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miR-17 inhibitor suppressed osteosarcoma tumor growth and metastasis via increasing PTEN expression

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ABSTRACT

MicroRNAs (miRNAs) play essential roles in cancer development and progression. Here, we investigated the role of miR-17 in the progression and metastasis of osteosarcoma (OS). miR-17 was frequently increased in OS tissues and cell lines. Inhibition of miR-17 in OS cell lines substantially suppressed cell proliferation, migration, and invasion. Phosphatase and tensin homolog (PTEN) was identified as a target of miR-17, and ectopic expression of miR-17 inhibited PTEN by direct binding to its 3′-untranslated region (3′-UTR). Expression of miR-17 was negatively correlated with PTEN in OS tissues. Together, these findings indicate that miR-17 acts as an oncogenic miRNA and may contribute to the progression and metastasis of OS, suggesting miR-17 as a potential novel diagnostic and therapeutic target of OS.

1. Introduction

Osteosarcoma (OS) is the most primary bone tumor in children and young adults [1]. It occurs predominantly around regions with active bone growth and repairation. Emerging evidence suggests OS was caused by genetic and epigenetic changes which interrupt osteoblast differentiation from mesenchymal stem cells [2]. Advances in OS therapy over the past decade have enhanced patient outcomes [3], and the 5-year survival rate of OS patients has been dramatically improved. However, outcome remains poor and most of them died of pulmonary metastases eventually [4]. Therefore, identification of the effector molecules or signal pathways responsible for regulating tumor growth and metastasis is critical for improving the OS treatment.

MicroRNAs (miRNAs) are a class of endogenous, noncoding, small RNA, approximately 22 nucleotides in length [5]. miRNAs play an essential role in the regulation of gene expression post-transcriptionally. miRNAs are frequently aberrantly altered in diverse cancers, including breast cancer [6], colon cancer [7], glioblastoma [8], lung cancer [9], and OS [10]. miRNAs function as either tumor suppressors or oncogenes depending on the role of their target genes. miR-17 belongs to the miR-17–92 cluster family and has been reported to be increased in many cancers [11–13], including OS [14]. miR-17 acts as oncogenic miRNA in some cancers [15–17], however, its role in OS progression and metastasis remains unclear.

In this report, we demonstrated that miR-17 inhibitor suppressed cell proliferation, migration, and invasion in both human osteosarcoma cell lines. Moreover, we showed that miR-17 negatively regulated PTEN by binding to the 3′-UTR of PTEN leading to inhibition of PTEN expression and activation of Akt pathway. Finally, we observed that expression of miR-17 was negatively correlated with PTEN in OS tissues. We supposed that miR-17 might be a promising therapeutic target in OS.

2. Materials and methods

2.1. OS tissues, cell lines and transfection

28 paired OS and matched normal non-tumor tissues were obtained from our Department. All the tissues were immediately stored in liquid nitrogen until use. This study was approved by the Ethics Committee of Union Hospital.

Human osteosarcoma cell lines U2OS, Saos-2, and MG-63 were cultured in DMEM, RPMI 1640, and DMEM mediums respectively, supplemented with 10% FBS, 100 mg/ml streptomycin, and 100 IU/ml penicillin at 37 °C with 5% CO_2. The human osteoblast cell line hFOB 1.19 was maintained in DMEM/F12 medium supplemented with 10% FBS, 100 mg/ml streptomycin, and 100 IU/ml penicillin at 37 °C with 5% CO_2. Transfection was performed when cells were grown to 80% confluence, using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

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2.2. RNA isolation and quantitative real-time PCR

Total RNA and miR were isolated using RNeasy Mini and miRNeasy Mini Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The expression of miR-17 was determined by quantitative real-time PCR (qRT-PCR) using TaqMan MicroRNA Assay Kits (ABI, Foster City, CA, USA) on a LightCycler 480 System II (Roche). PTEN primer: forward 5′-CCAGGACCAGAGGAAACCT-3′, reverse 5′-GCTAGCCTCTGGATTTGA-3′. GAPDH primer: forward 5′-ATGTCGTGGAGTCTACTGGC-3′, reverse 5′-TGACCTTGCCCACAGCCTTG-3′. The expression of PTEN was determined using SYGR green real time PCR (TAKARA, Tokyo Japan). The qRT-PCR data were normalized using the $2^{-\Delta\Delta CT}$ method relative to GAPDH or U6.

2.3. Plasmid construction

miR-17 and control mimics/inhibitors were obtained from Ribobio (Guangzhou, Guangdong, China). For luciferase reporter, the following primer was used: forward 5′-CGAGCTCGGACGAACTGGTGTAATG-3′, reverse 5′-CGACGCGTGTCCAGAGTCCAGCATAA-3′. The PCR fragment was inserted into pMir-Report vector (Ambion) within SacI and MluI restriction sites. Mutation was performed with a fast mutation kit (NEB, Ipswich, Canada).

2.4. MTT assay

1×10^4 transfected MG-63 cells were seeded into 96-well plate for 24 h. MTT (5 mg/mL) was added to each well. After 3 h, the cells were re-suspended in 200 μL of DMSO and shaken for 15 min. The absorbance value at 490 nm was recorded using a microplate reader (Perkin Elmer).

2.5. Colony formation assay

500 transfected MG-63 cells were seeded into 6-well plate and maintained in DMEM containing 10% FBS for 14 days. Then, cells were fixed and stained with methanol for 20 min, followed by 0.5% crystal violet for 15 min. Visible colonies were quantified using inverted microscope.

2.6. In vitro migration and invasion assay

Migration and invasion assays were performed using transwell chambers. For migration assay, 5×10^4 cells were seeded into the upper chamber of transwells (BD Bioscience, USA). For invasion assay, 1×10^5 cells were added into the upper chamber precoated with matrigel (BD Bioscience, USA). In both assays, cells were maintained in medium without serum in the upper chamber, and medium containing 10% FBS was added to the lower chamber as chemoattractant. After 24 h incubation, cells that did not migrate or invade through the membrane were wiped out. Then the membranes were fixed and stained with 0.5% crystal violet. Four random fields were counted per chamber using an inverted microscope (Olympus, Japan), and each experiment was repeated three times.

2.7. Luciferase activity assay

Luciferase activity assay was performed as previously described [18]. Briefly, HEK293 cells were cultured in 12-well tissue culture dishes (1×10^5 cells/well), and co-transfected with wild type or mutated 3′-UTRs of PTEN (WT and Mut respectively) luciferase reporter constructs and miR-17 or control mimic with Lipofectamine 2000. 24 h later, cell was harvested and luciferase activity was examined by Dual-Luciferase Reporter Assay Kit (Promega, Wisconsin, WI, USA).

2.8. Western blot

Proteins were extracted by SDS lysis buffer (Beyotime, Shanghai, China) according to the instructions. Protein concentration was quantified by the BCA protein assay kit (Santa Cruz, USA). Equal amounts of protein were separated by SDS–PAGE, transferred to onto PVDF membranes and blocked for 0.5 h at room temperature. Membranes were incubated with primary antibodies at 4 °C overnight, and further incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Blots were detected using an ECL detection system.

2.9. Statistical analysis

Data are presented as mean ± SD, analyzed by using SPSS 16.0. ANOVA or two-tail Student's *t* test was used to examine the statistical significance of differences, and $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. miR-17 was increased in OS tissues and cell lines

Expression of miR-17 in 28 OS tissues and matched normal tissues was determined by qRT-PCR. miR-17 was significantly increased in OS tissues compared with matched normal tissues (Fig. 1A). Moreover, expression of miR-17 in three OS cell lines,

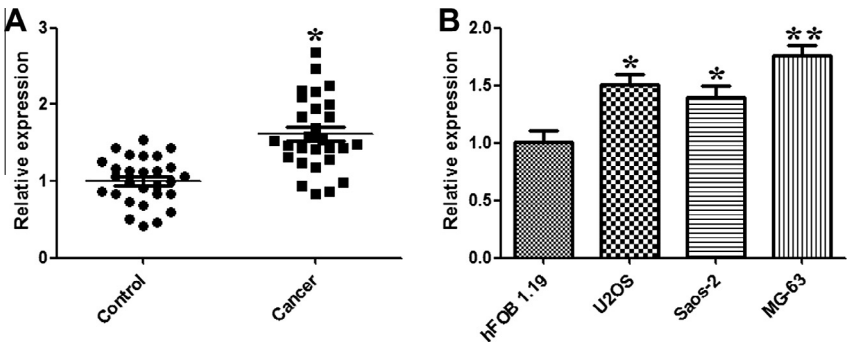

Fig. 1. miR-17 was increased in OS tissues and cell lines. (A) miR-17 was significantly increased in OS tissues compared with that in the corresponding normal tissues (NC). (B) miR-17 was significantly increased in three OS cell lines, U2OS, Saos-2, and MG-63 compared with that in human osteoblast cell line hFOB 1.19 cells. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

U2OS, Saos-2, and MG-63 was remarkably increased compared with that in human osteoblast cell line hFOB 1.19 cells (Fig. 1B).

3.2. Inhibition of miR-17 suppressed OS cell proliferation and motility

To examine the role of miR-17 in OS cell growth, MG-63 cells were transfected with miR-17 inhibitor (anti-miR-17) or control inhibitor (anti-miR-NC). Transfection of anti-miR-17 significantly inhibited the growth of MG-63 cells (Fig. 2A). Moreover, transfection of anti-miR-17 also remarkably suppressed colony formation of MG-63 (Fig. 2B). The effect of anti-miR-17 was validated by qRT-PCR (Fig. 2C).

To study the effect of miR-17 on the motility of OS cells, *in vitro* migration and invasion assays were performed. Transfection of anti-miR-17 significantly suppressed the *in vitro* migration and invasion abilities of MG-63 cells (Fig. 2D and E).

3.3. PTEN was a target of miR-17

TargetScan 6.2 was used to search the potential target gene of miR-17. PTEN was predicted to be a target of miR-17 (Fig. 3A). Luciferase activity assay showed that miR-17 significantly suppressed the WT 3′-UTR but not that of Mut 3′-UTR of PTEN luciferase activity in HEK293 cells (Fig. 3B). In addition, Overexpression of miR-17 significantly suppressed PTEN mRNA and protein levels, while inhibition of miR-17 showed opposite effects (Fig. 3C and D).

3.4. miR-17 was negatively correlated with PTEN in OS tissues

Expression of PTEN mRNA in 28 OS and the corresponding normal tissues was measured. Results showed that PTEN mRNA was significantly decreased in OS tissues compared with the corresponding normal tissues (Fig. 4A). Moreover, PTEN was inversely correlated with miR-17 level in OS tissues (Fig. 4B).

4. Discussion

Emerging studies have revealed that miRNAs participate in the progression of various cancers including OS via regulation of expression of multiple target genes involved in the progression and metastasis. Hence, identification of specific miRNAs and their targets involved in tumorigenesis would provide valuable insight for the diagnosis and therapy of patients with human malignancies. Our study indicated that miR-17 was elevated in OS tissues than in matched normal tissues. We also showed that miR-17 inhibitor suppressed proliferation, colony formation, migration, and invasion in MG63 cells. Taken together, our results suggest that miR-17 acts as an oncogene plays a role in the progression and metastasis of OS. However, its roles *in vivo* await further studies.

Next, our results revealed that PTEN is a direct target of miR-17 in OS cells, consistent with Li et al., PTEN was a direct target of miR-17 in glioblastoma cells [18]. PTEN is a well-established tumor suppressor, located on human chromosome region 10q23, and linked to cancer development and progression [19]. PTEN dephosphorylates Phosphatidylinositol 3,4,5-trisphosphate (PIP3), and antagonizes phosphatidylinositol 3-kinase (PI3K) signalling [20]. The PTEN/PI3K/Akt signalling pathway is highly involved in tumorigenesis and metastasis [21]. PTEN inhibits tumor cell growth and motility by blocking the PI3K/Akt pathway [22]. PTEN is decreased in some malignant cancers, resulting in Akt hyperactivation and promotion of cell proliferation, migration, invasion and angiogenesis [23]. Our results showed that miR-17 inhibitor suppressed tumor growth and metastasis, and miR-17 elevation was correlated with PTEN down-regulation in OS. These results indicate that the

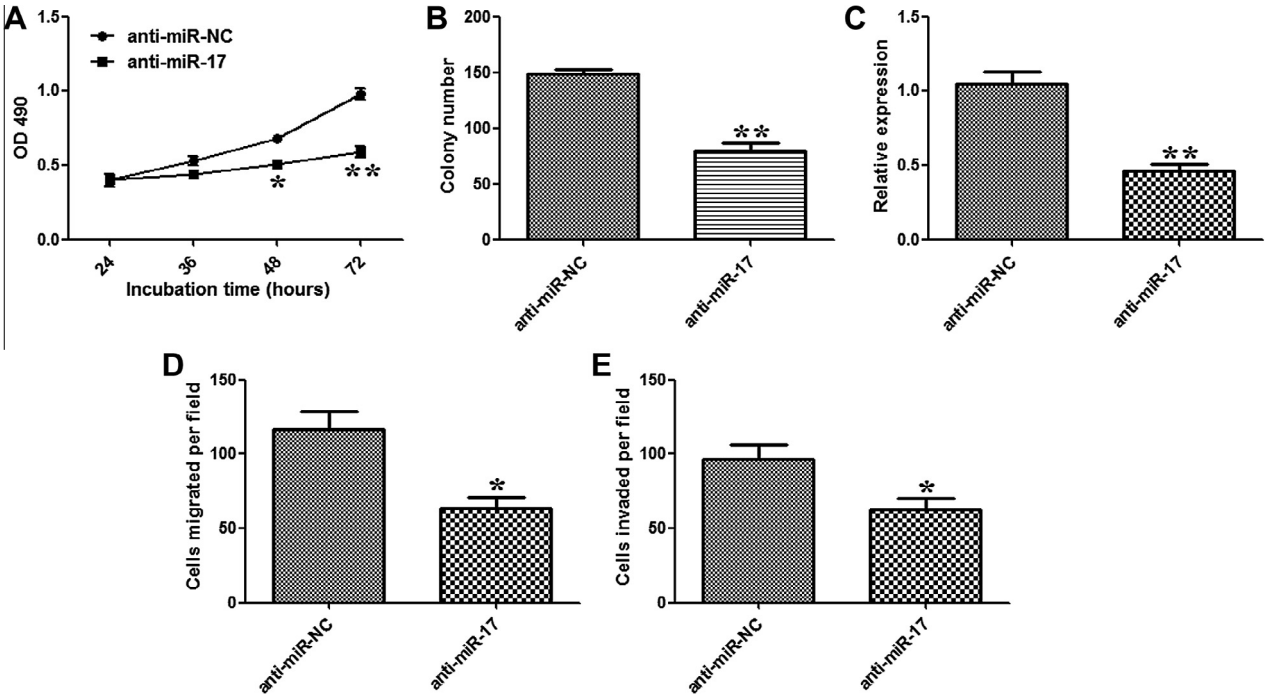

Fig. 2. Inhibition of miR-17 suppressed OS cell proliferation and motility. (A) MTT assay was performed to examine MG-63 cell proliferation. Transfection of anti-miR-17 significantly inhibited MG-63 cell proliferation. (B) Transfection of anti-miR-17 significantly inhibited MG-63 cells colony formation. (C) The expression of miR-17 was significantly decreased after anti-miR-17 transfection. (D) *In vitro* migration assay of MG-63 cells transfected with anti-miR-17 or anti-miR-NC. Transfection of anti-miR-17 significantly suppressed MG-63 cell migration. (E) *In vitro* invasion assay of MG-63 cells transfected with anti-miR-17 or anti-miR-NC. Transfection of anti-miR-17 significantly suppressed MG-63 cell invasion. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

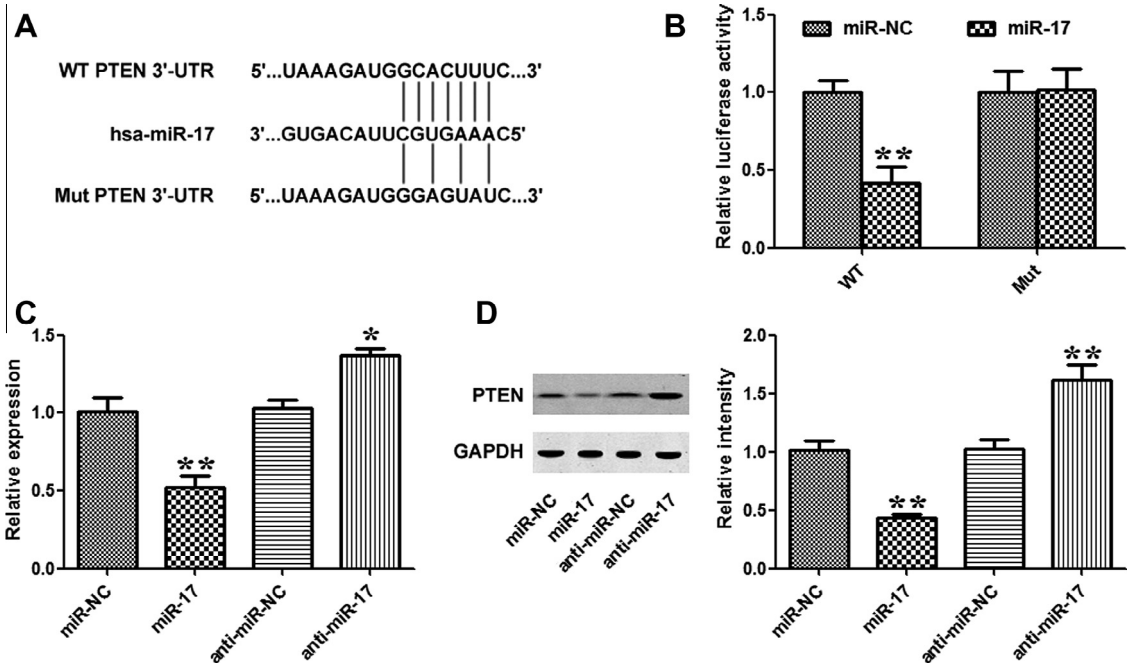

Fig. 3. PTEN was a target of miR-17. (A) Computational analysis showed that miR-17 potentially targeted PTEN. (B) HEK293 cells were co-transfected with miR-17 and WT or Mut 3′-UTR luciferase reporter construct. (C) Expression of PTEN mRNA was detected by qRT-PCR in MG-63 cells transfected with miR-17/miR-NC, or anti-miR-17/anti-miR-NC. (D) Protein level was detected by Western blot in MG-63 cells transfected with miR-17/miR-NC, or anti-miR-17/anti-miR-NC. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

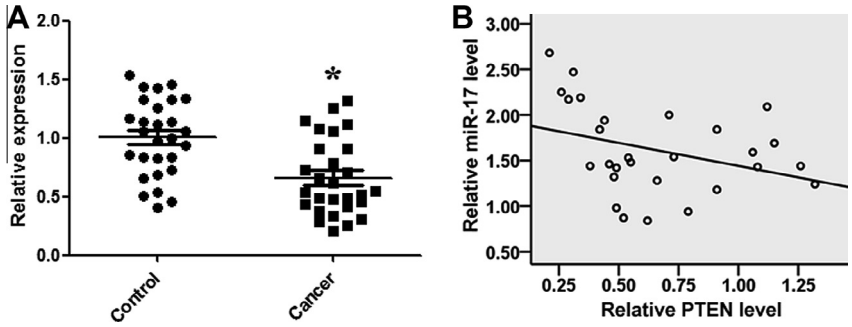

Fig. 4. miR-17 was negatively correlated with PTEN in OS tissues. (A) PTEN mRNA level was examined by qRT-PCR and, it was remarkably decreased in OS tissues. (B) PTEN mRNA level was inversely correlated with miR-17 level in OS tissues (Spearman's correlation analysis, $r = -0.417$; $P = 0.027$). $^{*}P < 0.05$ compared with the control group.

up-regulation of miR-17 in OS may induce cell growth, migration and invasion partially through inhibiting PTEN expression.

5. Conclusion

Collectively, we newly described the miR-17/PTEN link and provided a mechanism for PTEN dysregulation and contribution to OS cell growth, migration, and invasion. These results suggest that miR-17 may act as an oncogene in OS and represent a potential molecular target for OS therapy.

Conflict of interest

We declare that we have no conflict of interest.

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