 1169-1181.
19) Cho WJ, Shin JM, Kim JS, Lee MR, Hong KS, Lee JH, Koo KH, Park JW, Kim KS. miR-372 regulates cell cycle and apoptosis of ags human gastric cancer cell line through direct regulation of LATS2. Mol Cells 2009; 28: 521-527.
20) Yamashita S, Yamamoto H, Mimori K, Nishida N, Takahashi H, Haraguchi N, Tanaka F, Shibata K, Sekimoto M, Ishii H, Doki Y, Mori M. MicroRNA-372 is associated with poor prognosis in colorectal cancer. Oncology 2012; 82: 205-212.

21) Li G, Zhang Z, Tu Y, Jin T, Liang H, Cui G, He S, Gao G. Correlation of microRNA-372 upregulation with poor prognosis in human glioma. Diagn Pathol 2013; 8: 1.

22) Tian RQ, Wang XH, Hou LJ, Jia WH, Yang Q, Li YX, Liu M, Li X, Tang H. MicroRNA-372 is down-regulated and targets cyclin-dependent kinase 2 (CDK2) and cyclin A1 in human cervical cancer, which may contribute to tumorigenesis. J Biol Chem 2011; 286: 25556-25563.

23) Kong X, Qian X, Duan L, Liu H, Zhu Y, Qi J. microRNA-372 Suppresses Migration and Invasion by Targeting p65 in Human Prostate Cancer Cells. DNA Cell Biol 2016; 35: 828-835.

24) Yang Z, Chen Y, Fu Y, Yang Y, Zhang Y, Chen Y, Li D. Meta-analysis of differentially expressed genes in osteosarcoma based on gene expression data. BMC Med Genet 2014; 15: 80.

25) Rajasekaran SA, Huynh TP, Wolle DG, Espineda CE, Inge LJ, Skay A, Lassman C, Nicholas SB, Harper JF, Reeves AE, Ahmed MM, Leatherman JM, Mullin JM, Rajasekaran AK. Na,K-ATPase subunits as markers for epithelial-mesenchymal transition in cancer and fibrosis. Mol Cancer Ther 2010; 9: 1515-1524.

26) Delprat B, Schaer D, Roy S, Wang J, Puel JL, Geering K. FXYD6 is a novel regulator of Na,K-ATPase expressed in the inner ear. J Biol Chem 2007; 282: 7450-7456.

27) Gao Q, Chen X, Duan H, Wang Z, Feng J, Yang D, Song L, Zhou N, Yan X. FXYD6: a novel therapeutic target toward hepatocellular carcinoma. Protein Cell 2014; 5: 532-543.

28) Chen X, Sun M, Hu Y, Zhang H, Wang Z, Zhou N, Yan X. FXYD6 is a new biomarker of cholangiocarcinoma. Oncol Lett 2014; 7: 393-398.

29) Olstad OK, Gautvik VT, Reppe S, Rian E, Jemtland R, Ohlsson C, Bruland OS, Gautvik KM. Molecular heterogeneity in human osteosarcoma demonstrated by enriched mRNAs isolated by directional tag PCR subtraction cloning. Anticancer Res 2003; 23: 2201-2216.

30) Li ZM, Zhang HY, Wang YX, Wang WB. MicroRNA-137 is downregulated in human osteosarcoma and regulates cell proliferation and migration through targeting FXYD6. J Drug Target 2016; 24: 102-110.