ORIGINAL ARTICLE

MicroRNA-17-5p regulates the growth, migration and invasion of the human osteosarcoma cells by modulating the expression of PTEN

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Summary

Purpose: Osteosarcoma causes extensive human mortality and there is urgent need to develop novel therapies or to identify efficient therapeutic targets for its management. Herein the role and therapeutic potential of miR-17 was explored in osteosarcoma.

Methods: The normal hFOB.19 cell line and the osteosarcoma cell lines SAOS-2, HOS, 143B, T1-73 and mG63 were used in the present study. The expression analysis of miR-17 was carried out by quantitative Real-Time polymerase chain reaction (qRT-PCR). Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used for transfection. WST-1 assay was used for determination of cell proliferation and *autophagy was detected by transmission electron microscopy* (TEM). Wound healing and transwell assays were used for the determination of cell migration and invasion. Protein expression was determined by western blot analysis.

Results: The expression of miR-17 was significantly elevated in all the osteosarcoma cells. Suppression of miR-17 resulted in decrease of the viability and colony formation of

the SAOS-2 osteosarcoma cells. The inhibition of SAOS-2 cell proliferation upon miR-17 suppression was found to be due to induction of autophagy which was accompanied with enhancement in the expression of LC3B II and Beclin-1. Suppression of miR-17 was also accompanied by inhibition of the SAOS-2 cell migration and invasion. The in silico analysis showed that miR-187 targets PTEN in the SAOS-2 cells. The expression of PTEN was found to be downregulated in all the osteosarcoma cells and suppression of miR-17 expression caused enhancement in the expression of PTEN. Overexpression of miR-17 caused inhibition of the proliferation and colony formation of the SAOS-2 cells. Additionally, silencing of miR-17 could abolish the effects of miR-17 inhibition in the SAOS-2 cells.

Conclusion: MiR-17 may be proven a therapeutic target in the management of osteosarcoma.

Key words: osteosarcoma, microRNA, PTEN, proliferation, invasion

Introduction

nucleotides non-coding RNAs produced from the endogenous hairpin transcripts. In humans miRs are highly conserved and exhibit multiple isoforms [1]. They negatively control the expression of human genes and it has been reported that around 60% of the human protein-coding genes harbour miR-binding sites [2]. Since miRs control vital The miR-17-5p has been reported to be involved

MicroRNAs (miRs) are single stranded 19-25 cellular processes in humans, it is not surprising that control the development of diseases such as cancer [3]. The dysregulation of several miRs may contribute to the development of cancer was for the first time discovered in 2002 when deletion of miR-15 and miR-16 were reported to be involved in the development of lymphocytic leukemia [4].

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in the development of several types of cancers, for example miR-17-5p has been shown to induce the growth of nasopharyngeal carcinoma [5]. It has also been reported to be involved in the development of pancreatic cancer [6]. Nonetheless, the role of miR-17-5p (now onwards referred as miR-17) has not been thoroughly explored in osteosarcoma. Osteosarcomas are primary malignant bone tumors that are characterized by osteoid or immature bone development by the malignant cells [7]. The osteosarcoma is considered as one of the rare cancers accounting for around 1% of all the cancers detected in United States. Among all age groups, the osteosarcomas are more prevalent in children and adolescents and in United States, since around 55% of all the osteosarcomas occur in children and adolescents [8]. Osteosarcomas are considered fatal types of cancers owing to their ability to develop metastasis. It has been reported that around 80% of osteosarcomas show metastatic spread and hence there is urgent need for early detection, identification of efficient therapeutic targets and effective chemotherapy for their treatment [9]. This study was undertaken to investigate the role and therapeutic implications of miR-17 in osteosarcoma.

Methods

Cell lines and culture conditions

The normal hFOB.19 cell line and the osteosarcoma cell lines SAOS-2, HOS, 143B, T1-73 and MG63 were purchased from American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 2 mM glutamine. The cells were cultured in an incubator (Thermo Fisher Scientific, Inc.) at 37°C with 98% humidity and 5% CO₂. All transfections were carried out by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as per the manufacturer's protocol.

qRT-PCR analysis

The total RNA from the normal and the osteosarcoma cell lines was isolated by TRIzol Reagent (Invitrogen) following the manufacturer's instruction. The cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and amplified with Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen) using the CFX96 sequence detection system (Bio-Rad, Hercules, CA, USA). The expression was estimated by $2^{-\Delta\Delta Ct}$ method and actin was used as an internal control.

Cell transfection

The miR-17 inhibitor and miR-negative control (NC) were synthesized by RiboBio (Guangzhou, China). Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was then used for the transfection of the constructs into the SAOS-2 cells as per the manufacturer's instruc-tions. As the SAOS-2 cells reached 80% confluence, the appropriate concentrations of miR-17 inhibitor or NC were transfected into these cells.

Cell proliferation and colony formation assay

The viability of SAOS-2 cells was determined by WST-1 assay. In brief, SAOS-2 cells were cultured in 96well plates at the density of 2×10⁵ cells/well. The cells were then transfected with miR-NC or miR-17 inhibitor and again incubated for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for 4 h. The absorbance was then measured at 450 nm using a victor 3 microplate reader to determine the proliferation rate at 0, 12, 24, 48 and 96 h time intervals. Colony formation assay of the miR-17 overexpressing SAOS-2 cells was performed as described previously [10].

Detection of autophagy

Autophagy in miR-17 inhibitor and miR-NC transfected SAOS-2 cancer cells was demonstrated by transmission electron microscopy (TEM). In brief, the SAOS-2 cells were transfected with miR-NC or miR-17 inhibitor for 24 h. The cells were collected by trypsinization and subjected to washing with phosphate buffered saline (PBS) which was followed by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by treatment of the cells with ethanol and embedding in resin. Thin sections were then cut with an ultramicrotome and subjected to TEM.

Cell invasion assay

The effects of miR-17 suppression on the invasion ability of SAOS-2 cells was determined by transwell chambers with Matrigel. The SAOS-2 cells were transfected with miR-17 inhibitor and around 200 ml of cell culture were placed onto the upper chamber and only medium was placed in the bottom chamber. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chambers were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200× magnification.

Wound healing assay

The transfected SAOS-2 cells were cultured till 80% confluence. This was followed by removal of the Dulbecco's modified Eagle's medium (DMEM) and subsequent washing with PBS. Afterwards, a wound was scratched with a sterile pipette tip and a picture was taken. The plates were then incubated for about 24 h at 37°C and then a picture was taken again under an inverted microscope

Target identification

The miR-17 target was identified by TargetScan online software (http://www.targetscan.org).

Western blotting

The normal and the osteosarcoma cell lines were cultured at 37°C for 24h and then centrifuged at 12000 rpm. The cell pellet was washed with PBS and then suspended again in RIPA lysis buffer. Thereafter the concentrations of the proteins were determined and equal con-



Figure 1. qRT-PCR analysis showing the expression of miR-17 in normal hFO8b1.19 and osteosarcoma cell lines. The experiments were performed in triplicate and expressed as mean \pm SD (*p<0.05).

centrations of the proteins were loaded on SDS-PAGE gel (15%). The samples were transferred to polyvinylidene fluoride membranes and blocking was done using 5% skimmed milk powder. This was followed by membrane incubation with primary antibodies at 4°C for 24 h. Next, the membranes were incubated with horseradish peroxidase-linked secondary biotinylated antibodies for 2 h. The immunoreactive bands were observed by ECL-PLUS/Kit as per the manufacturer's guidelines.

Statistics

The experiments were repeated thrice and the values shown represent the mean of triplicates±SD. Student's t-test was used for statistical analysis. P<0.05 was taken as statistically significant difference.

Results

miR-17 is upregulated in osteosarcoma cells

The expression of profile of miR-17 in osteosarcoma cell lines and the normal bone cells was determined by qRT-PCR. The results showed that miR-17 was significantly elevated in the osteosarcoma cells relative to its expression in normal bone cells (Figure 1A). The expression of miR-17 was



Figure 2. A: Expression of miR-17in miR-NC and miR-17 inhibitor transfected SAOS-2 cells. **B:** Cell viability of miR-17-in miR-NC and miR-117 inhibitor transfected SAOS-2 cells. **C:** Colony formation assay of in miR-NC and miR-17 inhibitor transfected SAOS-2 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

observed to be up to 4.2-fold upregulated in the osteosarcoma cells. Additionally, the expression of miR-17 was found to be highly upregulated in the SAOS-2 cells.

Suppression of miR-17 suppresses the proliferation of SAOS-2 cells

To elucidate the role of miR-17 suppression on the proliferation of the osteosarcoma SAOS-2 cells, the cells were transfected with miR-17 inhibitor. The suppression of miR-17 in SAOS-2 cells was validated by qRT-PCR which showed around 8-fold decrease in the miR-17 expression (Figure



Figure 3. A: Electron microscopic analysis of miR-NC and miR-17 inhibitor transfected SAOS-2 cells showing the induction of autophagy. **B:** expression of LC3 B I, II, and Belcin-1 in miR-NC and miR-17 inhibitor transfected SAOS-2 cells. The results show that miR-17 inhibition induces autophagy in osteosarcoma cells. The experiments were performed in triplicate.



Figure 4. Wound healing assay showing the inhibition of cell migration in miR-17 inhibitor transfected SAOS-2 cells. The experiments were performed in triplicate.

1B). Next, the proliferation rate of miR-17 inhibitor transfected SAOS-2 cells was monitored at different time periods. The results showed that miR-17 suppression resulted in significant decrease in the proliferation rate of the SAOS-2 osteosarcoma cells (Figure 1C). The effects of miR-17 suppression were also examined on the colony formation potential of the SAOS-2 cells and it was found that miR-17 suppression inhibited the colony formation of the miR-17 cells by 64% (Figure 1D).

miR-17 induces autophagy in the SASO-2 cells

To assess the underlying mechanisms for the inhibition of proliferation induced upon the suppression of miR-17 in the SAOS-2 cells, TEM analysis was performed. The results showed that miR-17 suppression led to development of autophagosomes in the SAOS-2 cells, suggestive of autophagy (Figure 2). The induction of autophagy upon miR-17 suppression was further confirmed by examining the expression of apoptosis marker proteins. The results showed that the expression of LC3B-II and Beclin-1 increased while that of LC3B-I remained almost unaltered in SAOS-2 cells transfected with miR-17 inhibitor (Figure 3).

Suppression of miR-17 inhibits the migration and invasion of SAOS-2 cells

The impact of miR-17 suppression on the migration and invasion of the SAOS-2 cells was determined by wound healing and transwell assay. The results showed that miR-17 caused significant



Figure 5. Transwell assay showing inhibition of cell invasion in miR-17 inhibitor transfected SAOS-2 cells. The experiments were performed in triplicate and expressed as mean \pm SD (*p<0.05).

decrease in the migration of the SAOS-2 cells as depicted by the wound width (Figure 4). The results of the transwell assay showed that invasion of the SAOS-2 cells was inhibited by 55% upon miR-17 suppression (Figure 5). miR-17 targets PTEN in osteosarcoma cells

The target of miR-17 in SAOS-2 cells was identified to be PTEN by TargetScan analysis (Figure 6A). The qRT-PCR analysis revealed that the re-



Figure 6. A: TargetScan analysis showing PTEN as the target of miR-17-5p. **B:** Expression of PTEN in osteosarcoma cells and **C:** Expression of PTEN in miR-NC and miR-17 inhibitor transfected SAOS-2 cells. These results show that miR-17 exerts its effects by modulating the expression of PTEN. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).



Figure 7. A: Expression of PTEN in NC and pcDNA-PTEN transfected SAOS-2 cells. **B:** WST-1 assay showing the viability of the NC and pcDNA-PTEN transfected SAOS-2 cells. **C:** Colony formation assay of the NC and pcDNA-PTEN transfected SAOS-2 cells. **D:** WST-1 assay of miR-NC, miR-17 inhibitor and miR-17 inhibitor ± Si-PTEN transfected SAOS-2 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

sults of PTEN were significantly overexpressed in the all the osteosarcoma cells as compared to the normal bone cells (Figure 6B). However, suppression of miR-17 resulted in overexpression of PTEN in SAOS-2 cells (Figure 6C), confirming PTEN as the target of miR-17. Overexpression of PTEN in SAOS-2 cells caused inhibition of their proliferation and colony formation (Figure 7A and B). However, it was found that suppression of PTEN in SAOS-2 cells nullified the growth inhibitory effects of miR-17 suppression on SAOS-2 cell proliferation (Figure 7C).

Discussion

Osteosarcoma is prevalent in children and adolescents and causes significant mortality in humans [11]. The high metastasis rate, late diagnosis and the lack of therapeutic targets make the treatment of osteosarcoma ineffective [12]. The diverse roles that miRs play in human by controlling the expression of human genes suggests that miRs may prove useful therapeutic targets for treating human diseases including cancer [13]. In this study, we investigated the role of miR-17 in osteosarcoma and found the expression of miR-17 is significantly upregulated in osteosarcoma cells. Previously carried out studies have also shown that the expression of miR-17 is dysregulated in cancer cells. For instance, the upregulation of miR-17 has been reported in human hepatocellular carcinoma [14]. To determine the role of miR-17, it was suppressed in SAOS-2 osteosarcoma cells and what was observed was that miR-17 suppression resulted in significant decline in the proliferation rate of the SAOS-2 cells. These results are in agreement with previous investigations wherein miR-17 suppression has been observed to inhibit the growth of breast cancer cells [15]. Similarly, miR-17 has also been reported to regulate the growth of pancreatic

and lung cancer cells [16,17]. TEM analysis showed that suppression of miR-17 resulted in the induction of autophagy in the SAOS-2 cells which was also accompanied by the upregulation of LC3B II and Beclin-1 expression. The effects of miR-17 were also examined on SAOS-2 cell migration and invasion and it was found that miR-17 suppressed the migration and invasion of SAOS-2 cells indicating the implications of miR-17 in the management of metastatic cancers. These studies are in agreement with previous studies wherein miR-17 has been reported to inhibit the migration and invasion of gastric cancer cells [18]. The miRs exert their effects by suppressing the expression of the target genes and each miR has several targets [19]. Herein bioinformatic analysis together with dual luciferase assay showed that miR-17 exerts its effects by targeting PTEN. Additionally, the expression of PTEN was considerably increased in all the osteosarcoma cells and suppression of miR-17 could inhibit the expression of PTEN. Moreover, the overexpression of PTEN could nullify the effects of the miR-17 suppression on the proliferation of the SAOS-2 osteosarcoma cells.

Conclusion

The findings of the present study revealed that miR-17 is upregulated in osteosarcoma cells. Suppression of miR-17 in SAOS-2 osteosarcoma cells inhibited their proliferation by inducing autophagy and apoptosis. Suppression of miR-17 also suppressed the migration and invasion and enhanced the chemosensitivity of SAOS-2 osteosarcoma cells, indicative of the therapeutic implications of miR-17 in osteosarcoma treatment.

Conflict of interests

The authors declare no conflict of interests.

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