Original Article
MicroRNA-539 suppresses osteosarcoma cell invasion and migration *in vitro* and targeting Matrix metallopeptidase-8

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Abstract: microRNAs (miRNA) are a class of small, non-coding RNA that involved in different cancer-related processes. Previous studies have been indicated miR-539 as a tumor suppressor during tumorigenesis. However, the role of miR-539 in osteosarcoma is still unclear. In this study, we demonstrate miR-539 was downregulated in osteosarcoma tissues compared to adjacent normal tissue. Functional study suggests miR-539 inhabits the osteosarcoma cell proliferation, invasion and migration. We also identified that MMP8 was a direct target of miR-539 by the luciferase activity assay. These findings provide evidence that miR-539 plays a key role in inhibiting osteosarcoma cell invasion and migration and can regulating MMP8 expression in osteosarcoma cells. These strongly suggest that exogenous miR-539 may have therapeutic value in treating osteosarcoma.

Keywords: microRNA (miRNA), miR-539, osteosarcoma, Matrix metallopeptidase-8 (MMP-8)

Introduction

Although it accounts for less than 0.5% of all types of cancer, osteosarcoma (OS) is the most frequent primary malignancy of the bone and occurs mainly in adolescents and young adults [1]. By combining surgery with multiagent chemotherapy, the 5-year cumulative survival rate of primary OS has significantly improved to 60-90% in the past three decades [2, 3]. Unfortunately, as a result of approximately 80% of patients eventually developing metastatic disease following surgical treatment, pulmonary metastasis in OS patients is a major cause of fatal outcome [4, 5]. Therefore, the identification of effector molecules and signaling pathways that exhibits a close relationship with tumor progression and metastasis in order to improve the existing OS treatment is crucial.

MiRNAs are a class of small, noncoding RNA molecules ranging in size from 16 to 27 bases; usually between 21 and 23 bases for most miRNAs [6]. MiRNA is crucial in the regulation of diverse target mRNAs at the level of mRNA degradation or translation [7, 8]. A large number of miRNAs are located inside or close to chromosomal fragile sites that are frequently lost or amplified in cancers [9]. miRNAs have been characterized as oncogenes, tumor suppressors or as components of regulatory pathways critical for tumorigenesis; therefore miRNAs play an important role in tumorigenesis and metastasis.

Recently, miR-539 has been reported to be upregulated in failing heart, and suppresses O-GlcNAcase expression [10]. Another study demonstrates miR-539 were downregulated in OS compared with the osteoblast cell line using microarray method [11]. However, the role of miR-539 in OS proliferation, invasion and migration remains elusive.

In the present study, we found that miR-539 functions as a tumor suppressor in OS, which upregulates miR-539 expression in the OS cell line MG-63 reduces cell proliferation, migration and invasion *in vitro*. We screen the potential target of miR-539 by microRNA.org and TargetScan.human6.2. The results revealed that MMP8 may be a target of miR-539. Then we confirmed miR-539 directly target MMP by
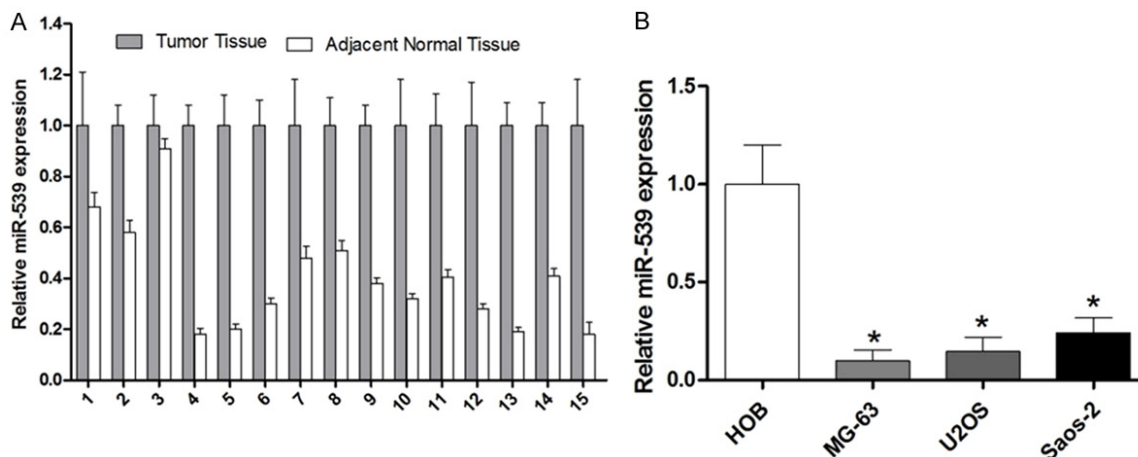
Figure 1. miR-539 expression in OS tissues and cell lines. A. Relative expression of miR-539 in 15 paired tissues in comparison with corresponding adjacent normal tissues. B. The miR-539 expression was significantly lower in OS cell lines compared with osteoblast HOB cell lines. bars, SD; *P<0.05.

interacted with its 3'UTR. Therefore, we speculate that miR-539 may suppress OS invasion and metastasis by targeting MMP8. Together, our data indicate that miR-539 plays an important role in regulating OS cell proliferation, migration and invasion and may serve as a potential therapeutic target.

Materials and methods

Patient samples

OS specimens and adjacent normal tissues were collected in the First Affiliated Hospital of Harbin Medical University, China, from November 2008 to January 2014. All the patients recruited into the present study did not receive radiotherapy or chemotherapy or any other treatment before and after operation. Surgical specimens of the tumor resection were collected, and lumps of tumors as well as adjacent normal tissues, which were at least 2 cm distal to tumor margins, were snap-frozen in liquid nitrogen. The use of tissue samples were approved by the ethical committees of the First Affiliated Hospital of Harbin Medical University.

Cell culture and transfection

The human OS cell line MG-63, U2OS, Saos2 and osteoblast HOB cell lines (Shanghai Cell Bank, Chinese Academy of Sciences) was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and incubated at 37°C in 5% CO_2. MG-63 cells were seeded in six-well plates at 30% confluence

one day prior to transfection. Transfection with miR-539 or miRNA control was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Transfection complexes were prepared according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed using gene-specific reverse primers and reverse transcriptase (Takara, Japan), and the resulting cDNAs were PCR-amplified on an ABI 7500 thermocycler (Applied Biosystems). GAPDH were used to standardize the amounts of RNA in each sample.

Detection of cell phenotypes

The effect of miR-539 on proliferation of OS cells was evaluated by the MTT assay. MG-63 cells were plated in 96-well culture plates (3×10^3 per well). After 24 h incubation, the cells were transfected with miR-539 mimics or miRNA control for 12, 24 and 48 hours. Then the MTT (0.5 mg/ml; Sigma-Aldrich, USA) was added to each well (20 μl/well). After 4 hours of additional incubation, MTT solution was discarded and 200 ml of DMSO (Sigma, USA) was added and the plates shaken gently. The absorbance was measured on an ELISA reader at a wavelength of 570 nm. For colony formation assay, cells were counted and seeded in 12-well plates (in triplicate) at 100 cells per
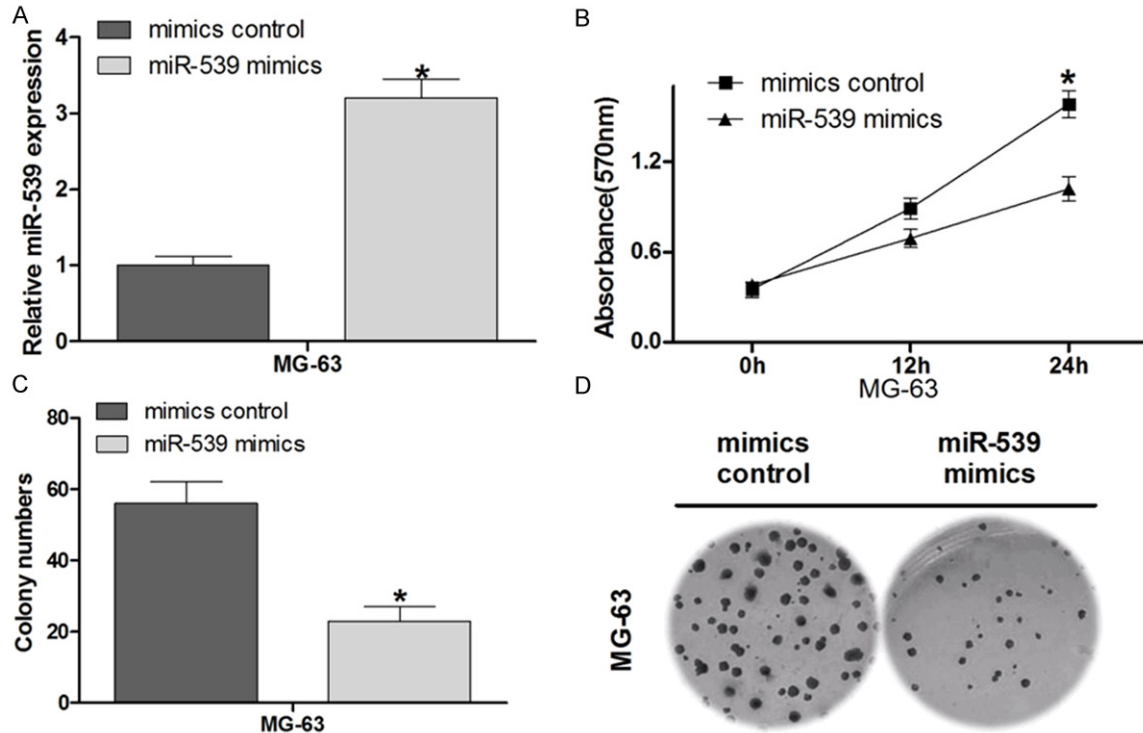
Figure 2. Overexpression of miR-539 inhibits in vitro growth of MG-63 cells. A. MG-63 cells were transfected with miR-539 or miRNA controls, respectively. qRT-PCR was used to measure the miR-539 expression after transfetion. B. At 24 h after transfection, MTT assay was performed to determine the proliferation of MG-63 cells. Data represent the mean ± SD from three independent experiments. C, D. Colony formation assay was performed to determine the long term proliferation of MG-63 cells. Representative results of colony formation of MG-63 cells transfected with miR-539 or miRNA control. The results were reproducible in three independent experiments.

well. Fresh culture medium was replaced every 3 days. The number of viable cell colonies was determined after 14 days and colonies were fixed with methanol, stained with crystal violet, photographed and counted. Each experiment was performed in triplicate.

Luciferase activity assay

MG-63 cells were cotransfected with the pGL3 vectors containing the 3'-UTR of MMP8 and miR-539 or the miRNA control. Luciferase activity was measured 36 h after transfection. The firefly luciferase activity was then normalized to the Renilla luciferase activity.

Transwell invasion assay in vitro

Invasion assays were performed in triplicate using Transwell invasion chambers (Costar 3422, Corning Inc., NY, USA) coated with Matrigel (50 μl per filter) (BD, USA) as described in the manufacturer's instructions. MG-63 cells

were transfected with either miR-539 or the miRNA control oligonucleotide, cultured for 48 h and then transferred to the top of the Matrigel-coated invasion chambers in 1% fetal calf serum DMEM/F12 (2×10⁴ cells/ well). DMEM/F12 containing 10% fetal calf serum was added to the lower chambers. Following incubation for 24 h, cells that remained on the top of the filter were removed and cells that migrated to the lower surface were fixed in 90% alcohol followed by crystal violet staining. The values for invasion were obtained by counting three fields per membrane and represented as the average of six independent experiments made over multiple days.

Wound healing migration assay

When MG-63 cells transfected with miR-539 or miRNA control oligonucleotides were grown to confluence, a scratch in the cell monolayer was made with a micropipette tip. Following incubation of the cells under standard conditions for
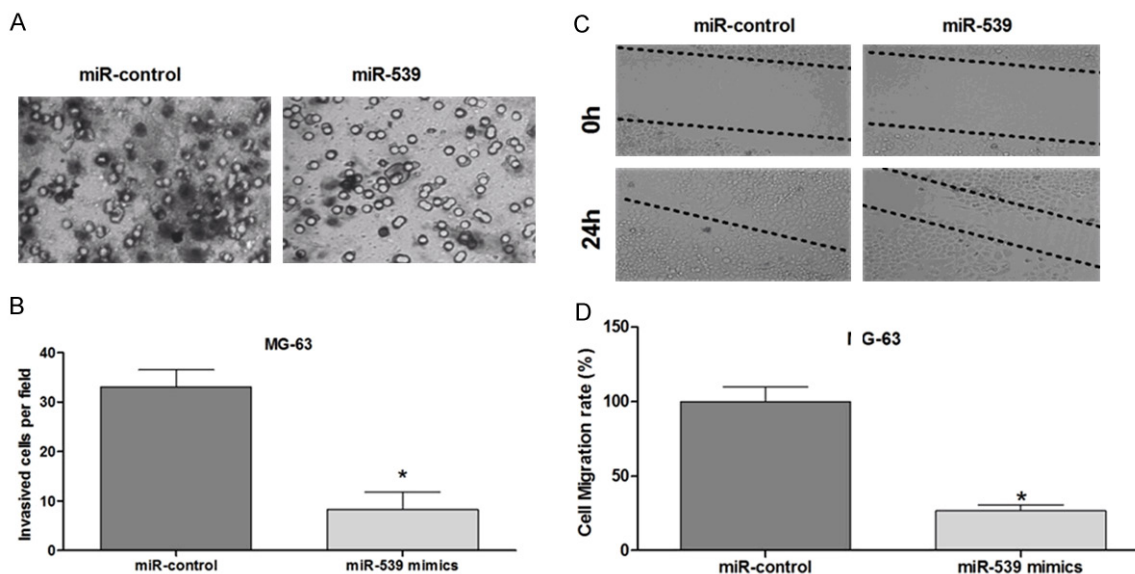
Figure 3. Ectopic miR-539 expression inhibits invasion and migration of MG-63 cells. A. A representative image of the three experiments is shown for each group. The cell invasion of MG-63 was inhibited via the upregulation of miR-539 using the Transwell invasion assay. B. Quantification of cell invasion. Bars, SD; *P<0.05. This indicated that cell invasion is significantly inhibited by overexpression of miR-539. C. A representative image of the three experiments is shown for each group. The migration rate of miR-539 transfected MG-63 cells significantly lower than that of the control group. D. Quantification of cell migration. Bars, SD; *P<0.05.

24 h, the plates were washed twice with fresh medium and images were captured at different times. The migration potential was estimated by counting the cells that migrated from the wound edge. The cell migration rate was obtained by counting three fields per area and represented as the average of six independent experiments made over multiple days.

Western blot analysis

MG-63 cells in the exponential growth phase were transfected with miR-539 for 48 h. Total proteins were isolated from the transfected cells. Protein concentrations were measured using a Micro-BCA protein assay kit (Beyotime, Beijing, China). Proteins were resolved by 10% SDS-PAGE gel, transferred to the nitrocellulose membrane and blocked in 5% non-fat dry milk in Tris-buffered saline pH 7.4, containing 0.05% Tween-20. They were subsequently blotted with a rabbit polyclonal antibody against MMP8 (1:1000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and goat anti-rabbit IgG (1:3000, Santa Cruz), with actin used as a loading control. Signals were detected by secondary antibodies labeled with horseradish peroxidase (HRP). All western blot analyses were performed three times.

Statistical analysis

Data were expressed as the means ± SD of at least three experiments. A value of P<0.05 was considered to indicate a statistically significant result. All analyses were performed using SPSS version 17.0 (Statistical Package for the Social Sciences, Chicago, IL, USA).

Results

Expression of miR-539 in OS tissue and cell lines

Here, we used quantitative real-time PCR (qRT-PCR) to measure miR-539 expression levels in 15 pairs of OS tissues and adjacent normal tissues, which showed that miR-539 was down-regualted in the OS tissues (**Figure 1A**). In addition, we showed that miR-539 expression was downregulated in MG-63, U2OS and Saos2 cells compared with osteoblast HOB cells (**Figure 1B**).

Effect of miR-539 on OS cell proliferation

To assess the biological role of miR-539 in OS cells, we investigated the effect of overexpression of miR-539 on cell viability and prolifera-
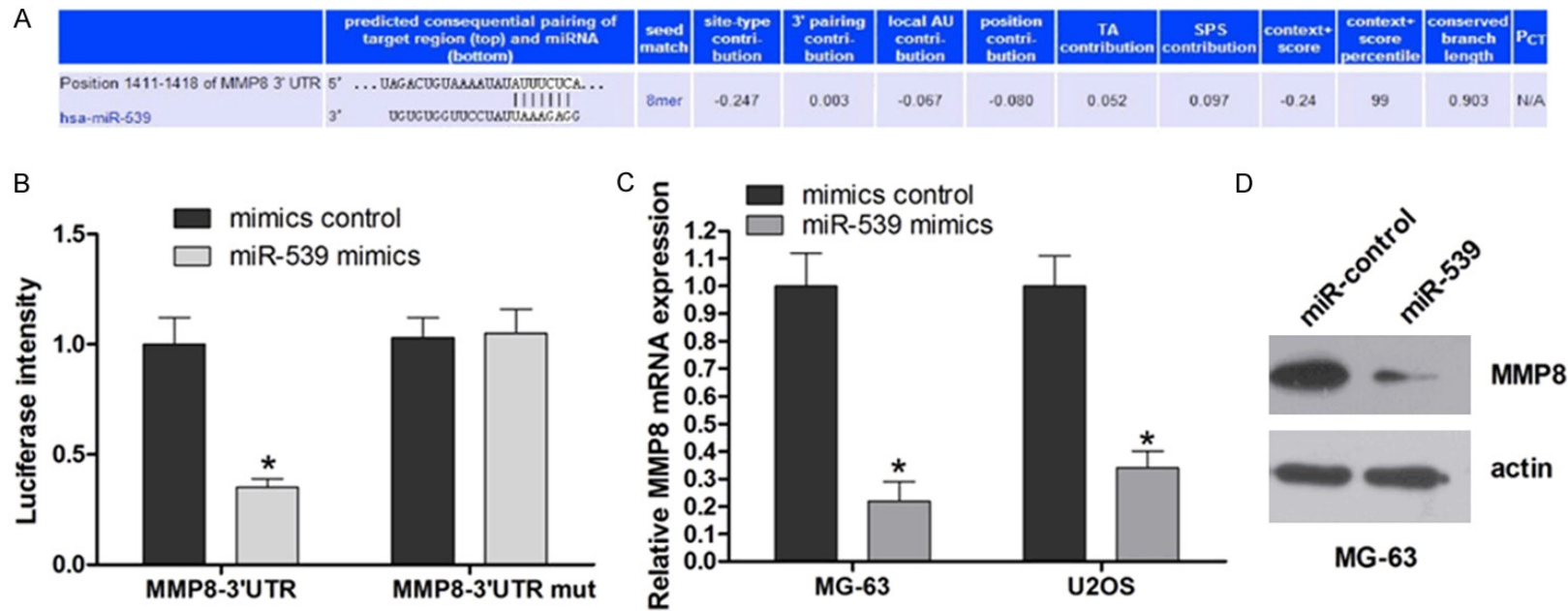
Figure 4. MiR-539 directly targets MMP8 mRNA and inhibits its expression. A. The putative miR-539-binding sites in the 3'-UTR of MMP8 mRNA was shown. B. The wild type (MMP8 3'-UTR) or mutant (MMP8 3'-UTR-mut) reporter plasmids was co-transfected into MG-63 with miR-539 or miRNA control. The normalized luciferase activity in the control group was set as relative luciferase activity. C. The expression of MMP8 mRNA was analyzed by real time PCR assay; actin was used as an internal control. D. The expression of MMP8 protein was analyzed by western blot assay, while actin was used as an internal control. All experiments were at least repeated in triplicate.

tion. Firstly, qRT-PCT results confirmed miR-539 expression level was significant elevated by miR-539 transfection. MTT assay revealed that cell growth was significantly impaired in MG-63 cells transfected with miR-539 (**Figure 2A**, **2B**). Similarly, the results of colony-formation assays revealed that clonogenic survival was decreased following overexpression of miR-539 in OS cells (**Figure 2C**, **2D**).

miR-539 inhibits cell invasion and migration in vitro

To further investigate the effect of miR-539 on MG-63 cell invasion and migration, we employed the Transwell invasion assay and wound healing assay. MG-63 cells were transfected with miR-539 or miRNA control. We observed a significant inhibition of invasion into Matrigel in miR-539 transfected cells (**Figure 3A**, **3B**; $P<0.05$). We also indicated that the number of migrated cells transfected with miR-539 was significantly fewer than the number transfected with miRNA control (**Figure 3C**, **3D**; $P<0.05$). These results suggest that the upregulation of miR-539 inhibits the invasion and migration of OS cells.

miR-539 directly targets and inhibits MMP8 protein expression

In order to explore the potential target for miR-539, miRanda and Targetscan were used and a large number of different genes were predicted. Among these candidate miRNAs, MMP8 attracted our attention immediately (**Figure 4A**). To understand whether the effect of miR-539 on MMP8 is specific, we performed the 3'UTR luciferase reporter assay, which showed miR-539 had an obvious effect of inhibiting the luciferase intensity of wild-type 3'UTR luciferase reporter (**Figure 4B**). However, the inhibitory effect of miR-539 was reduced in the presence of mutant 3'UTR luciferase reporter (**Figure 4B**). Then we tested whether miR-539 could influence the endogenous MMP8 expression. Enforced expression of miR-539 induced a reduction of endogenous MMP8 mRNA expression in MG-63 and U2OS cells. Meanwhile, miR-539 also inhibits MMP8 protein level in MG-63 cells (**Figure 4D**). These results provide evidence that miR-539 directly targets the 3'UTR of MMP8 mRNA, resulting MMP8

mRNA degradation and suppressing its expression.

Discussion

Recent study report that the aberrant expression of miRNAs contributes to the proliferation, invasion or metastatic behavior of human cancer [12]. MiRNAs have been demonstrated to targeting multiple genes thus regulating several biological processes. Moreover, the aberrant expression of miRNAs in human cancer cells causes destruction of miRNA-mediated mRNA networks. A recent study has demonstrated the miRNA expression signature associated with OS-characterizing pathogenesis, clinical metastasis and chemotherapy response [13]. Another study indicated miR-26b inhibits proliferation, migration, invasion and apoptosis induction via the downregulation of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase-3 driven glycolysis in osteosarcoma cells [14]. However, the mechanism of miRNA in OS development is complex and still need to be explored. Previous study reported that the miR-539 was deregulated in OS detected by microarray assay [11]. In this study, we investigate the downregulation of miR-539 in OS tissue and cell lines by qRT-PCR, and find ectopic expression of miR-539 inhibits MG-63 cell proliferation, invasion and migration.

Matrix metalloproteinase-8 (MMP8) was a member of the matrix metalloproteinase (MMP) family of proteins. This protein is involved in the breakdown of extracellular matrix in embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. It was once thought to be produced exclusively by polymorphonuclear leukocytes, but more recent studies have shown that various other cell types including stem/progenitor cells express this protease [15, 16]. However, in human cancers, Matrix metalloproteinase 8 was revealed to be tumor- or metastasis-suppressive in vivo [17-19]. Subsequently, MMP-8 expression was found to be a marker of a lower incidence of lymph node metastasis and, consequently, to confer a better prognosis in human breast carcinoma [20]. In human melanoma, function-inactivating mutations in MMP8 have been found at high frequency and wild-type but not mutant forms suppressed tumor formation in soft agar as-

says, supporting its role as a tumor suppressor [21]. In this study, the prediction of the MMP8 gene as a potential target of miR-539 was performed by microRNA.org and TargetScan. human6.2. To identify whether miR-539 can genuinely regulate the expression of MMP8, the MMP8 3'-UTR was cloned into the pGL3 vector, placing the 3'-UTR with the majority of potential miRNA binding sites downstream of the coding sequence of luciferase. We found that the overexpression of miR-539 significantly reduced the luciferase activity from the reporter construct containing the MMP8 3'-UTR. This indicates that MMP8 is a direct miR-539 target. Although this may not fully explained the miR-539 induced OS cell proliferation, invasion and migration inhibition, there were hundreds of predicted targets of miR-539 revealed in the TargetScan prediction and a single miRNA has been proven to target multiple mRNAs in order to regulate gene expression[22], it is probable that other targets of miR-539 may also participate in OS proliferation migration and invasion. Furthermore, miR-539 may also target different targets in different types of cancer. Additionally, the tumor microenvironment may influence tumor progression, invasion and migration. Therefore, further studies are needed to identify the entire role of miR-539 in OS tumorigensis.

Our present study indicated that MMP8 is negatively regulated by miR-539 through a special binding site in the MMP8 3'-UTR. Moreover, miR-539 inhibits cell proliferation, invasion and migration in MG-63 cells. These results suggest that miR-539 may serve as a target in the discovery of effective therapies for OS.

Disclosure of conflict of interest

None.

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References

[1] Mirabello L, Troisi RJ and Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer 2009; 115: 1531-1543.

[2] Cho Y, Jung GH, Chung SH, Kim JY, Choi Y and Kim JD. Long-term survivals of stage IIb osteosarcoma: a 20-year experience in a single institution. Clin Orthop Surg 2011; 3: 48-54.

[3] Lewis VO. What's new in musculoskeletal oncology. J Bone Joint Surg Am 2009; 91: 1546-1556.

[4] Marina N, Gebhardt M, Teot L and Gorlick R. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist 2004; 9: 422-441.

[5] Wada T, Isu K, Takeda N, Usui M, Ishii S and Yamawaki S. A preliminary report of neoadjuvant chemotherapy NSH-7 study in osteosarcoma: preoperative salvage chemotherapy based on clinical tumor response and the use of granulocyte colony-stimulating factor. Oncology 1996; 53: 221-227.

[6] Fang Z, Du R, Edwards A, Flemington EK and Zhang K. The sequence structures of human microRNA molecules and their implications. PLoS One 2013; 8: e54215.

[7] Cipolla GA. A non-canonical landscape of the microRNA system. Front Genet 2014; 5: 337.

[8] Ameres SL and Zamore PD. Diversifying microRNA sequence and function. Nat Rev Mol Cell Biol 2013; 14: 475-488.

[9] Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M and Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci U S A 2004; 101: 2999-3004.

[10] Muthusamy S, DeMartino AM, Watson LJ, Brittian KR, Zafir A, Dassanayaka S, Hong KU and Jones SP. MicroRNA-539 is up-regulated in failing heart, and suppresses O-GlcNAcase expression. J Biol Chem 2014; 289: 29665-29676.

[11] Hu H, Zhang Y, Cai XH, Huang JF and Cai L. Changes in microRNA expression in the MG-63 osteosarcoma cell line compared with osteoblasts. Oncol Lett 2012; 4: 1037-1042.

[12] Nelson KM and Weiss GJ. MicroRNAs and cancer: past, present, and potential future. Mol Cancer Ther 2008; 7: 3655-3660.

[13] Jones KB, Salah Z, Del Mare S, Galasso M, Gaudio E, Nuovo GJ, Lovat F, LeBlanc K, Palatini J, Randall RL, Volinia S, Stein GS, Croce CM, Lian JB and Aqeilan RI. miRNA signatures associate with pathogenesis and progression of osteosarcoma. Cancer Res 2012; 72: 1865-1877.

[14] Du JY, Wang LF, Wang Q and Yu LD. miR-26b inhibits proliferation, migration, invasion and apoptosis induction via the downregulation of 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase-3 driven glycolysis in osteosarcoma cells. Oncol Rep 2015; 33: 1890-1898.

[15] Turu MM, Krupinski J, Catena E, Rosell A, Montaner J, Rubio F, Alvarez-Sabin J, Cairols M and Badimon L. Intraplaque MMP-8 levels are increased in asymptomatic patients with carotid plaque progression on ultrasound. Atherosclerosis 2006; 187: 161-169.

[16] Xiao Q, Zhang F, Lin L, Fang C, Wen G, Tsai TN, Pu X, Sims D, Zhang Z, Yin X, Thomaszewski B, Schmidt B, Mayr M, Suzuki K, Xu Q and Ye S. Functional role of matrix metalloproteinase-8 in stem/progenitor cell migration and their recruitment into atherosclerotic lesions. Circ Res 2013; 112: 35-47.

[17] Montel V, Kleeman J, Agarwal D, Spinella D, Kawai K and Tarin D. Altered metastatic behavior of human breast cancer cells after experimental manipulation of matrix metalloproteinase 8 gene expression. Cancer Res 2004; 64: 1687-1694.

[18] Agarwal D, Goodison S, Nicholson B, Tarin D and Urquidi V. Expression of matrix metalloproteinase 8 (MMP-8) and tyrosinase-related protein-1 (TYRP-1) correlates with the absence of metastasis in an isogenic human breast cancer model. Differentiation 2003; 71: 114-125.

[19] Balbin M, Fueyo A, Tester AM, Pendas AM, Pitiot AS, Astudillo A, Overall CM, Shapiro SD and Lopez-Otin C. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet 2003; 35: 252-257.

[20] Gutierrez-Fernandez A, Fueyo A, Folgueras AR, Garabaya C, Pennington CJ, Pilgrim S, Edwards DR, Holliday DL, Jones JL, Span PN, Sweep FC, Puente XS and Lopez-Otin C. Matrix metalloproteinase-8 functions as a metastasis suppressor through modulation of tumor cell adhesion and invasion. Cancer Res 2008; 68: 2755-2763.

[21] Palavalli LH, Prickett TD, Wunderlich JR, Wei X, Burrell AS, Porter-Gill P, Davis S, Wang C, Cronin JC, Agrawal NS, Lin JC, Westbroek W, Hoogstraten-Miller S, Molinolo AA, Fetsch P, Filie AC, O'Connell MP, Banister CE, Howard JD, Buckhaults P, Weeraratna AT, Brody LC, Rosenberg SA and Samuels Y. Analysis of the matrix metalloproteinase family reveals that MMP8 is often mutated in melanoma. Nat Genet 2009; 41: 518-520.

[22] Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R and Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. Nature 2008; 455: 58-63.