JBUON 2020; 25(1): 472-478
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

MicroRNA-187 suppresses the proliferation migration and invasion of human osteosarcoma cells by targeting MAPK7

Min Liu[1,2], Lifen Wu[2], Chunyuan Cai[2], Liangle Liu[2], Youjia Xu[1]

[1]Department of Orthopaedics, the Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215101, China; [2]Department of Orthopaedics, Rui'an People's Hospital & the Third Affiliated Hospital of Wenzhou Medical University, Rui'an , Zhejiang 325200, China.

Summary

Purpose: *Osteosarcoma is one of the rare but fatal malignancies. The high metastatic rate, late diagnosis, emergence of drug resistance against drugs such as doxorubicin, and the lack of therapeutic targets obstructs the treatment of osteosarcoma. This study was undertaken to investigate the role and therapeutic potential of miR-187 in human osteosarcoma cells.*

Methods: *The WST-1 proliferation assay was used for investigation of cell viability. Transfections were carried out by Lipofectamine 2000 reagent. The qRT-PCR was used for expression analysis. DAPI, acridine orange (AO)/ethidium bromide (EB) and Annexin V/propidium iodide (PI) assay were used for apoptosis. Western blot analysis was used for the determination of protein expression.*

Results: *The expression of miR-187 was significantly downregulated in human osteosarcoma cells. Out of all osteosarcoma cell lines the SAOS-2 showed the lowest expression of miR-187 and therefore this cell line was selected for further studies. Overexpression of miR-187 caused significant inhibition in the proliferation of SAOS-2 osteosarcoma cells. The miR-187-triggered growth inhibition was found to be mainly due to induction of G2/M phase cell cycle arrest of the SAOS-2 cells. The G2/M cell cycle arrest was also accompanied by depletion of Cyclin-B1 expression. Additionally, miR-187 enhanced the chemosensitivity of the osteosarcoma cells to doxorubicin. The wound healing and transwell assay showed that miR-187 overexpression resulted in the suppression of migration and invasion of the SAOS-2 osteosarcoma cells. In silico analysis showed that miR-187 exerts its effects by inhibiting mitogen activated protein kinase 7 (MAPK7). The expression of MAPK7 was found to be significantly upregulated in osteosarcoma cells and overexpression of MAPK7 could nullify the effects of miR-187 on the proliferation of the osteosarcoma cells.*

Conclusion: *Taken together, miR-187 may bear therapeutic implications in the treatment of osteosarcoma.*

Key words: *osteosarcoma, microRNA, cell cycle arrest, MAPK7, migration, invasion*

Introduction

Osteosarcomas are primary malignant tumors of bone that are characterized by osteoid or immature bone development [1]. Osteosarcoma is considered as one of the rare cancers accounting for around 1% of all the malignancies detected in United States. Among all age groups, osteosarcomas are more prevalent in children and adolescents and in United States, around 55% of all the osteosarcomas occur in children and adolescents [2]. Despite being rare, osteosarcomas are considered fatal malignancies owing to their capacity to develop metastasis. It has been reported that around 80% of osteosarcomas create metastasis and hence there is urgent need for early detection, identification of efficient therapeutic targets and effective chemotherapy for the treatment this disease [3]. Over the last few decades, research endeavours have been directed to explore the roles of microRNAs (miRs) in human cells. The miRs control the majority of the human genes and are thus involved in vital cellular

Corresponding author: Youjia Xu, MD. Department of Orthopaedics, the Second Affiliated Hospital of Soochow University, Sanxiang Rd no.1055, Gusu District, Suzhou, Jiangsu 215101, China.
Tel & Fax: +086 0512 68282030, Email: youjia25@yahoo.com
Received: 12/04/2019; Accepted: 02/05/2019

functions [4]. They have been found to regulate the expression of target genes via post transcriptional regulation [5]. The dysregulation of miR expression has been shown to be responsible for the development of deadly diseases such as cancer. Thus, miRs exhibit therapeutic implications in treating a wide range of human diseases [6]. The miR-187 has been shown to be downregulated in several types of cancers such as renal cell carcinoma [7]. This miR has been shown to target disabled homolog-2 to control the progression of ovarian cancer [8]. In colorectal cancer, miR-187 has been reported to play a role in growth and metastasis [9]. It has also been reported to suppress the metastasis of hepatocellular carcinoma by modulating the expression of S100A4 [10]. Also, it has been reported to inhibit the proliferation of non-small lung cancer cells by targeting CYP1B1 [11]. This study investigated the role and therapeutic potential of miR-187 in osteosarcoma.

Methods

Cell lines and culture conditions

The normal hFOB.19 cells and osteosarcoma cell lines (SAOS-2, HOS, 143B, T1-73 and MG63) were purchased from American Type Culture Collection (Manassas, VA, USA). Out of all osteosarcoma cell lines the SAOS-2 showed the lowest expression of miR-187 and, therefore, this cell line was selected for further studies. The cell lines were maintained in Dulbecco's modified Eagle's medium 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 a CO_2 incubator (Thermo Fisher Scientific, Inc.) at 37°C with 98% humidity and 5% CO_2. All transfections were carried out by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as per the manufacturer's protocol.

The qRT-PCR analysis

The total RNA from the normal and the osteosarcoma cell lines was isolated by TRIzol Reagent (Invitrogen) following the manufacturer's instructions. The complementary (c)DNA 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-187 mimics and negative control (NC) were synthesized by RiboBio (Guangzhou, China). Transfection was then carried out by the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instruc¬tions. As the SAOS-2 cells reached 80% confluence, the appropriate concentrations of miR-187 mimics or NC was transfected into these cells.

The WST-1 proliferation and colony assays

The proliferation rate of SAOS-2 cells was monitored by WST-1 assay. In brief, SAOS-2 cells were cultured in 96-well plates at a density of 2×10^5 cells/well. The cells were then transfected with miR-NC or miR-187 mimics 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-187 overexpressing SAOS-2 cells was performed as described previously [12].

Cell cycle analysis

The transfected SAOS-2 cells were cultured for 24 h at 37°C. The cells were then washed with phosphate buffered saline (PBS). Afterwards, the SAOS-2 cells were stained with PI and the distribution of the cells in different cell cycle phases was assessed by FACS flow cytometer.

Cell invasion assay

The effects of miR-187 overexpression on the invasion ability of SAOS-2 cells was determined by transwell assay with Matrigel. The SAOS-2 cells were transfected with miR-187 mimics and around 200 ml cell cultures were placed onto the upper chamber and empty in Dulbecco's modified Eagle's 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 Dulbecco's modified Eagle's medium 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.

Dual-luciferase reporter assay

The miR-187 target was identified by TargetScan online software (http://www.targetscan.org). The miR-187 mimics or NC were co-transfected with Plasmid pGL3-MAPK7´-UTR-WT or pGL3-MAPK7´-UTR-MUT into SAOS-2 cells. Dual-luciferase reporter assay (Promega) was carried out at 48 h after transfection. *Renilla* luciferase was used for normalization.

Western blotting

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

equal concentrations of the proteins were loaded on 10% SDS-PAGE. 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 biotinylated secondary antibodies for 2 h. The membranes were washed in phosphate-buffered saline (PBS) and the immunoreactive bands were observed by ECL-PLUS/Kit as per the manufacturer's guidelines.

Statistics

The experiments were performed in triplicate and the values represent the mean ± standard deviation (SD). P<0.05 was considered as significant difference. Student's t-test using Graph Pad prism 7 software was used for the statistical analyses.

Results

miR-187 is downregulated in osteosarcoma cells

For the determination of the miR-187 expression in osteosarcoma, the expression of miR-187 was examined in 5 different osteosarcoma cell lines as well as the normal cells by qRT-PCR. The results showed that miR-187 was significantly suppressed in the osteosarcoma cells relative to its expression in normal astrocytes (Figure 1A). The expression of miR-187 was 8-fold lower in the osteosarcoma cells. Additionally, the expression of miR-187 was found to be highly downregulated in the SAOS-2 cells.

miR-187 suppresses the proliferation and colony formation of SAOS-2 cells

To elucidate the role of miR-187 in the proliferation of the osteosarcoma SAOS-2 cells, the cells were transfected with miR-187 mimics. The overexpression of miR-187 in SAOS-2 cells was validated by qRT-PCR, which showed around 5-fold enhancement in the miR-187 expression (Figure 1B). Next, the proliferation rate of miR-187 overexpressing SAOS-2 cells was monitored at different time periods. The results showed that miR-187 overexpression resulted in significant decrease in the proliferation rate of the SAOS-2 cells (Figure

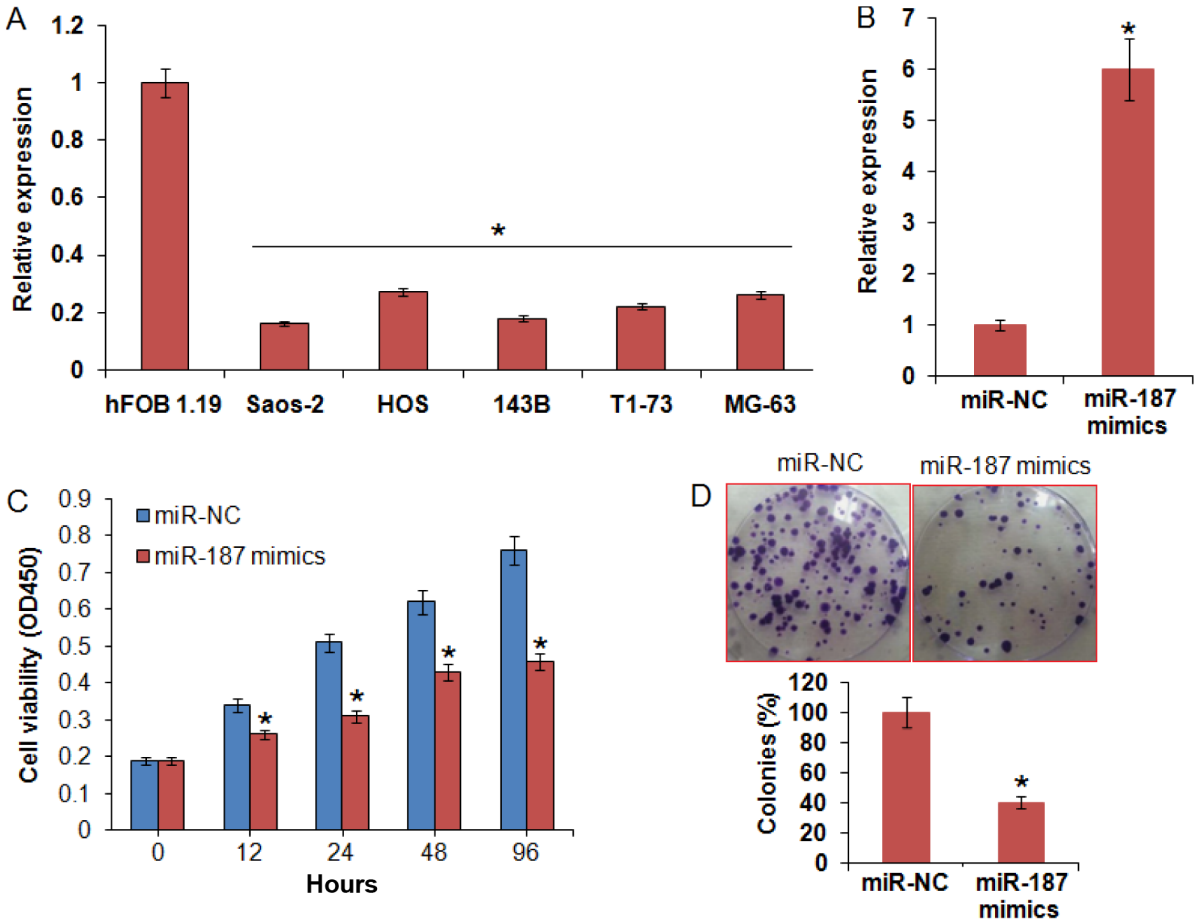

Figure 1. A: Expression of miR-187 in different osteosarcoma cell lines and hFOB1.19 cells. **B:** Expression of miR-187 in miR-NC and miR-87 mimics transfected SAOS-2 cells. **C:** Cell viability of miR-187 in miR-NC and miR-87 mimics transfected SAOS-2 cells. **D:** Colony formation of miR-187 in miR-NC and miR-87 mimics transfected SAOS-2 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

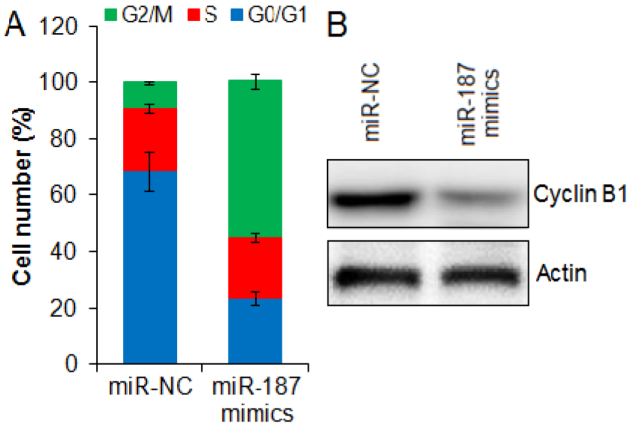

Figure 2. A: Cell cycle distribution of miR-NC and miR-187 mimics transfected SAOS-2 osteosarcoma cells as determined by flow cytometry **B:** Expression of cyclin B1 in miR-NC and miR-187 mimics transfected SAOS-2 osteosarcoma cells as determined by western blot analysis. The Figure shows that miR-187 induces G2/M cell cycle arrest and inhibits Cyclin B1 expression. The experiments were performed in triplicate and expressed as mean ± SD.

1C). The effects of miR-187 overexpression were also examined on the colony formation potential of the SAOS-2 cells and it was found that miR-187 overexpression inhibited the colony formation of the miR-187 cells by 62% (Figure 1D).

miR-187 induces G2/M arrest of the SAOS-2 cells

Assessment of the underlying mechanisms was performed for the inhibition of proliferation induced upon the overexpression of miR-187 in the SAOS-2 cells. The results showed that miR-187 caused remarkable increase in the percentage of the G2/M phase cells, suggestive of the G2/M phase cell cycle arrest. The percentage of the G2/M phase cells was 55% in miR-187 mimics transfected SAOS-2 cells as compared to the 9.2% in the miR-NC transfected cells (Figure 2A). The G2/M arrest induced by the overexpression of miR-187 cells was also accompanied by inhibition of the cyclin B1 expression (Figure 2B).

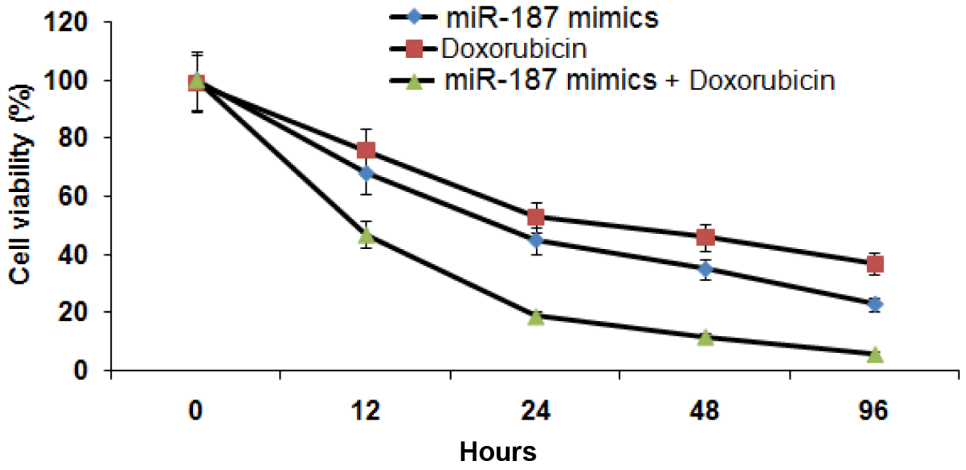

Figure 3. miR-187 overexpression enhances the doxorubicin resistance of the SAOS-2 osteosarcoma cells as depicted by WST-1 assay. The experiments were performed in triplicate and expressed as mean ± SD ($p<0.05$).

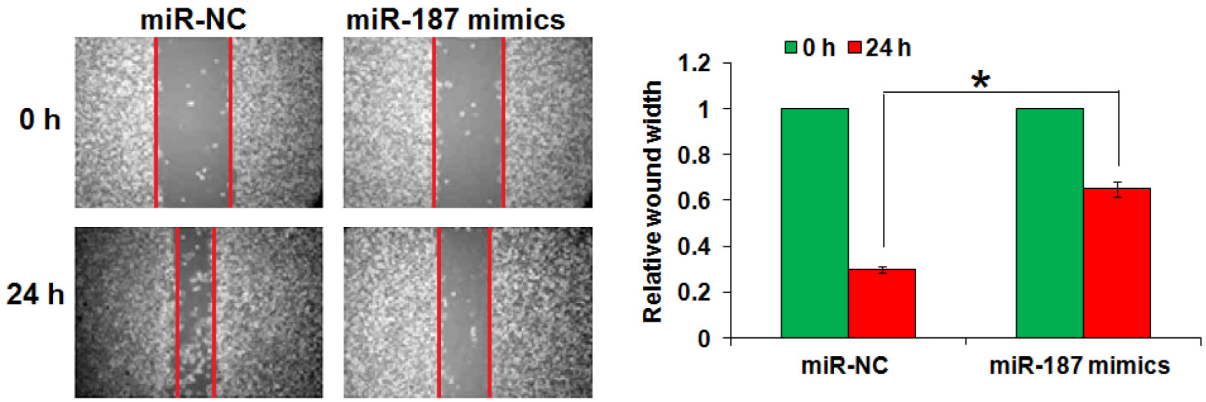

Figure 4. Wound healing assay showing cell migration in miR-NC and miR-187 mimics transfected SAOS-2 osteosarcoma cells as determined by Western blot analysis. The experiments were performed in triplicate and expressed as mean ± SD (*$p<0.05$).

miR-187 enhances the doxorubicin sensitivity of SAOS-2 cells

The effects of miR-187 were examined on the sensitivity of the osteosarcoma SAOS-2 cells to doxorubicin. The osteosarcoma SAOS-2 cells were transfected with miR-187 mimics or treated with 5 μM doxorubicin or transfected with miR-187 mimics plus treated with 25 μM doxorubicin and subjected to WST-1 assay. The results showed that effects of miR-187 mimics and doxorubicin treatment were more profound on the SAOS-2 cell proliferation than miR-187 mimics or doxorubicin individually (Figure 3), suggesting miR-187 overexpression enhances the drug sensitivity of osteosarcoma cells.

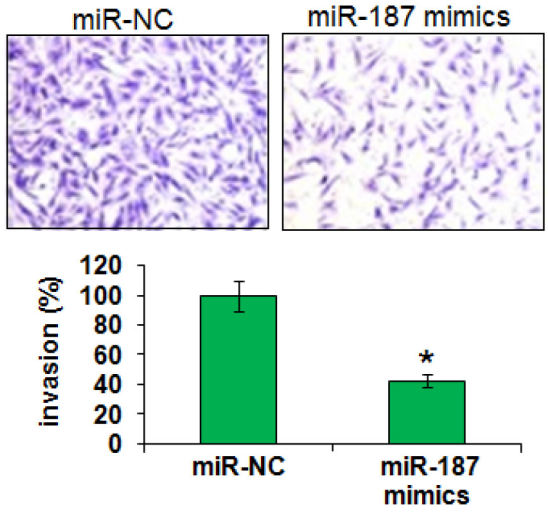

Figure 5. Transwell assay showing cell invasion in miR-NC and miR-187 mimics transfected SAOS-2 osteosarcoma cells as determined by western blot analysis. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

miR-187 suppresses the migration and invasion of osteosarcoma cells

The effects of miR-187 on metastasis of the SAOS-2 cells were determined by wound healing and transwell assay. The results showed that miR-187 caused significant 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 60% upon miR-187 overexpression (Figure 5).

miR-187 targets MAPK7 in osteosarcoma cells

The TargetScan revealed that MAPK7 acts as the target of miR-187 in osteosarcoma cells (Figure 6A). MAPK7 was further confirmed as the target of miR-187 by dual luciferase assay (Figure 6B). The western blotting results further revealed that the MAPK7 was significantly overexpressed in all the osteosarcoma cells as compared to the normal astrocytes (Figure 6C). Nonetheless, overexpression of miR-187 resulted in suppression of MAPK7

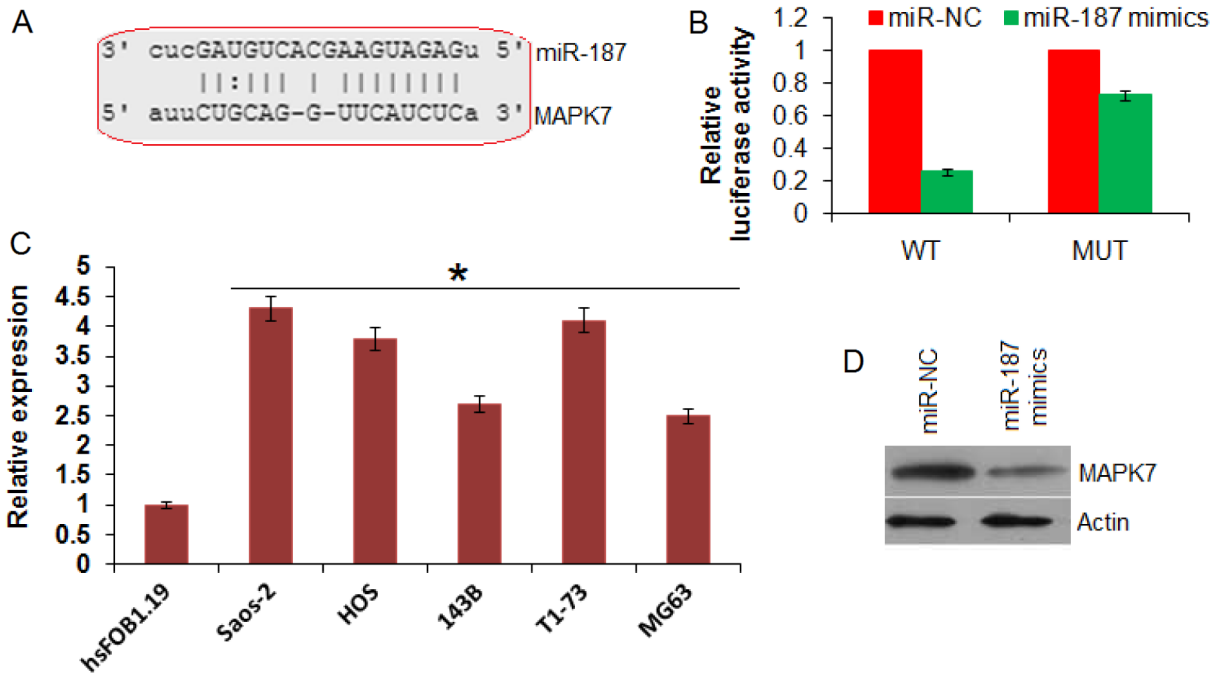

Figure 6. A: TargetScan analysis showing MAPK7 as the target of miR-187. **B:** Dual luciferase assay. **C:** Expression of MAPK7 in osteosarcoma and normal cell lines and **D:** Western blot showing the expression of MAPK7 miR-NC and miR-187 mimics transfected SAOS-2 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

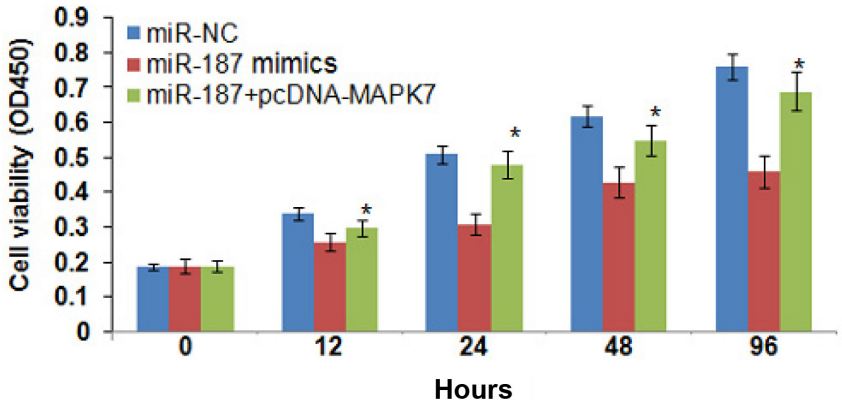

Figure 7. WST-1 assay showing overexpression of MAPK7 rescues the growth inhibitory effects of miR-189 mimics on the SAOS-2 osteosarcoma cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05 for miR-187 mimics Vs miR-187 + pcDNA-MAPK7).

expression in SAOS-2 cells (Figure 6D), confirming MAPK7 as the target of miR-187.

Next, it was found that overexpression of MAPK7 in SAOS-2 cells could promote the growth of the SAOS-2 cells and nullified the growth inhibitory effects of miR-187 overexpression on SAOS-2 cell proliferation (Figure 7).

Discussion

Osteosarcoma is one of the rare but fatal diseases common in children and adolescents [13]. The high metastasis rate, late diagnosis, emergence of drug resistance against the drugs such as doxorubicin, and the lack of therapeutic targets obstruct the treatment of osteosarcoma [14]. The wide array of roles that miRs carry out in humans by controlling the expression of human genes suggests that miRs may prove useful therapeutic targets for treating human diseases including cancer [15]. In this study, we investigated the role of miR-187 in osteosarcoma. It was found the expression of miR-187 is significantly downregulated in osteosarcoma cells. Previous studies have also shown that the expression of miR-187 is dysregulated in cancer cells. For example, the expression of miR-187 has been shown to be downregulated in renal cancer [7]. miR-187 was overexpressed in SAOS-2 osteosarcoma cells. It was observed that miR-187 overexpression resulted in significant decline in the proliferation rate of the SAOS-2 cells. These results are in concordance with previous studies wherein miR-187 has been reported to suppress the growth of colorectal cancer cells by controlling the expression of CD276 [16]. In yet another study, it has been shown to regulate the expression of FOXA2 to control the proliferation of gastric cancer cells [18]. Cell cycle analysis was performed to assess if overexpression of miR-187 causes arrest of the SAOS-2 cells. It was found that overexpression of miR-187 caused accumulation of the G2/M phase cells, indicative of cell cycle arrest. The G2/M arrest was also accompanied by suppression of cyclin B1 expression in the SAOS-2 cells. Previous studies have also shown that miRs cause arrest of different cancer cells, for example, miR-185 and miR-107 have been shown to cause the arrest of the lung cancer cells [19]. Similarly, miR-122 has been shown to cause the cell cycle arrest of hepatocellular carcinoma cells [20]. The emergence of drug resistance in osteosarcoma against the anticancer drugs such as doxorubicin make it more difficult to treat this disease [21]. Herein, we also investigated the effects of miR-187 overexpression on the sensitivity of the osteosarcoma SAOS-2 cells to the anticancer drug doxorubicin and the results showed that miR-187 enhanced the chemosensitivity of SAOS-2 cells to doxorubicin. These results are in agreement with the results of other authors that miR-187 has been shown regulate the sensitivity of lymphoma cells to bortezomib [22]. The effects of miR-187 were also examined on the SAOS-2 cell migration and invasion capability and it was found that this miR suppressed the migration and invasion of SAOS-2 cells, indicating the implications of the miR-187 in the management of metastatic cancers. These results are in agreement with previous studies [9,10] wherein miR-187 has been reported to inhibit the metastasis of hepatocellular and colorectal cancer cells [9,10]. The miRs exert their effects by suppressing the expression of the target genes and each miR has several targets [23]. Herein, bioinformatic analysis together with dual luciferase assay showed that miR-187 exerts its effects by targeting MAPK7. Additionally, the expression of MAPK7 was considerably increased in all the osteosarcoma cells and overexpression of miR-187 could inhibit the expression of MAPK7.

Moreover, the overexpression of MAPK7 could nullify the effects of the miR-187 overexpression on the proliferation of SAOS-2 osteosarcoma cells.

Conclusion

Taken together, the findings of the present study revealed that miR-187 is downregulated in osteosarcoma cells. Overexpression of miR-187 in SAOS-2 osteosarcoma cells inhibited their proliferation by inducing autophagy, apoptosis and G2/M cell cycle arrest. Overexpression of miR-187 also suppressed the migration and invasion and also enhanced the chemosensitivity of SAOS-2 osteosarcoma cells, indicative of the therapeutic implications of miR-187 in osteosarcoma treatment.

Conflict of interests

The authors declare no conflict of interests.

References

1. Anderson ME. Update on survival in osteosarcoma. Orthoped Clin 2016;47:283-92.
2. Durfee RA, Mohammed M, Luu HH. Review of osteosarcoma and current management. Rheumatol Ther 2016;3:221-43.
3. Harrison DJ, Geller DS, Gill JD, Lewis VO, Gorlick R. Current and future therapeutic approaches for osteosarcoma. Expert Rev Anticancer Ther 2018;18:39-50.
4. Ramassone A, Pagotto S, Veronese A, Visone R. Epigenetics and microRNAs in cancer. Int J Molec Sci 2018;19:459.
5. Ji W, Sun B, Su C. Targeting microRNAs in cancer gene therapy. Genes 2017;8:21.
6. Svoronos AA, Engelman DM, Slack FJ. OncomiR or tumor suppressor? The duplicity of microRNAs in cancer. Cancer Res 2016;76:3666-70.
7. Zhao J, Lei T, Xu C et al. MicroRNA-187, down-regulated in clear cell renal cell carcinoma and associated with lower survival, inhibits cell growth and migration though targeting B7-H3. Biochem Biophys Res Commun 2013;438:439-44.
8. Chao A, Lin CY, Lee YS et al. Regulation of ovarian cancer progression by microRNA-187 through targeting Disabled homolog-2. Oncogene 2012;31:764.
9. Zhang F, Luo Y, Shao Z et al. MicroRNA-187, a downstream effector of TGFβ pathway, suppresses Smad-mediated epithelial–mesenchymal transition in colorectal cancer. Cancer Lett 2016;373:203-13.
10. Dou C, Liu Z, Xu M et al. miR-187-3p inhibits the metastasis and epithelial–mesenchymal transition of hepatocellular carcinoma by targeting S100A4. Cancer Lett 2016;381:380-90.
11. Mao M, Wu Z, Chen J. MicroRNA-187-5p suppresses cancer cell progression in non-small cell lung cancer (NSCLC) through down-regulation of CYP1B1. Biochem Biophys Res Commun 2016;478:649-55.
12. Borowicz S, Van Scoyk M, Avasarala S et al. The soft agar colony formation assay. JoVE (Journal of Visualized Experiments) 2014;92:e51998.
13. Lindsey BA, Markel JE, Kleinerman ES. Osteosarcoma overview. Rheumatol Ther 2017;4:25-43.
14. Ferrari S, Serra M. An update on chemotherapy for osteosarcoma. Expert Opin Pharmacother 2015;16:2727-36.
15. Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. Annu Rev Med 2009;60:167-79.
16. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Molec Med 2014;20:460-9.
17. Wang ZS, Zhong M, Bian YH et al. MicroRNA-187 inhibits tumor growth and invasion by directly targeting CD276 in colorectal cancer. Oncotarget 2016;7:44266.
18. Li C, Lu S, Shi Y. MicroRNA-187 promotes growth and metastasis of gastric cancer by inhibiting FOXA2. Oncol Rep 2017;37:1747-55.
19. Takahashi Y, Forrest AR, Maeno E, Hashimoto T, Daub CO, Yasuda J. MiR-107 and MiR-185 can induce cell cycle arrest in human non small cell lung cancer cell lines. PLoS One 2009;4:e6677.
20. Xu Y, Xia F, Ma L et al. MicroRNA-122 sensitizes HCC cancer cells to adriamycin and vincristine through modulating the expression of MDR and inducing cell cycle arrest. Cancer Lett 2011;310:160-9.
21. Chou AJ, Gorlick R. Chemotherapy resistance in osteosarcoma: current challenges and future directions. Expert Rev Anticancer Ther 2006;6:1075-85.
22. Yan ZX, Wu LL, Xue K et al. MicroRNA187 overexpression is related to tumor progression and determines sensitivity to bortezomib in peripheral T-cell lymphoma. Leukemia 2014;28:880.
23. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215-33.