MicroRNA-2682-3p inhibits osteosarcoma cell proliferation by targeting *CCND2*, *MMP8* and *Myd88*

FAN ZHANG*, YANJIE ZHU*, GUOXIN FAN* and SHUO HU

Department of Orthopedics, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072, P.R. China

Received June 29, 2017; Accepted May 31, 2018

DOI: 10.3892/ol.2018.9029

Abstract. Osteosarcoma is the most common primary bone malignancy in children and young adults. It is associated with dysregulation of certain microRNAs (miRNAs/miRs), which provides a target for osteosarcoma therapy. *miR-2682-3p* expression in osteosarcoma cell lines and tissues was assayed by reverse transcription-quantitative polymerase chain reaction and was upregulated or downregulated by transfection with miRNA mimics or inhibitors. *miR-2682-3p* was downregulated in osteosarcoma tissues and cell lines, and overexpression of *miR-2682-3p* inhibited tumor growth. Further studies revealed that cyclin D1 (*CCND)2,* matrix metalloproteinase *(MMP)8,* and myeloid differentiation primary response (*Myd)88* were the direct targets of *miR-2682-3p* in osteosarcoma cells. Overexpression of *miR-2682-3p* promoted osteosarcoma cell apoptosis by targeting *CCND2, MMP8*, and *Myd88*, and vice-versa. Therefore, *miR-2682-3p* may act as a tumor suppressor gene, the downregulation of which contributed to the progression and metastasis of osteosarcoma, to provide a potential therapy target for patients with osteosarcoma.

Introduction

Osteosarcoma, an aggressive malignant neoplasm arising from primitive transformed cells of mesenchymal origin, is the most common type of human primary bone sarcoma and a leading cause of cancer death in children and adolescents (1-5). The treatment for osteosarcoma is unsatisfactory and new targets for the treatment of osteosarcoma are urgently needed (6-9).

Correspondence to: Professor Shuo Hu, Department of Orthopedics, Shanghai Tenth People's Hospital, Tongji University School of Medicine, 301 Yanchang Road, Shanghai 200072, P.R. China
E-mail: hushuo10hospital@163.com

*Contributed equally

Key words: osteosarcoma, microRNA-2682-3p, CCND2, MMP8, Myd88

The mechanisms on the formation and development of osteosarcoma have been studied for a long time (6,7,9). Recent evidence has shown that gene expression is regulated by microRNAs (miRNAs/miRs), which are small noncoding RNAs (about 22 nt in length) that play crucial roles in regulating tumor growth by binding to the 3' untranslated region (UTR) of target mRNAs, which represses their translation (9-12). Hundreds of target mRNAs have been associated with osteosarcoma, but the underlying mechanisms are unclear (13-18).

One example is miR-2682-3p (19). Recently, we studied whether the dysregulation of miR-2682-3p is involved in osteosarcoma. This study is aimed at determining the role of miR-2682-3p in osteosarcoma cell growth and the target genes for miR-2682-3p. In the present study, miR-2682-3p was decreased in osteosarcoma tissues and cell lines, and overexpression of miR-2682-3p inhibited osteosarcoma cell proliferation. Moreover, *in vitro* experiments proved that downregulation of miR-2682-3p promoted tumor proliferation; these experiments also identified cyclin D1 (CCND)2, matrix metalloproteinase 8 (MMP8), and Myd88 as the direct targets of miR-2682-3p in osteosarcoma cells. Our findings suggest the involvement of miR-2682-3p in osteosarcoma cell apoptosis induced by CCND2, MMP8, and Myd88.

Materials and methods

Ethics statement. All patients participated in the study provided written informed consent, and the study was approved by the Ethics Committee of Shanghai Tenth People's Hospital (20).

Tissues and cell culture. Twelve paired osteosarcoma tissues were obtained from Shanghai Tenth People's Hospital. Human normal osteoblast cells (hFOB) and osteosarcoma cell lines (Saos-2, MG-63, and U2OS) were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium at 37˚C in an atmosphere of 5% CO_2 (9,17).

Cell proliferation assay. Cells were cultured in 96-well microplates at $1x10^4$ per well for 3 days after transfection. CCK-8 (Dojindo, Kumamoto, Japan) were used to analyze the viability of the cells (9). The viable cells were counted by absorbance measurement at 450 nm using an auto-microplate reader.

Colon formation assay. U2OS and MG63 cells were counted and diluted to 100 cells/ml. Aliquots (2 ml) of each suspension were added to wells of six-well culture plates. The medium was refreshed every 3 days until cell clones could be observed with the naked eye.

RNA extraction and quantitative PCR. Total RNA was extracted from the cells or tissues using the miRNA isolation kit (Ambion, Auston, TX, USA). Reverse transcriptions were performed using an RNA PCR kit (Takara Bio, Shiga, Japan) in accordance with manufacturer's instructions (9). To quantify gene transcripts, real-time PCR was performed using SYBR-Green Premix Ex Taq (Takara Bio) on LightCycler 480 (Roche, Basel, Switzerland). U6 and glycerladehyde-3-phosphate dehydrogenase (GAPDH) were used as the normalizing controls for quantifying miRNA and mRNA, respectively (9).

Oligonucleotide and transfection. miR-2682-3p mimics and scrambled miRNAs (Shanghai GenePharma Co., Ltd., Shanghai, China) were transfected into cells using DharmaFECT1 reagent (Dharmacon, Austin, TX, USA) according to the manufacturer's instructions (9).

Western blot analysis. Tissues or cells were prepared using ice-cold lysis buffer (50 mM Tris-HCl, pH 7.0, 1% w/v SDS, 10% glycerol), then were centrifuged at 4˚C. Proteins in each supernatant were quantified. The proteins were separated by 10% SDS-PAGE and were blotted to PVDF membranes (Amersham BioSciences, Buckinghamshire, UK) (9). After blocking using 5% nonfat milk for 1 h, the membranes were incubated with antibodies. After using horseradish peroxidase-linked secondary antibodies (Cell Signaling Technology, Inc., Beverley, MA, USA), proteins bands were visualized (9).

Luciferase reporter assay. Primers were designed in accordance with the pGL3-CCND2, and MMP8, and Myd88 gene mRNA sequence. MG-63 cells were cultured in 96-well plates for 48 h at $1x10^4$ cells per well, and then were transfected with miR-2682-3p mimics or scramble, 10 ng pGL3, and pGL3-CCND2, MMP8 and Myd88-3'UTR or pGL3-CCND2, and MMP8 and Myd88-3'UTR Mut plasmid per well using Lipofectamine 3000 (9). The Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) was used to calculate relative luciferase activity of the cells after 48 h. Normalized firefly luciferase activity for each construct was compared with that of the pmirGLO Vector no insert (NO) control (9).

Statistical analysis. Data are presented as the mean ± SD from three separate experiments. Student's t-test was used to compare the differences between the two groups, and ordinary one-way ANOVA followed by Dunnett's multiple comparisons test was used to analyze the differences in multigroups. The Pearson's correlation analysis was used to verify the relevance and the log-rank test was used to compare the statistical significance difference. A P-value <0.05 was considered statistically significant. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses.

Results

miR-2682-3p expression is decreased in osteosarcoma tissues and cell lines. *miR-2682-3p* expression was lower in tumor tissues than in normal tissues (Fig. 1). *miR-2682-3p* expression was decreased in the osteosarcoma tissues and cell lines of the patients (Fig. 1A and B). Moreover, *miR-2682-3p* expression was lower in the three osteosarcoma cell lines (Saos-2, MG-63, and U2OS) than in normal osteoblast cells (FOB) (Fig. 1C).

Overexpression of miR-2682-3p inhibited osteosarcoma cell proliferation. Increased *miR-2682-3p* expression was confirmed by qRT-PCR (Fig. 2A and B); overexpression of *miR-2682-3p* decreased the proliferation of tumor cells (MG-63, and U2OS) (Fig. 2C and D).

Downregulation of miR-2682-3p promoted tumor proliferation. Decreased *miR-2682-3p* expression, which was confirmed by qRT-PCR (Fig. 3A and B), promoted the proliferation of tumor cells (MG-63 and U2OS) (Fig. 3C and D).

CCND2, MMP8, and Myd88 are the direct targets of miR-2682-3p in osteosarcoma cells. CCND2, MMP8, and Myd88 were predicted to be miR-2682-3p targets (Fig. 4A). The mRNA levels of CCND2, MMP8, and Myd88 were inhibited in the miR-2682-3p mimic groups but not in the control groups (Fig. 4B), the mutation of 3'UTR conversely (Fig. 4C).

MMP8, CCND2, and Myd88 were upregulated in osteosarcoma and inversely correlated with miR-2682-3p expression. MMP8, CCND2, and Myd88 were upregulated in osteosarcoma tissues. Moreover, the upregulation of MMP8, CCND2, and Myd88 and miR-2682-3p expression were inversely correlated in osteosarcoma tissues of patients (Fig. 5A and B).

miR-2682-3p restrained osteosarcoma cell proliferation by targeting CCND2, MMP8 and Myd88. *CCND2, MMP8*, and *Myd88* promoted osteosarcoma cell proliferation. The *miR-2682-3p* mimic that was added into tumor cells inhibited osteosarcoma cell proliferation and invasion. Consistent with our data, the survival rate was significantly decreased in the *miR-2682-3p* groups. However, in the co-transfected group, the survival rate was significantly increased, indicating that *CCND2, MMP8* and *Myd88* partly rescued the effect of *miR-2682-3p* on osteosarcoma (Fig. 5C).

Discussion

Accumulating evidence has shown that *miR-2682-3p* expression is downregulated in osteosarcoma tissues and cell lines (17). The data obtained in this study show that overexpression of *miR-2682-3p* inhibits cell proliferation in MG-63 and U2OS cells. Moreover, *CCND2, MMP8*, and *Myd88* are considered to be direct targets of miR-2682-3p. When *miR-2682-3p* mimic and CCND2, MMP8, and Myd88 were added into tumor cells, the effect of *miR-2682-3p* was partly rescued in osteosarcoma. These results indicate that miR-2682-3p is a potential tumor suppressor gene of osteosarcoma. However, further investigations of the role of miR-2682-3p *in vivo* are needed.

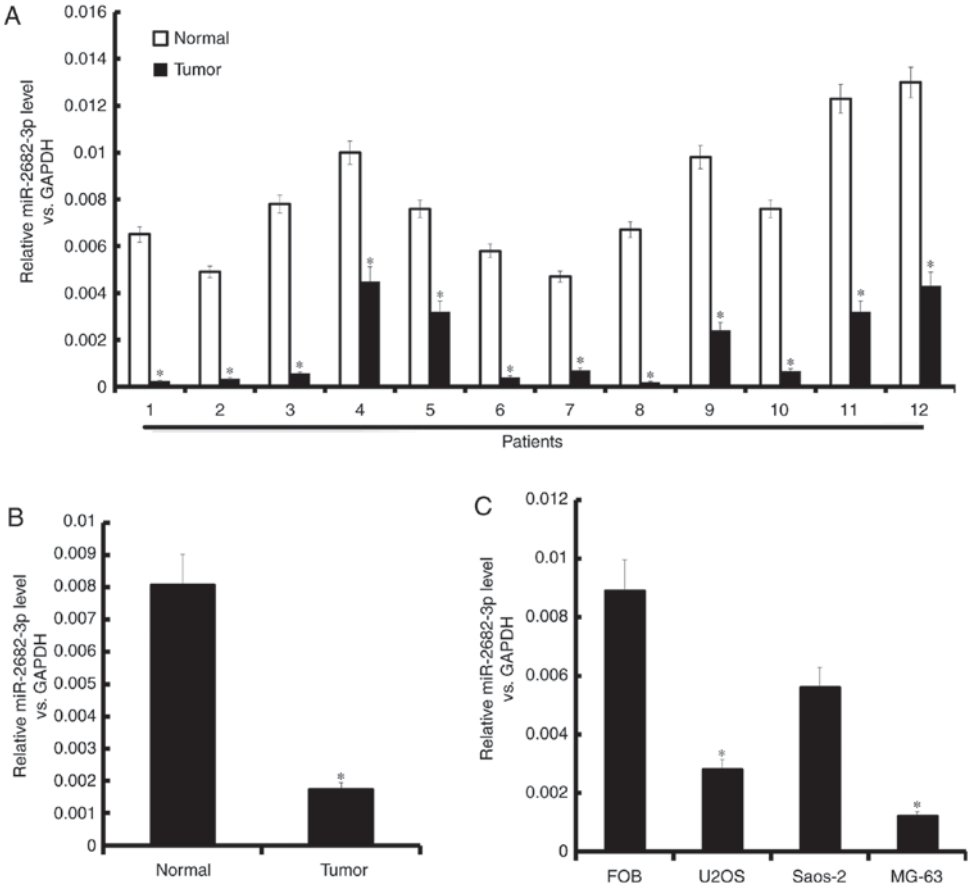

Figure 1. *miR-2682-3p* expression is decreased in osteosarcoma tissues and cell lines. (A) *miR-2682-3p* expression was determined by qRT-PCR in 12 paired osteosarcoma tissues of patient; The expression was uniformized to U6 snRNA. (B) Relative *miR-2682-3p* expression levels in osteosarcoma tissues and normal tissues; (C) *miR-2682-3p* expression analyzed in normal osteoblast cells (hFOB) and Saos-2, MG-63, and U2OS cell lines. *P<0.05. miR, microRNA.

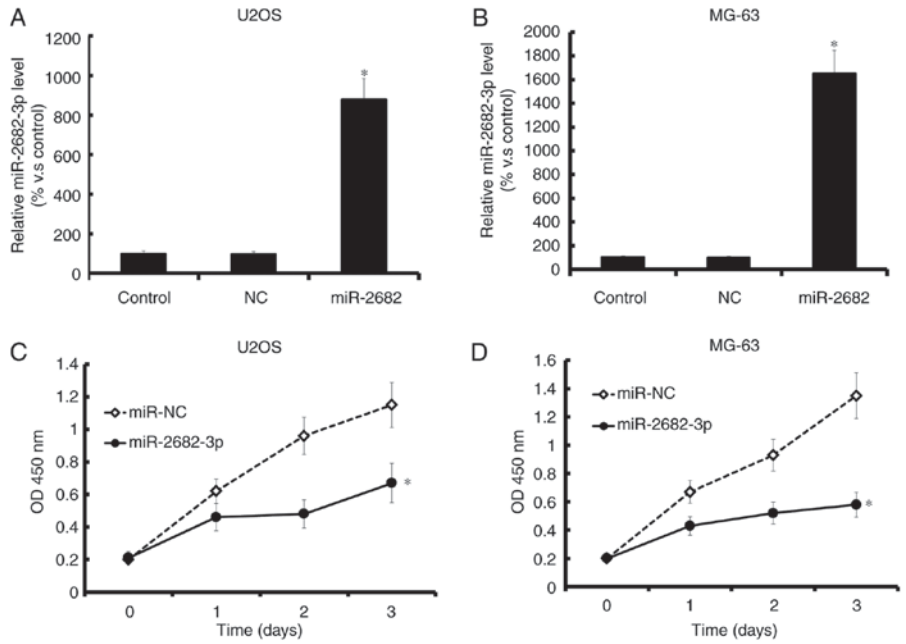

Figure 2. Overexpression of *miR-2682-3p* inhibited osteosarcoma cell proliferation. (A and B) qRT-PCR analysis of *miR-2682-3p* expression after the transfection of *miR-2682-3p* mimics or no change or control. (C and D) CCK-8 assay used to evaluate the proliferation of MG-63 and U2OS cells after being transferred with *miR-2682-3p* mimics or no change or control; relative ratio of invasive cells per field (right). *P<0.05. miR, microRNA.

Increasing evidence indicates that miR-2682-3p is involved in the progression of some cancers, such as osteosarcoma (9). The same study recently suggested that CCND2, MMP8, and MyD88 can promote cancer metastasis and that the

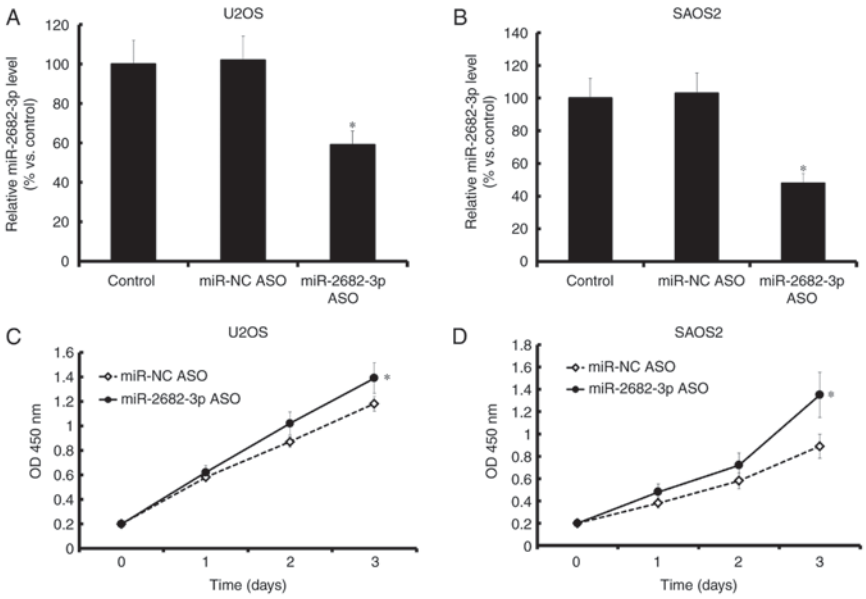

Figure 3. Decreased *miR-2682-3p* expression promoted osteosarcoma proliferation. (A and B) qRT-PCR analysis on *miR-2682-3p* expression after the transfection of *miR-2682-3p* mimics or no change or control. (C and D) CCK-8 assay evaluated the proliferation of MG-63 and U2OS cells after being transferred with *miR-2682-3p* mimics or no change or control. *P<0.05.

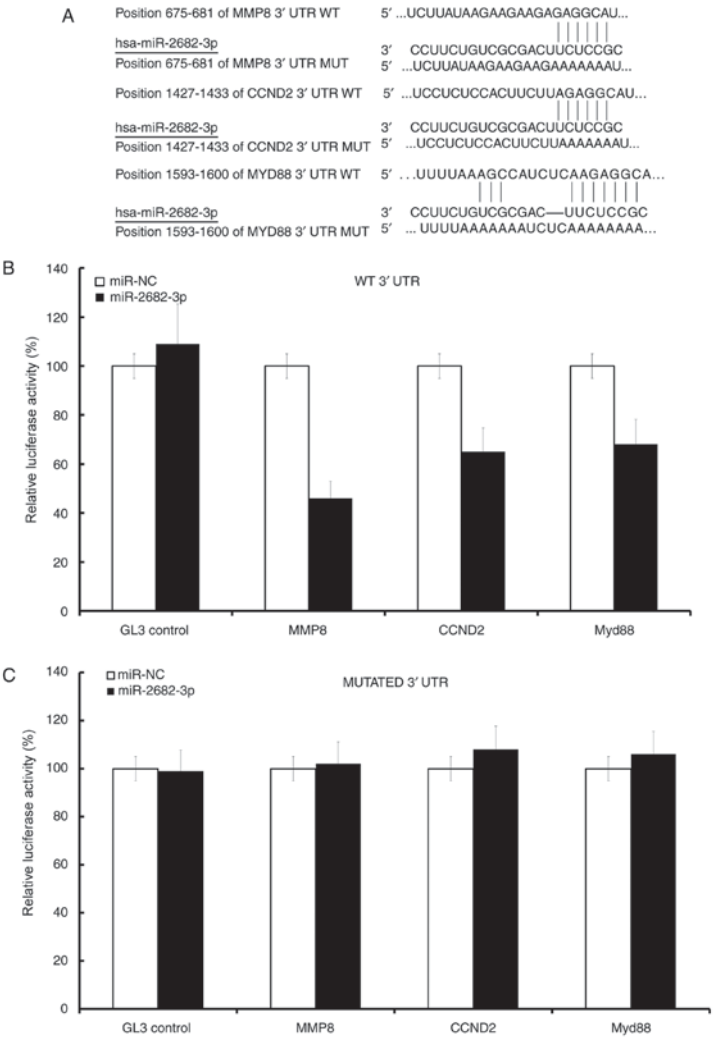

Figure 4. *miR-2682-3p* directly targeted the CCND2, MMP8 and Myd88 in osteosarcoma. (A) Possible *miR-2682-3p* binding site at the 3'-UTR of *CCND2, MMP8* and *Myd88* mRNA, prediction by TargetScan. (B) Cells were transfected with wild-type 3'-UTR reporter or mutant constructs, with 20 nM *miR-2682-3p* mimics or controls. (C) Luciferase was normalized by the ratio of firefly and *Renilla* luciferase signals; relative ratio of invasive cells per field, the mutation of 3'UTR conversely. miR, microRNA; MMP8, matrix metalloproteinase 8; Myd88, myeloid differentiation primary response 88.

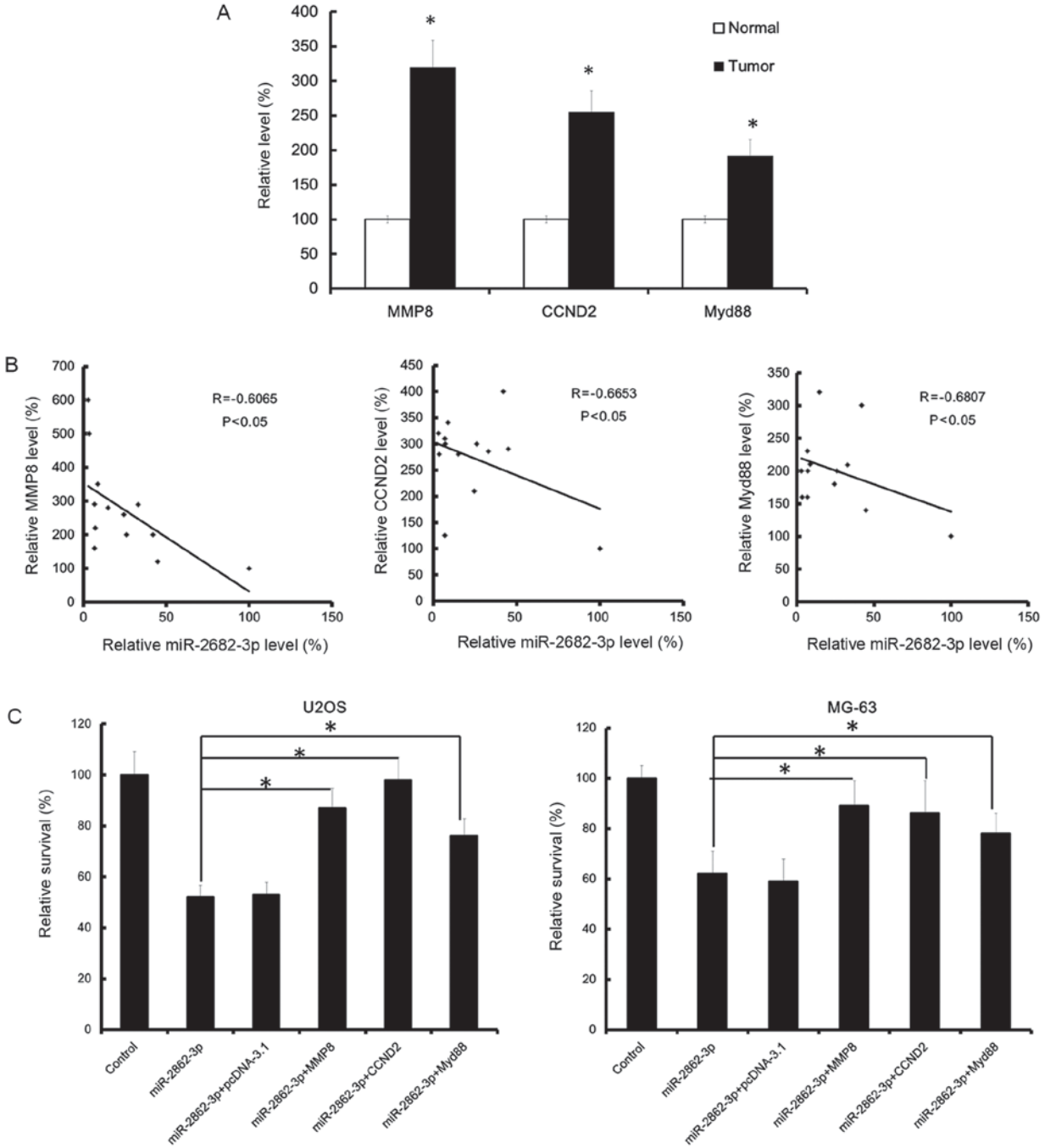

Figure 5. *miR-2682-3p* restrained osteosarcoma cell proliferation by targeting *CCND2*, *MMP8* and *Myd88*. (A) qRT-PCR analysis of *CCND2*, *MMP8* and *Myd88* expression in osteosarcoma tissues. Upregulated mRNA expression levels of *CCND2*, *MMP8* and *Myd88* in osteosarcoma. Expression levels of *CCND2*, *MMP8* and *Myd88* normalized to GAPDH. (B) Inverse correlation between MMP8, CCND2 and Myd88 upregulation and *miR-2682-3p* expression in osteosarcoma patients. (C) Decreased survival rates in osteosarcoma cells by overexpression of *miR-2682-3p* (U2OS, MG-63). Significant increases in the survival rates of the co-transfected groups relative to those of cells overexpressing *miR-2682-3p*. Relative ratio of invasive cells per field (right). *P<0.05. miR, microRNA; MMP8, matrix metalloproteinase 8; Myd88, myeloid differentiation primary response 88.

proliferation of many cancer cells can be induced by regulating the composition of extracellular matrix (9). However, the underlying mechanisms by which CCND2, MMP8, and Myd88 stimulate MG-63 cell growth still need to be clarified. The present results show that one of the tumor suppressor miRNAs is *miR-2682-3p* in osteosarcoma.

In conclusion, miR-2682-3p expression was decreased in osteosarcoma tissues and cell lines. Overexpression of miR-2682-3p leads to the inhibition cell proliferation of osteosarcoma. CCND2, MMP8, and Myd88 are potential targets of miR-2682-3p. Thus, miR-2682-3p has potential value as a new target for the treatment of osteosarcoma in future.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FZ analyzed and interpreted the patient data regarding the osteosarcoma. YZ performed the cytology examination of the osteosarcoma. GF was a major contributor in writing the manuscript and helped with the experiment and statistical analysis. SH as the leader of this study, designed the experiment and reviewed the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients participated in the study provided written informed consent, and the study was approved by the Ethics Committee of Shanghai Tenth People's Hospital.

Consent for publication

All the patients and researchers consented for publication.

Competing interests

The authors declare that they have no competing interests.

References

1. Gorlick R and Khanna C: Osteosarcoma. J Bone Miner Res 25: 683-691, 2010.
2. Ritter J and Bielack SS: Osteosarcoma. Ann Oncol 21 (Suppl 7): Vii320-Vii325, 2010.
3. Kager L, Zoubek A, Dominkus M, Lang S, Bodmer N, Jundt G, Klingebiel T, Jürgens H, Gadner H and Bielack S; COSS Study Group: Osteosarcoma in very young children: Experience of the cooperative osteosarcoma study group. Cancer 116: 5316-5324, 2010.
4. Botter SM, Neri D and Fuchs B: Recent advances in osteosarcoma. Curr Opin Pharmacol 16: 15-23, 2014.
5. Moore DD and Luu HH: Osteosarcoma. Cancer Treat Res 162: 65-92, 2014.
6. Gianferante DM, Mirabello L and Savage SA: Germline and somatic genetics of osteosarcoma-connecting aetiology, biology and therapy. Nat Rev Endocrinol 13: 480-191, 2017.
7. Anderson ME: Update on survival in osteosarcoma. Orthop Clin North Am 47: 283-292, 2016.
8. Szewczyk M, Lechowski R and Zabielska K: What do we know about canine osteosarcoma treatment? review. Vet Res Commun 39: 61-67, 2015.
9. Niu G, Li B, Sun L and An C: MicroRNA-153 inhibits osteosarcoma cells proliferation and invasion by targeting TGF-β2. PLoS One 10: e0119225, 2015.
10. Rupaimoole R and Slack FJ: MicroRNA therapeutics: Towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 16: 203-222, 2017.
11. Deng K, Wang H, Guo X and Xia J: The cross talk between long, non-coding RNAs and microRNAs in gastric cancer. Acta Biochim Biophys Sin (Shanghai) 48: 111-116, 2016.
12. Jothy SL, Chen Y, Vijayarathna S, Kanwar JR and Sasidharan S: MicroRNAs: Association with radioresistant and potential uses of natural remedies as green gene therapeutic approaches. Curr Gene Ther 15: 15-20, 2015.
13. Zou P, Ding J and Fu S: Elevated expression of microRNA-19a predicts a poor prognosis in patients with osteosarcoma. Pathol Res Pract 213: 194-198, 2017.
14. Lian D, Wang ZZ and Liu NS: MicroRNA-1908 is a biomarker for poor prognosis in human osteosarcoma. Eur Rev Med Pharmacol Sci 20: 1258-1262, 2016.
15. Chen J, Yan D, Wu W, Zhu J, Ye W and Shu Q: MicroRNA-130a promotes the metastasis and epithelial-mesenchymal transition of osteosarcoma by targeting PTEN. Oncol Rep 35: 3285-3292, 2016.
16. Zhang S, Zhao Y and Wang L: MicroRNA-198 inhibited tumorous behaviors of human osteosarcoma through directly targeting ROCK1. Biochem Biophys Res Commun 472: 557-565, 2016.
17. Waresijiang N, Sun J, Abuduaini R, Jiang T, Zhou W and Yuan H: The downregulation of miR-125a-5p functions as a tumor suppressor by directly targeting MMP-11 in osteosarcoma. Mol Med Rep 13: 4859-4864, 2016.
18. Zhang J, Hou W, Chai M, Zhao H, Jia J, Sun X, Zhao B and Wang R: MicroRNA-127-3p inhibits proliferation and invasion by targeting SETD8 in human osteosarcoma cells. Biochem Biophys Res Commun 469: 1006-1011, 2016.
19. Duan J, Shi J, Fiorentino A, Leites C, Chen X, Moy W, Chen J, Alexandrov BS, Usheva A, He D, *et al*: A rare functional noncoding variant at the GWAS-implicated MIR137/MIR2682 locus might confer risk to schizophrenia and bipolar disorder. Am J Hum Genet 95: 744-753, 2014.
20. Hu J, Lv G, Zhou S, Zhou Y, Nie B, Duan H, Zhang Y and Yuan X: The Downregulation of MiR-182 is associated with the growth and invasion of osteosarcoma cells through the regulation of TIAM1 expression. PLoS One 10: e0121175, 2015.