RESEARCH ARTICLE

MicroRNA-802 Promotes Osteosarcoma Cell Proliferation by Targeting p27

Zhong-Qing Cao[1], Zan Shen[2], Wei-Yi Huang[2]*

Abstract

MicroRNAs have been demonstrated to regulate proliferation and apoptosis in many types of cancers, but biological functions in osteosarcomas remain relatively unknown. Here, we found expression of miR-802 to be up-regulated in osteosarcoma tissues in comparison with adjacent normal tissues. Enforced expression of miR-802 was able to promote cell proliferation in U2OS and MG63 cells, while miR-802 antisense oligonucleotides (antisense miR-802) inhibited cell proliferation. At the molecular level, our results further revealed that expression of p27, a negative cell-cycle regulator, was negatively regulated by miR-802. Therefore, the data reported here indicate that miR-802 is an important regulator in osteosarcoma, our findings contributing to a better understanding of important mis-regulated miRNAs in this tumour type.

Keywords: MicroRNA-802 - p27 - osteosarcoma - cell proliferation

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Introduction

Osteosarcoma is a primary mesenchymal tumor characterized histologically by malignant tumor cells that directly produce osteoid or immature bone (Resnick et al., 2005; Klein et al., 2006). It has become one of the most common primary malignant bone tumors in childhood and adult (Eppert et al., 2005; Tan et al., 2009). Clearly, the impact of identifying factors that govern proliferation and progression is useful in the management of osteosarcoma. Several genetic alterations have been found to be tightly associated with osteosarcoma, indicating the potential application of gene therapy in osteosarcoma treatment (DuBois et al., 2009; Amankwah et al., 2013).

MicroRNA (miR) is a small non-coding RNA molecule found in plants and animals, which functions in transcriptional and post-transcriptional regulation of gene expression (Bartel et al., 2004; Chen et al., 2007). Recent studies have demonstrated that miRNAs play critical role in cell proliferation, apoptosis and metastasis (Esquela-Kerscher et al., 2006; Bushati et al., 2007). Mis-regulation of some miRNAs in diverse types of cancer is associated with tumor progression and treatment (Esquela-Kerscher et al., 2006; Bushati et al., 2007). Previous studies have shown that miR-802 regulates human angiotensin II type 1 receptor expression in intestinal epithelial C2BBe1 cells (Sansom et al., 2010). Besides, miR-802 mediates the stimulatory effect of a high-potassium diet on renal outer medullary potassium channel (ROMK) channel activity by suppressing caveolin-1 expression, which leads to increased surface expression of ROMK channels in the distal nephron (Lin et al., 2012). Moreover, a recent study showed that obesity-induced overexpression of miR-802 impairs glucose metabolism through silencing of Hnf1β (Kornfeld et al., 2013). However, whether miR-802 was involved in the development of osteosarcoma remain unexplored, and here we will investigate its role in osteosarcoma cell proliferation.

Materials and Methods

Cell culture and tissue samples

Osteosarcoma cells (U2OS and MG63) were obtained from American Type Culture Collection (Rockville, MD). Cells were culture in Dulbecco's modified Eagle's medium (DMEM; Sigma. St. Louis, MO) supplemented with 10% fetal bovine serum. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Tumor tissues and adjacent non-tumor normal tissues were collected from routine therapeutic surgery at our department. All samples were obtained with informed consent and approved by the hospital institutional review board.

Analysis of miRNA expression using TaqMan RT-PCR

Total RNA from tissue samples and cell lines was isolated by using the miRNA Isolation Kit (Ambion, USA). Expression of mature miRNAs was assayed using Taqman MicroRNA Assay (Applied Biosystems) specific for hsa-miR-802. Briefly, 10 ng of total RNA were reverse transcribed to cDNA with specific stem-loop RT primers. Quantitative real-time PCR was performed by using an Applied Biosystems 7900 Real-time PCR System and a

[1]*Department of Orthopedics, St. Luke's Hospital,* [2]*Department of Oncology, First Affiliated People's Hospital, Shanghai Jiaotong University, Shanghai, China *For correspondence: weiyi_huang@126.com*

TaqMan Universal PCR Master Mix. All the primers were obtained from the TaqMan miRNA Assays. Small nuclear U6 snRNA (Applied Biosystems) was used as an internal control.

Plasmid construction and transfection

For miR-802 expression plasmid, human miR-802 precursor was cloned into pSilencer 4.1 (Ambion, Austin, TX). The negative control plasmid consists of a scrambled sequence (Ambion). To inhibit miR-802 function, an Ambion miRNA inhibitor for miR-802 was used, along with the negative control. For transfection, a complex of Lipofectamine 2000 (Invitrogen, CA, USA) and 25 nM miRs mentioned above was prepared following the manufacturer's instructions.

BrdU Assays

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. All experiments were performed in triplicate. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA)

Western blotting

Cells or tissues were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM 2-ME, 2% w/v SDS, 10% glycerol). After centrifugation at 20000× g for 10 min at 4 °C, proteins in the supernatants were quantified and separated by 10% SDS PAGE, transferred to NC membrane (Amersham Bioscience, Buckinghamshire, U.K.). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Cell Signaling). The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to manufacturer's instructions. Anti-p27 antibodies were purchased from Cell signaling (USA). Protein levels were normalized to total GAPDH, using a mouse anti-GAPDH antibody (Santa Cruz, USA).

Luciferase reporter assay

Total cDNA from MG63 cells was used to amplify the 3'UTR of p27 by PCR. The p27 3'UTR was cloned into pMir-Report (Ambion), yielding pMir-Report-p27. Mutations were introduced in potential miR-802 binding sites using the Quick-change site-directed mutagenesis Kit (Stratagene). Cells were transfected with the pMir-Report vectors containing the 3'-UTR variants, and miR-802 precursor, control plasmids for 36 hours. The pRL-SV40 vector (Promega) carrying the Renilla luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

Data are expressed as the mean±SEM from at least three separate experiments. Differences between groups were analyzed using Student's t-test. A value of $p < 0.05$ was considered statistically significant.

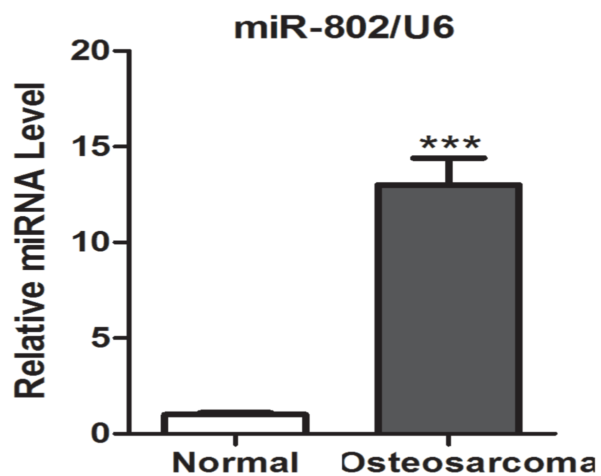

Figure 1. Expression Levels of miR-802 in Osteosarcoma Tissues. miR-802 expression was determined by TaqMan real-time PCR in human osteosarcoma tissues and adjacent noncancerous tissues (Normal). ***p<0.001 compared with normal tissues

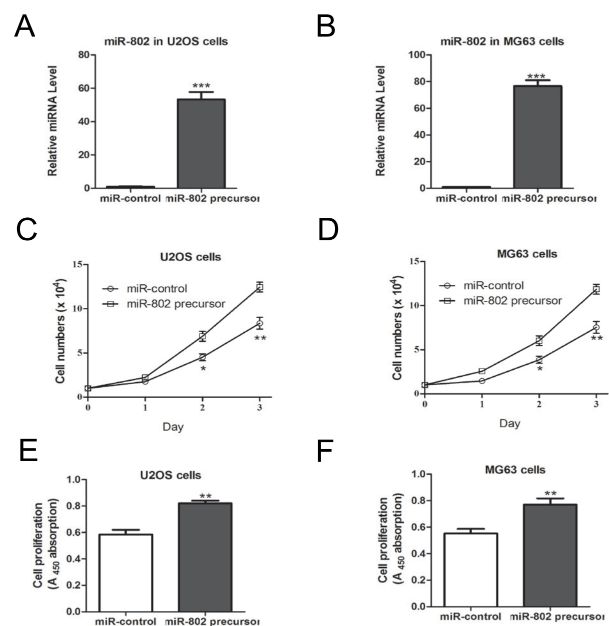

Figure 2. Overexpression of miR-802 Promotes Osteosarcoma Cell Proliferation. (A-B) Expression of miR-802 was determined in U2OS (A) and MG63 (B) cells after transfection of miR-802 precursor or negative control (miR-control). ***p<0.001 compared with miR-control. (C-D) The growth curve of U2OS (C) and MG63 (D) cells after transfection of miR-802 precursor or negative control (miR-control).*p<0.05, **p<0.01 compared with miR-control. (E-F) The cell proliferative potential (BrdU) was determined in U2OS (E) and MG63 cells (F) after transfection of miR-802 precursor or negative control (miR-control). A450 absorption was assayed after transfection for 24 hr. **p<0.01 compared with miR-control

Results

miR-802 expression levels were up-regulated in patients with osteosarcoma

Firstly, to examine whether the miR-802 is differentially expressed in human osteosarcoma, its expression level was determined using TaqMan real-time PCR in 28 pairs of human osteosarcoma tissues and pair-matched adjacent noncancerous tissues. Our results demonstrated that the

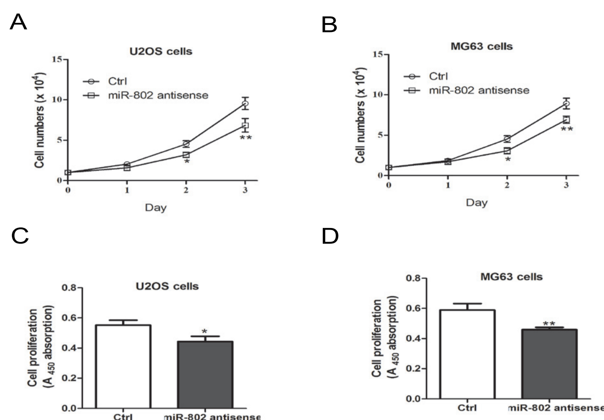

Figure 3. miR-802 Antisenses Inhibits the Proliferation of Osteosarcoma Cells. (A-B) The growth curve of U2OS and MG63 cells after miR-802 antisense transfection compared to negative control (Ctrl). *p<0.05, **p<0.01 compared with Ctrl. (C-D) The cell proliferative potential (BrdU) was determined in U2OS and MG63 cells transfected with miR-802 antisense or negative control (Ctrl). A450 absorption was assayed after transfection for 24 hr. *p<0.05, **p<0.01 compared with Ctrl

expression level of miR-802 was significantly increased in osteosarcoma tissues in comparison with the adjacent noncancerous tissues (Figure 1).

miR-802 overexpression promotes cell proliferation

In order to assess the effects of miR-802 on osteosarcoma cell growth, miR-802 precursor was transfected into U2OS and MG63 cells and cell growth was examined. miR-802 precursor was found to be able to up-regulate miR-802 expression (Figure 2A and 2B) and significantly increased cell number and promoted proliferation in cells (Figure 2C-2F).

Inhibition of miR-802 represses the proliferation of osteosarcoma cells

As described above, miR-802 plays a critical role in the proliferation of osteosarcoma cells. However, it remained unknown whether inhibiting miR-802 would reduce cell proliferation. Therefore, both cells were transfected with miR-802 antisense. We discovered that ectopic expression of the hsa-miR-802 antisense reduced the growth of U2OS and MG63 cells, compared to NC-transfected cells (Figure 3A-3D).

miR-802 directly targets the p27 in osteosarcoma cells

Using a stringent bioinformatics approach, we identified 12 putative human miR-802 target genes (data not shown), among which the gene encoding p27 harbored a potential miR-802 binding site (Figure 4A). Overexpression of miR-802 led to a reduction of luciferase activity when the reporter construct contained the p27 3'UTR (Figure 4B). In contrast, mutation of the conserved miR-802 binding motif abrogated the reduced luciferase expression (Figure 4B). Moreover, overexpression of miR-802 in osteosarcoma cells led to reduced p27 protein expression (Figure 4C-4D). Consistently, inhibition of miR-802 led to an increased expression of p27 contents (Figure 4E-4F), further indicating that p27 is a target of miR-802 in osteosarcoma cells.

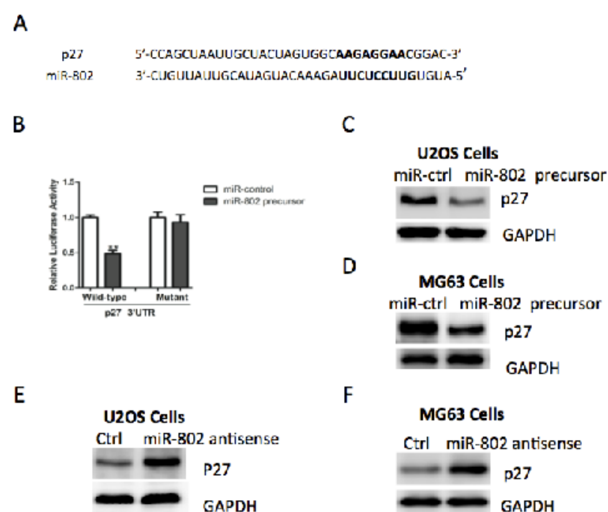

Figure 4. miR-802 Negatively Regulates p27 Expression in Osteosarcoma Cells. (A) Computer prediction of miR-802 binding sites in the 3'UTRs of human p27 genes. Potential binding site was highlighted in bold. **p<0.01 compared with miR-control. (B) Luciferase reporter assays in U2OS cells. Cells were transfected with 100 ng of wild-type 3'-UTR-reporter or mutant constructs together with 100 nM of miR-802 precursor or controls. (C-D) Western blotting analysis of p27 in U2OS and MG63 cells transfected with miR-802 precursor or negative control (miR-ctrl). (E-F) Western blotting analysis of p27 in U2OS and MG63 cells transfected with miR-802 antisense or negative control (Ctrl)

Discussion

Here, we demonstrated that miR-802 expression is upregulated in osteosarcoma tissues. Besides, for the first time, we identified that miR-802 regulated p27 expression through targeting its 3'UTR. Collectively, these findings suggest that down-regulation of miR-802 may promote the initiation and progression of osteosarcoma.

It has been reported that several miRNAs were mis-regulated in osteosarcoma tissues or cells, such as miR-34, miR-21, miR-140, miR-223 and miR-195 (He et al., 2009; Song et al., 2009; Wu et al., 2011; Mao et al., 2012; Li et al., 2012; Zhao et al., 2013). For instance, expression of miR-34s was decreased in tumor samples, and MiR-34 genes underwent minimal deletions and epigenetic inactivation in osteosarcomas (He et al., 2009). Besides, miR-34s affect the expression of its target genes partially in a p53-dependent manner (He et al., 2009). Besides, miR-21 was found to be significantly overexpressed in osteosarcoma tissues (Wu et al., 2011). Knockdown of miR-21 greatly decreased cell invasion and migration of MG-63. At the molecular level, RECK (reversion-inducing-cysteine-rich protein with kazal motifs), a tumor suppressor gene, was a direct target of miR-21 (Wu et al., 2011). Moreover, microRNA-195 was shown to suppress osteosarcoma cell invasion and migration in vitro by targeting fatty acid synthase (FASN) (Mao et al, 2012). Therefore, miRNAs expression has a key role in regulating cellular processes in osteosarcoma, which need to further investigated in the future.

In the present study, our results demonstrated that miR-802 enhanced osteosarcoma cell proliferation through negatively regulation of p27. Numerous studies

have identified p27 as a key tumor suppressor in multiple tumors (Micel et al., 2013). In animal models, loss of p27 is associated with infrequent spontaneous pituitary tumors and intestinal adenomas (Damo et al., 2005). Importantly, decreases in p27 protein expression have been found in 60% of human carcinomas, and are associated in breast cancer with poor prognosis (Zhang et al., 2013). In osteosarcoma, p27 expression was also associated with poor differentiation and prognosis (Thomas et al., 2013), suggesting that p27 could be a promising therapeutic target in human cancers, including osteosarcoma.

In conclusion, the key finding of the current study is that miR-802 can promote the proliferation of osteosarcoma cell lines by targeting p27. This data indicates that miR-802 plays an essential role in the regulation of osteosarcoma cell proliferation and may function as an onco-microRNA. Understanding the precise role played by miR-802 progression will not only advance our knowledge of osteosarcoma biology, but also will help determine if miR-802 has potential as a novel therapeutic target for the treatment of osteosarcoma.

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