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MiR-582-5p inhibits the growth and invasion of osteosarcoma cell by targeting NOVA1

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Abstract. – OBJECTIVE: To investigate the role of microRNA-582-5p (miR-582-5p) on osteosarcoma (OS) cell behaviors and provide potential therapeutic targets for OS.

MATERIALS AND METHODS: Expression levels of miR-582-5p in OS cell lines and normal cell lines were measured by quantitative real-time PCR. Cell proliferation, migration and invasion capacities were assessed by cell counting kit-8 assay, wound-healing assay, and transwell invasion assay, respectively. Downstream target of miR-582-5p was confirmed by Luciferase activity reporter assay and Western blotting assay.

RESULTS: MiR-582-5p expression was downregulated in OS cell lines compared with normal cell lines. Overexpression of miR-582-5p inhibits OS cell proliferation, migration, and invasion capacities. Neuro-oncological ventral antigen 1 (NOVA1) was chosen as target gene of miR-582-5p by bioinformatics analysis and Luciferase reporter assay. Moreover, overexpression of NOVA1 could impair tumor suppression role of miR-582-5p on OS cell behaviors.

CONCLUSIONS: MiR-582-5p exerts tumor-suppressive role on OS cell behaviors *via* targeting NOVA1 *in vitro*, which will help us to understand the mechanisms underlying OS progression.

Key Words:
MiR-582-5p, NOVA1, Osteosarcoma, Growth, Invasion.

Introduction

Osteosarcoma (OS), a cancer type originated from bone, represents approximately 0.2% of all human cancers[1]. 5-year overall survival for OS patients has been elevated to about 70%; however, OS treatment often fail due to chemoresistance and metastasis[2,3]. Although numerous cancer-related genes have been identified for OS, mechanisms underlying OS progression remain to be elucidated[4].

MicroRNAs (miRNAs) are crucial regulators in numerous cellular processes including growth and metastasis[5-7]. Several studies[8,9] have illustrated that miRNAs play crucial roles in OS initiation and progression. Therefore, targeting miRNAs has been revealed to be a promising measure against OS.

MiR-582-5p has been recognized to function as tumor suppressor in several cancers. Of note, miR-582-5p reduced expression was found in bone metastasis tissues compared with non-metastasis prostate cancer tissues[10]. Moreover, overexpression of miR-582-5p was shown to inhibit prostate cancer cell invasion and migration[10]. Besides that, miR-582-5p expression was found downregulated in non-small cell lung cancer and correlated with advance tumor stage and poor overall survival of cancer patients[11]. Jin et al[12] on gastric cancer revealed that miR-582-5p was lowly expressed and its overexpression inhibits cancer cell proliferation, promotes cell apoptosis, and arrests cell cycle at G0/G1 phase through regulating AKT serine/threonine kinase 3. However, its expression and biological role in OS remains unclear.

Neuro-oncological ventral antigen 1 (NOVA1) is RNA-binding protein and reported to function as oncogene in human cancer. NOVA1 expression was found upregulated in melanoma, and the knockdown of NOVA1 inhibits cancer cell growth, migration, and invasion via targeting forkhead box O3a[13]. In hepatocellular carcinoma, NOVA1 was found to interact with GABAA Receptor-γ2 to promote cancer progression *in vivo*[14].

In this study, we aimed to investigate the role of miR-582-5p in OS and the associated mechanisms. We then examined the effects of miR-582-5p on OS

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cell proliferation, migration, and invasion. Besides, the possibility of NOVA1 as a direct target of miR-582-5p was validated by Luciferase activity reporter assay and Western blot assay. Our research will provide basis for in-depth exploration of mechanisms underlying OS progression.

Materials and Methods

Cell Lines and Cell Transfection

OS cell lines (U2OS and MG-63) and normal human osteoblastic cell line (hFOB1.19) purchased at the Cell Bank of Chinese Academy of Sciences (Shanghai, China) were incubated at Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) at a 37°C incubator with 5% CO_2.

To elevate the expression level of miR-582-5p, miR-582-5p mimic and the corresponding control (miR-NC) were purchased from GenePharma (Shanghai, China). To elevate the expression of NOVA1, the coding sequence for NOVA1 was inserted into pcDNA3.1 (pNOVA1) by GenScript (Nanjing, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

RNA Extraction and Quantitative Real Time-PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the provided protocols. Complementary DNA was synthesized from RNA using TaKaRa Reverse Transcription Kit (Haimen, Jiangsu, China). qRT-PCR was conducted using SYBR Green Mix (Invitrogen, Carlsbad, CA, USA) at ABI 7500 system (Applied Biosystems, Foster City, CA, USA) with U6 snRNA as the internal control. Relative miR-582-5p expression levels were calculated using $2^{-\Delta\Delta CT}$ method. The primers used were: miR-582-5p (F) 5'-GCGGTTACAGTTGTTCAACC-3', and (R) 5'-CTCAACTGGTGTCGTGGA-3'; U6 snRNA (F) 5'-CTCGCTTCGGCAGCACA-3', and (R) 5'-AACGCTTCACGAATTTGCGT-3'.

Protein Isolation and Western Blot

Total protein was extracted using radio immunoprecipitation assay lysis (RIPA) buffer (Beyotime, Haimen, Jiangsu, China) supplemented with protease inhibitor (Beyotime) according to the provided protocols. After isolated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the protein samples were transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were incubated with primary antibodies (anti-NOVA1: ab183024; anti-GAPDH: ab181602; both from Abcam, Cambridge, MA, USA) at 4°C overnight after blocked with fat-free milk. Membranes were then incubated with secondary antibody (ab6721, Abcam, Cambridge, MA, USA) at room temperature for 4 h. Subsequently, band signals were developed with BeyoECL kit (Beyotime) according to the standard protocol.

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 (Beyotime) was conducted to measure cell proliferation rate. Briefly, 3×10^3 cells were seeded into 96-well plate and incubated for 0, 24, 48 or 72 h. Then, CCK-8 reagent was added and incubated for 4 h before measuring the absorbance at 450 nm using microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Wound-Healing Assay

Cell migration was measured using scratch wound assay. Cells were grown to confluence and then scratched by a plastic tip. Distance at 0 and 24 h after scratch generation was measured under light microscope.

Transwell Invasion Assay

Cell invasion assay was conducted using transwell chamber consisting of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) pre-coated 8-mm membrane filter inserts (Corning Inc., Corning, NY, USA). 1.5×10^5 cells in FBS-free medium were added to upper chamber, and lower chamber was filled with FBS supplemented medium. After 48 h of incubation, invasive cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet and counted under a microscope.

Luciferase Activity Reporter Assay

TargetScan was used to predict the targets of miR-582-5p and identified NOVA1 was a putative target of miR-582-5p. The wild-type 3'-UTR of NOVA1 was cloned into pmirGLO and named as wt-NOVA1 3'-UTR. The mutant 3'-UTR of NOVA1 was generated from wt-NOVA1 3'-UTR using site-direct mutagenesis kit (TaKaRa, Otsu, Shiga, Japan) and named as mt-NOVA1 3'-UTR. Cells were co-transfected with 200 ng Luciferase vector and miR-582-5p mimic or miR-NC. After

transfection for 48 h, cells were collected to measure relative Luciferase activity using Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

SPSS13.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Differences between groups were analyzed using paired Student's *t*-test. Multi-group comparison was conducted using one-way analysis of variance and Tukey post-hoc test. *p*-value less than 0.05 was considered as statistically significant.

Results

MiR-582-5p Was Reduced Expression In OS

To determine whether miR-582-5p was dysregulated in OS cell lines, qRT-PCR was conducted to measure miR-582-5p expression in OS cell lines. As shown in Figure 1, expression level of miR-582-5p in OS cell lines was reduced compared with the normal cell line. Our results indicated that miR-582-5p has a crucial role in OS.

MiR-582-5p Acts As Tumor Suppressor In Regulating OS Cell Behaviors

To examine the biological roles of miR-582-5p in OS, miR-582-5p mimic and miR-NC were transfected to OS cell lines. qRT-PCR showed the introduction of miR-582-5p mimic significantly increased the levels of miR-582-5p in OS cells (Figure 2A). CCK-8 assay showed the viability of OS cells was significantly inhibited by miR-582-5p mimic compared with miR-NC (Figure 2B). Besides that, wound-healing assay and transwell invasion assay were performed to determine the migration and invasion abilities. As shown in Figure 2C and 2D, these results indicated that overexpression of miR-582-5p inhibits OS cell migration and invasion. Collectively, these results indicated that miR-582-5p functions as tumor suppressor in OS progression.

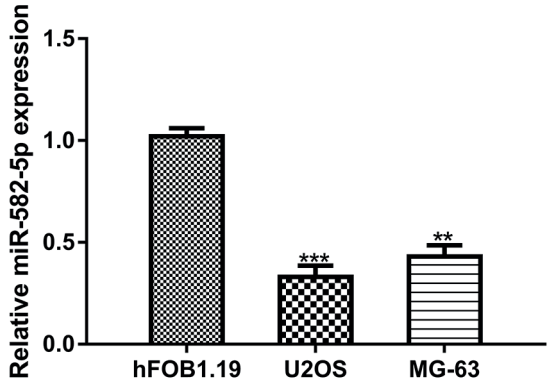

Figure 1. Relative expression level of miR-582-5p in OS cell lines (U2OS and MG-63) and normal human osteoblastic cell line (hFOB1.19). MiR-582-5p: microRNA-582-5p; OS: osteosarcoma.

MiR-582-5p Directly Target NOVA1

Bioinformatic algorithm revealed that NOVA1 was a putative target of miR-582-5p (Figure 3A). To confirm this prediction, Luciferase activity reporter assay was conducted. It was found miR-582-5p mimic transfection decreased the Luciferase activity in cells transfected with wt-NOVA1 3'-UTR (Figure 3B). However, introduction of miR-582-5p mimic did not have influence on the Luciferase activity of mt-NOVA1 3'-UTR (Figure 3B). Western blot showed overexpression of miR-582-5p decreased NOVA1 expression in OS cell lines (Figure 3C).

Alteration of NOVA1 Expression Affects the Effects of MiR-582-5p on OS Cells

To further confirm NOVA1 as a function target of miR-582-5p, miR-582-5p mimic and pNOVA1 was co-transfected into OS cell lines. Expression of NOVA1 after transfection was estimated by Western blot (Figure 4A). *In vitro* functional assays revealed that overexpression of NOVA1 partially reversed the effects of miR-582-5p on OS cell proliferation, migration, and invasion (Figure 4B-4D).

Discussion

Metastasis often results in the poor overall survival of cancer patients diagnosed with OS[15]. Abnormally expression of miRNAs has been found associated with tumor progression through functioning as either tumor suppressor or oncogene[8,9,16-18]. In this study, we investigated miR-582-5p expression in OS cell lines. Our results showed that miR-582-5p expression was downregulated in OS cell lines compared with normal cell line. Subsequently, the biological roles of miR-582-5p in OS. We found miR-582-5p overexpression by miR-582-5p mimic could suppress OS cell proliferation, migration, and invasion *in vitro*. These results suggested miR-582-5p may function as a

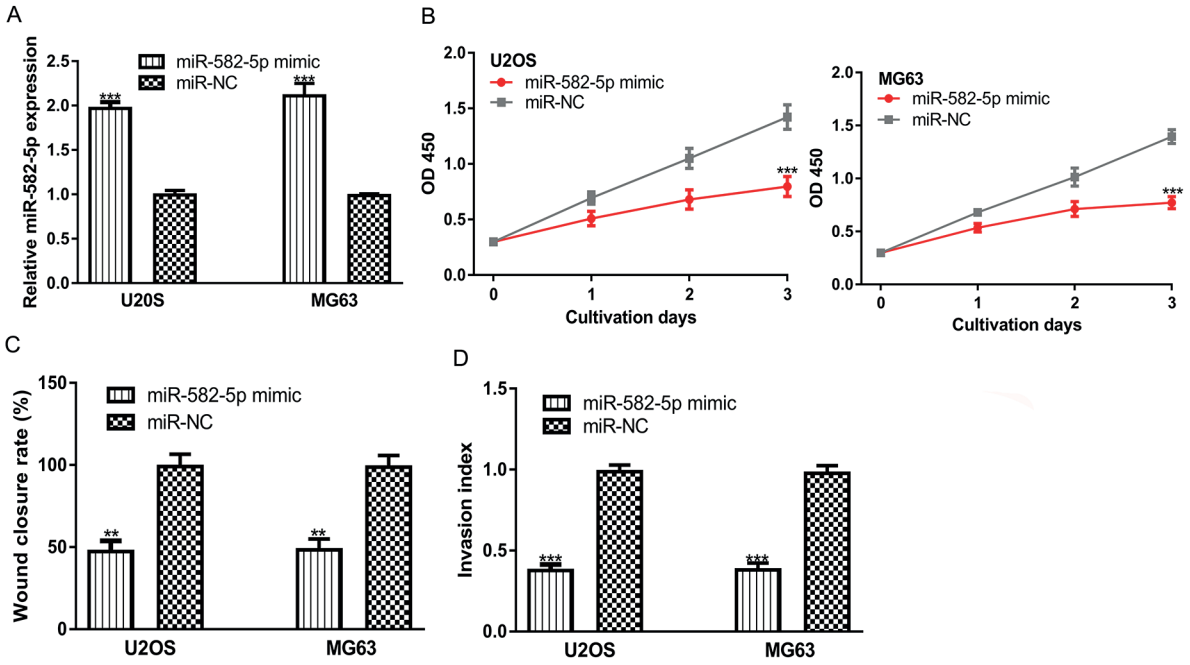

Figure 2. MiR-582-5p overexpression inhibited OS cells proliferation, migration and invasion. After transfection with miR-582-5p mimic or miR-NC. (**A**) MiR-582-5p expression in OS cells, (**B**) Cell proliferation, (**C**) Cell migration, and (**D**) Cell invasion was detected. miR-582-5p: microRNA-582-5p; OS: osteosarcoma; miR-NC: negative control miRNA.

tumor suppressor in OS. MiR-582-5p plays a tumor suppressive role in human cancers including prostate cancer, non-small cell lung cancer, and gastric cancer. Our results presented here are in line with the previous findings, which will help to establish the biological role of miR-582-5p in human cancers[10-12].

Several targets of miR-582-5p have been proposed in previous researches[10-12]. In this work, by bioinformation software analysis, we also found NOVA1 may be a target of miR-582-5p. Through Luciferase activity reporter assay, we found the transfection of miR-582-5p mimic could decrease the Luciferase activity of cells transfected with wt-NOVA1 3'-UTR but did not have influence on mt-NOVA1 3'-UTR. This result indicated that miR-582-5p could directly interact with the 3'-UTR of NOVA1. The Western blot analysis results suggested that NOVA1 protein expression could be downregulated by miR-582-5p mimic, indicating NOVA1 was indeed a target for miR-582-5p. Furthermore, the functional assays revealed that the overexpression of NOVA1 could partially reverse the effects of miR-582-5p on OS cell proliferation, migration, and invasion. These results demonstrated that NOVA1 was a functional target of miR-582-5p.

Conclusions

Taken together, our results for the first time showed miR-582-5p expression was significantly reduced in OS cell lines. In addition, miR-582-5p functions as tumor suppressor in OS by targeting the expression of NOVA1. The novel miR-582-5p/NOVA1 axis identified in this work will with no doubt help to understand the mechanisms underlying the progression of OS, which will also help to establish novel therapeutic targets for OS.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

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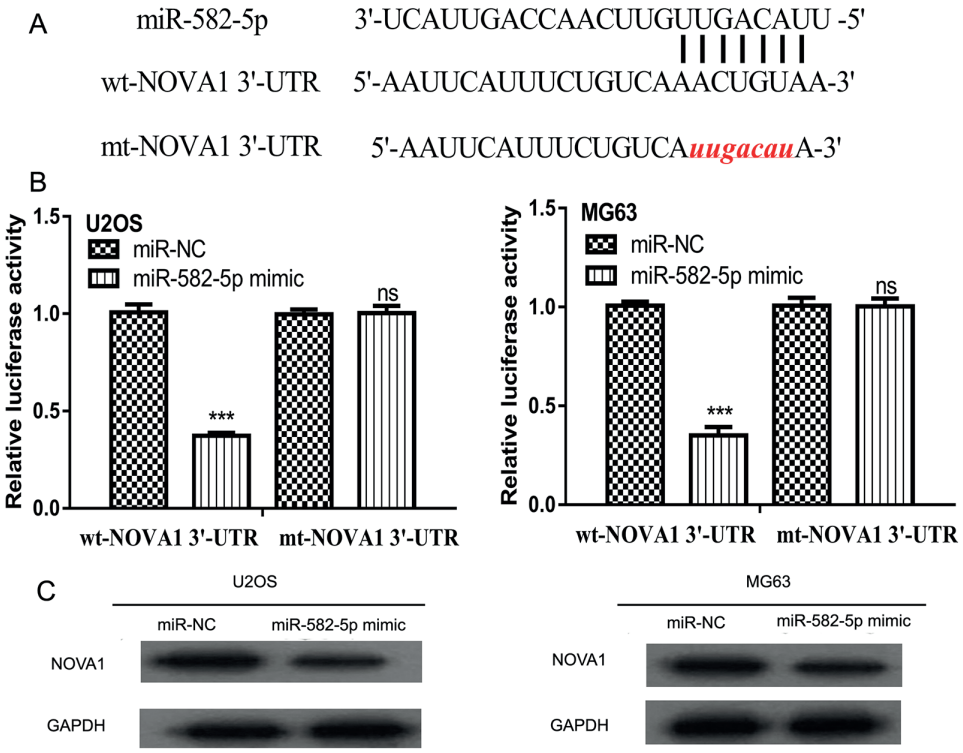

Figure 3. NOVA1 was the target gene of miR-582-5p. **A**, NOVA1 was selected as the potential target of miR-582-5p via using bioinformatics analysis. **B**, Luciferase activities of OS cells transfected with wild type or the mutated NOVA1 3'-UTR together with miR-582-5p mimic or miR-NC. **C**, NOVA1 protein expression level of OS cells transfected with miR-582-5p mimics or miR-NC. MiR-582-5p: microRNA-582-5p; OS: osteosarcoma; miR-NC: negative control miRNA; wt: wild-type; mt: mutant; UTR: untranslated region; NOVA1: Neuro-oncological ventral antigen 1.

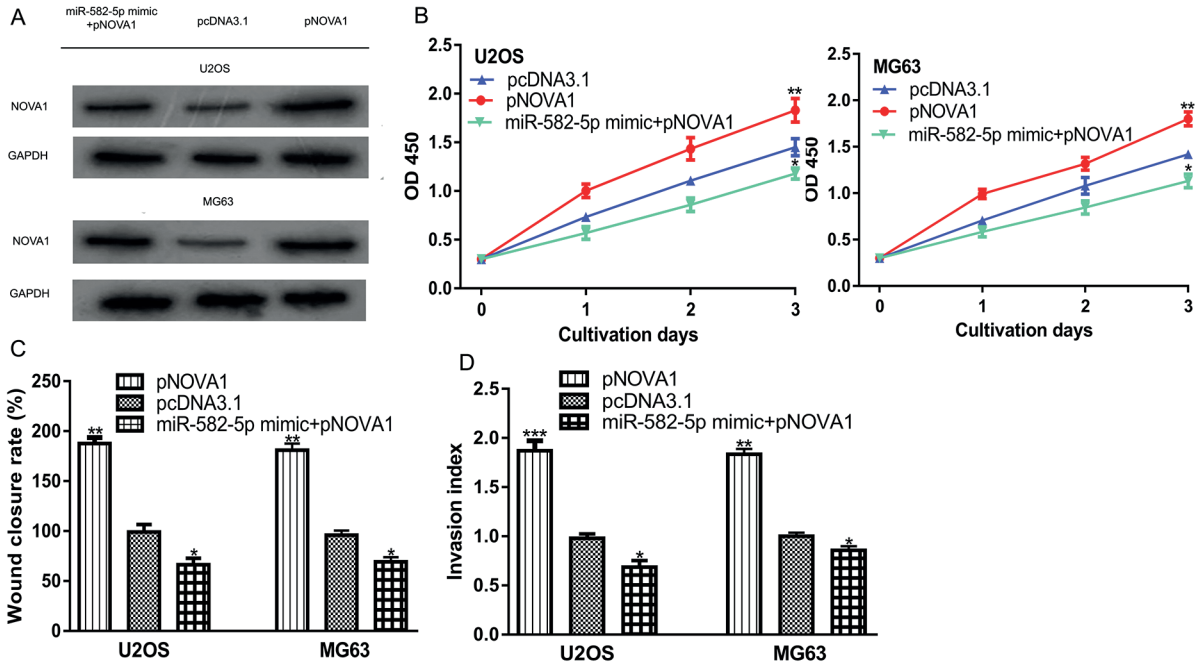

Figure 4. Restoration of NOVA1 impaired tumor suppression role of miR-582-5p. After transfection with pNOVA1, pcDNA3.1, miR-582-5p mimic and pNOVA1. (**A**) NOVA1 expression in OS cells, (**B**) Cell proliferation, (**C**) Cell migration, and (**D**) Cell invasion was detected. MiR-582-5p: microRNA-582-5p; OS: osteosarcoma; NOVA1: Neuro-oncological ventral antigen 1.

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