

RESEARCH ARTICLE

miRNA-218 Inhibits Osteosarcoma Cell Migration and Invasion by Down-regulating of TIAM1, MMP2 and MMP9

Jie Jin¹, Lin Cai^{1*}, Zhi-Ming Liu², Xue-Song Zhou²

Abstract

Deregulated miRNAs participate in osteosarcoma genesis. In this study, the expression of miRNA-218 in human osteosarcomas, adjacent normal tissues and Saos-2 human osteosarcoma cells was first assessed. Then the precise role of miRNA-218 in osteosarcoma cells was investigated. Upon transfection with a miR-218 expression vector, the proliferation of Saos-2 human osteosarcoma cells determined using the ATPlite assay was significantly suppressed, while migration of Saos-2 cells detected by wound healing and invasion determined using transwells were dramatically inhibited. Potential target genes of miR-218 were predicted and T-cell lymphoma invasion and metastasis 1 (TIAM1) and matrix metalloproteinase 2 (MMP2) and 9 (MMP9) were identified. This was confirmed by western blotting, which showed that miR-218 expression inhibited TIAM1, MMP2 and MMP9 protein expression. Collectively, these data suggest that miR-218 acts as a tumor suppressor in osteosarcomas by down-regulating TIAM1, MMP2 and MMP9 expression.

Keywords: miRNA-218 - osteosarcoma - TIAM1 - proliferation - migration - invasion - MMP

Asian Pacific J Cancer Prev, **14** (6), 3681-3684

Introduction

Osteosarcoma is the eighth most common form of childhood cancer, comprising 2.4% of all malignancies in pediatric patients, and approximately 20% of all primary bone cancers. It is an aggressive malignant neoplasm arising from primitive transformed cells of mesenchymal origin that exhibit osteoblastic differentiation and produce malignant osteoid (Ottaviani et al., 2009). The mechanisms of formation and development of osteosarcoma have been studied for a long time. Recently, more and more evidence showed that miRNAs play important roles in regulating tumor growth.

MicroRNAs (miRNAs) are small endogenous non-coding RNAs (~22nt) that bind to partially complementary recognition sequences of target mRNAs, either preventing translation or causing degradation (Osman et al., 2012; Nana-Sinkam et al., 2013). Accumulating evidence has shown that miRNAs have crucial functions in specific cellular processes such as differentiation, morphogenesis, and tumorigenesis. In human cancer, miRNAs can function as oncogenes (Qin et al., 2013) or tumor suppressor genes (Hagman et al., 2013) during tumorigenesis. Gene expression profiling studies have demonstrated that miRNA expression is an excellent biomarker associated with specific tumor subtypes and clinical outcomes (Dieckmann et al., 2012; Takahashi et al., 2012).

Recently, it has been shown that miR-218 is deregulated in several types of cancers, and downregulation of miR-218 correlates with worse prognosis; all these studies

indicated that miR-218 acts as a tumor suppressor. Previous studies showed that miR-218 inhibits the cell cycle progression and promotes apoptosis in colon cancer by downregulating BMI1 polycomb ring finger oncogene (He et al., 2013). Sujatha Venkataraman et al. reported that miR-218 acts as a tumor suppressor by targeting multiple cancer associated genes including CDK6, Rictor and CTSB (Venkataraman et al., 2013). Study also showed that miRNA-218 inhibit the head and neck squamous cell carcinoma by targeting laminin-332 (Kinoshita et al., 2012). Data from Liu et al. indicated that miR-218 can reverse high invasiveness of glioblastoma cells by targeting oncogenic transcription factor LEF1 (Liu et al., 2012). Study from Alajez et al. (2011) demonstrated that miR-218 suppresses nasopharyngeal cancer progression by regulating the surviving and SLIT2-ROBO1 pathways. All of the data above indicated that miR-218 serves as an effective tumor suppressor by targeting multiple genes in types of tumors.

Studies showed that TIAM1 can be targeted and regulated by different miRNAs including miR-21, miR-22, miR-183, miR-31 and miR-10b. In our present study, TargetScan was used and indicated that TIAM1 is a potential target gene of miR-218, so we subsequently investigated the effects of miR-218 on the proliferation, migration and invasion of human osteosarcoma cells-Saos-2. And the results from this study demonstrated that the miR-218 can suppresses the proliferation, migration and invasion of Saos-2 cells in vitro by targeting TIAM1, MMP2 and MMP9 genes.

¹Department of Orthopedics, Zhongnan Hospital of Wuhan University, ²Department of Orthopedics, Tongren Hospital of Wuhan, Wuhan, China *For correspondence: cailin_wuhu2012@163.com

Materials and Methods

Cell culture

The human Saos-2 osteosarcoma cells was purchased from ATCC and cultured in DMEM culture medium supplemented with 15% fetal calf serum and 100 U/ml penicillin, 100 µg/ml streptomycin in a 37°C humidified and 5% CO₂ incubator.

Human tissues

Paired resected surgical specimens from primary tumor and adjacent nontumor sites were obtained from osteosarcoma patients who underwent surgery at the Surgically resected paired 12 osteosarcoma specimens (before the administration of neoadjuvant chemotherapy) and 12 adjacent normal bone tissues were acquired from the Zhongnan Hospital of Wuhan University from 2011 to 2013. All the specimens were obtained after obtaining written informed consent according to a protocol approved by Institutional Review Board of the affiliated Zhongnan Hospital of Wuhan University.

Real-time quantitative RT-PCR

Total RNA was purified from tissues and cultured cell lines using the RNeasy Plus mini kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using the PrimeScript reverse transcription-PCR (RT-PCR) kit (TaKaRa, Shiga, Japan). Real-time PCR was performed using SYBR Premix Ex Taq TM II kit (TaKaRa) according to the manufacturer's protocol on an MX3005P QPCR system (Stratagene, La Jolla, CA, USA). Stem-loop RT-PCR (TaqMan MicroRNA assays; P/N: 000521 for miR-218; Applied Biosystems, Foster City, CA, USA) was used to quantify miRNAs according to earlier published conditions (13). RNU48 (assay ID: 001006; Applied Biosystems) was used to normalize the data for quantification of miR-218 expression. TaqMan probes and primers for GAPDH (P/N: Hs02758991_g1) was obtained from Applied Biosystems. Primers for ACTB (P/N: ACTB 533F 37546-020, ACTB 653R 37546-021) were obtained from Sigma genetics. We used the $\Delta\Delta C_t$ method to calculate the fold-change.

MicroRNA (miRNA) transfection

miR-218 precursor sequences were released from full-length constructs with NheI and NotI and subcloned into the complementary sites of pcDNA3.1/Hygro(p) (Invitrogen, Carlsbad, CA, USA). These constructs (or the empty parental vector as a control) were then stably transfected into Saos-2 cells using Lipofectamine 2000 reagent (Invitrogen) and stable transfectants were selected by growth in hygromycin.

Wound healing assay

Saos-2 cells were cultured in a 6-well-culture-plate. 24 h later, when cells reached 90% confluence, a single wound was created in the center of the well by removing the attached cells with a sterile plastic pipette tip. The debris was removed by serum free medium. After 24 h of culturing, the cells which migrated into the wounded area were visualized and photographed under an inverted

microscope. Each experiment was performed at least three times independently.

Transwell assay

Transwell assay was performed to estimate the effects of miR-218 on the invasion of Saos-2 cells. Matrigel (5 mg/ml to 1 mg/ml) was diluted in serum free-cold RPMI 1640 medium and put 100 µl of the diluted matrigel into upper chamber of 24-well transwell. Incubated the transwell at 37 °C for 4 h for gelling. Cells were harvested by Trypsin/EDTA and resuspended in media containing 1% FBS at a density of 106/ml. Gently wash gelled matrigel with warmed serum free RPMI 1640. Put 100 µl of the cell suspension onto the matrigel. 600 µl of RPMI 1640 containing 5 µg/ml fibronectin was filled in the lower chamber as an adhesive substrate. After 24 h of incubation, transwells were removed from the 24-well plate and stained with Diff-Quick solution. The noninvaded cells on the top of the transwell were scraped off using a cotton swab and the invaded cells were counted under light microscope.

ATPlite assay for cells proliferation

Saos-2 cells, control vector or miR-218-transfected Saos-2 cells were plated in 96 wells culture plate for 24 h. Then the ATP content was measured using Luminescence ATP Detection Assay System (PerkinElmer Life Sciences, MA, USA). In brief, 50 µl of mammalian cell lysis buffer was added to each well and shake for 5 min. Then 50 µl of substrate solution was added to wells and the plate was shaken at 700 rpm for another 5 min. Dark adapt the plate for 10 min and measure the luminescence.

SDS-PAGE and Western Blot

For preparation of cell extracts, the control cells or miR-218-transfected cells were washed thrice with ice-cold PBS, and lysed in lysis buffer (Tris-HCl 50 mM pH 8.0, NaCl 150 mM, EDTA 1 mM, Triton X-100 1%, PMSF 100 µg/ml) on ice for 30 min. After centrifuging at 10 000 × g for 5 min at 4°C, the supernatants were analyzed by 10% SDS-PAGE and then electrophoretically transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA). After 1 h of blocking with 5% BSA at room temperature, the membranes were incubated with either anti-TIAM1 monoclonal antibody, anti-MMP2, MMP9 or anti-β-actin monoclonal antibody (Sigma, Deisenhofen, Germany), and then reacted with HRP-conjugated secondary antibody. Protein bands were visualized with enhanced chemiluminescence reagent (Pierce, Rockford, IL).

Statistical analysis

The 2-tailed Student/test was used for statistical analysis. All data are presented as the mean ± standard error of the mean (s.e.m.). A *P*-value of less than 0.05 was considered to be significant.

Results

miR-218 was downregulated in the human osteosarcoma cancer tissues and Saos-2 cells

To confirm miR-218 expression was different between

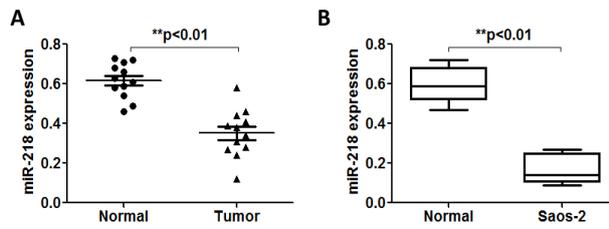


Figure 1. miR-218 is Down-regulated in Osteosarcoma and Human Osteosarcoma Cell Lines. (A) The expression of mature miR-218 was determined by TaqMan real-time PCR in 12 pairs of osteosarcoma and adjacent noncancerous tissue (Normal). (B) The relative expression level of mature miR-218 was determined in human osteosarcoma cell lines Saos-2 and adjacent noncancerous tissue (Normal)

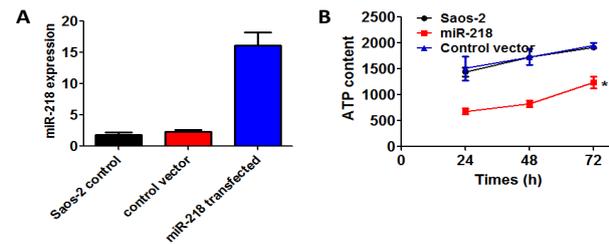


Figure 2. Expression of miR-218 in Saos-2 Cells Transfected with or Without miR-218 Vectors and Proliferation of Cells. (A) miR-218 levels were detected by real-time PCR in Saos-2 cells transfected with miR-218 vectors (miR-218), untransfected Saos-2 cells (Saos-2) and control vector transfected Saos-2 cells (Control vector). (B) proliferation of cells determined using ATPlite assay

tumor and nontumor tissues, we examined 12 pairs of human osteosarcoma samples. As shown in Figure 1A, the miR-218 was about 2-fold down-regulated in the osteosarcoma tissues compared with the adjacent normal tissues (**p<0.01). In this study, we also examined the miR-218 expression in Saos-2 cells using qRT-PCR and found that it was weakly expressed (Figure 1B, **p<0.01).

Overexpression of miR-218 and proliferation of Saos-2 cells

To study the effects of miR-218 on Saos-2 cells, we transfected a miR-218 expression vector into this cell line. Our data showed that the expression level of transfected miR-218 was 8.63 ± 0.74 fold higher than that of control vector transfected or parental cells (Figure 2A). Then we compared the proliferation change after miR-218 transfection by ATPlite assay. As shown in Figure 2B, the proliferation of Saos-2 cells was significantly inhibited by miR-218 (*p<0.05).

Effect of miR-218 on migration and invasion of osteosarcoma Saos-2 cells

Studies indicated that miR-218 inhibited the proliferation of Saos-2 cells. We subsequently investigated the migration and invasion of Saos-2 cells after miR-218 transfection. The effect of miR-218 on the migration of Saos-2 cells was determined by wound healing assay demonstrated that miR-218 significantly inhibited the migration of SAOS-2 cells compared with the SAOS-2 only or control vector transfected cells (Figure 3A). And the invasion of miR-218-transfected Saos-2 cells was also dramatically suppressed (Figure 3B).

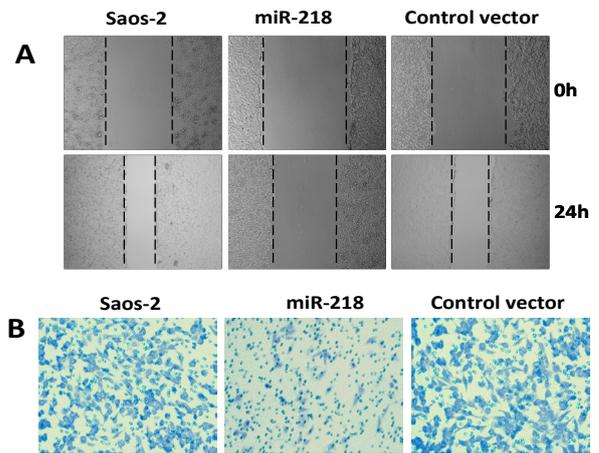


Figure 3. Effect of miR-218 on the Migration and Invasion of Saos-2 Cells. (A) Scratch wound-healing assay was conducted in miR-218-transfected Saos-2 cells and control cells. Migration distance was measured at 0, 24 hours after cells were scratched. (B) Saos-2 cells transfected with miR-218 or control cells were plated on Matrigel-coated membranes in the upper chamber of transwells. Cells penetrating the membrane were fixed and 0.1% crystal violet stained after 24 h as described in Experimental procedures

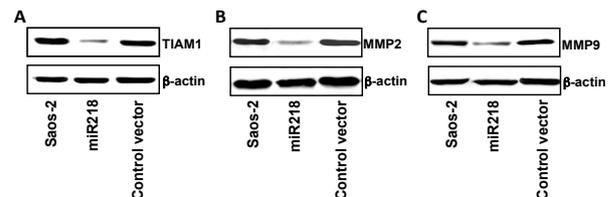


Figure 4. miR-218 Inhibit the Expression of TIAM1, MMP2 and MMP9. Western blot was used to detect the levels of TIAM1 (A), MMP2 (B) and MMP9 (C) in the miR-218 transfected Saos-2 cells or control cells

miR-218 suppressed the expression of TIAM1, MMP2 and MMP9 in SAOS-2 cells

TargetScan revealed that the TIAM1 is a potential target of miR-218, so we finally investigated the expression of TIAM1 in Saos-2 cells after miR-218 transfection. Consistent with the prediction, TIAM1 was dramatically inhibited by miR-218 as shown in Figure 4A. Furthermore, we analyzed expression of MMP-2 and MMP-9, which play critical roles in cancer cell invasion and metastasis. As shown in Figures 4B and 4C, the expression of MMP-2 and MMP-9 in Saos-2 cells was obviously inhibited by miR-218.

Discussion

There are types of treatments currently used or being tested in clinical trials for patients with osteosarcoma meant to help improve the therapeutic effects. microRNAs are small 19-22 nt of RNA that participate in the regulation of cell differentiation, cell cycle progression and apoptosis. They are critical factors in tumorigenesis and migration in many types of tumors including osteosarcoma. Reports have claimed that amounts of miRNAs act as tumor suppressor in osteosarcoma. Mao JH et al. demonstrated that miR-195 inhibit the osteosarcoma cells migration and invasion by targeting FASN (Mao et al., 2012). Study in Wu X et al. revealed that the proliferation of human

osteosarcoma was suppressed by downregulating Eag1 (Wu et al., 2013). Data from Long XH et al. indicated that miR-424 suppresses osteosarcoma cell migration and invasion via targeting fatty acid synthase (Long et al., 2013). Another study claimed that the proliferation and invasion of osteosarcoma cells can be inhibited by miR-376c by targeting the expression of transforming growth factor- α (Jin et al., 2013).

miR-218 has been extensively studied in types of cancer in vivo and in vitro. Data from previously reported showed that miR-218 acts as a tumor suppressor by not only targeting focal adhesive pathway, but also caveolin-2, ECOP, LEF1, mTOR, IKK-b, Robo1 receptor and survivin. But there is no study about the effect of miR-218 on the human osteosarcoma cells. Tiam1 was identified first in 1994 by in vitro selection for invasiveness in T-lymphoma cells (Habets et al., 1994). Accordingly, Tiam1 has been shown to increase invasion in T-lymphoma cells, as well as to increase cellular migration in fibroblasts, and to promote motility in some neuronal cells (Kawauchi et al., 2003). In contrast, Tiam1 has been demonstrated to increase cellular adhesion in some epithelial cell populations. Thus, Tiam1 has multiple roles in regulating cellular functions, likely dependent on the cell type, the substratum, transformation status of the cells, and the activation state of small G proteins in a given cell. Increasing evidence has focused on Tiam1's regulation, as well as Tiam1's role in cancer progression and metastasis. Recent studies also showed that TIAM1 can be negatively regulated by miR-10b (Moriarty et al., 2010), miR-22, miR-183 and miR-31 (Li et al., 2012) in types of cancer cells. But very few studies have reported the relationship between TIAM1 and miR-218 in human osteosarcoma cells.

In this study, we found that the miR-218 was weakly expressed in human osteosarcoma tissues and Saos-2 human osteosarcoma cells. Then we prepared a miR-218 overexpression Saos-2 cells by transfection of miR-218 vectors. ATPlite assay revealed that miR-218 significantly inhibited the proliferation of Saos-2 cells. And the migration and invasion of Saos-2 cells were also dramatically suppressed by miR-218, while there is no difference between Saos-2 cells and control vector transfected Saos-2 cells. Further study found proliferation associated TIAM1 gene, migration and invasion related MMP2, MMP9 were significantly inhibited by miR-218. To date, there are no studies showing the association between miR-218 and osteosarcoma.

In conclusion, our data suggest that the pharmacological manipulation of miR-218 expression may be a novel tool for the osteosarcoma treatment.

References

- Alajez NM, Lenarduzzi M, Ito E, et al (2011). MiR-218 suppresses nasopharyngeal cancer progression through downregulation of survivin and the SLIT2-ROBO1 pathway. *Cancer Res*, **71**, 2381-91.
- Dieckmann KP, Spiekermann M, Balks T, et al (2012). MicroRNAs miR-371-3 in serum as diagnostic tools in the management of testicular germ cell tumours. *Br J Cancer*, **107**, 1754-60.
- Habets GG, Scholtes EH, Zuydgeest D, et al (1994). Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. *Cell*, **77**, 537-49.
- He X, Dong Y, Wu CW, et al (2013). MicroRNA-218 inhibits cell cycle progression and promotes apoptosis in colon cancer by downregulating BMI1 polycomb ring finger oncogene. *Mol Med*, **18**, 1491-8.
- Hagman Z, Haffidadóttir BS, Ceder JA, et al (2013). miR-205 negatively regulates the androgen receptor and is associated with adverse outcome of prostate cancer patients. *Br J Cancer*, **108**, 1668-76.
- Jin Y, Peng D, Shen Y, et al (2013). MicroRNA-376c Inhibits Cell Proliferation and Invasion in Osteosarcoma by Targeting to Transforming Growth Factor-Alpha. *DNA Cell Biol*, **32**, 302-9.
- Kawauchi T, Chihama K, Nabeshima Y, et al (2003). The in vivo roles of STEF/Tiam1, Rac1 and JNK in cortical neuronal migration. *EMBO J*, **22**, 4190-201.
- Kinoshita T, Hanazawa T, Nohata N, et al (2012). Tumor suppressive microRNA-218 inhibits cancer cell migration and invasion through targeting laminin-332 in head and neck squamous cell carcinoma. *Oncotarget*, **3**, 1386-400.
- Li J, Liang S, Jin H, et al (2012). Tiam1, negatively regulated by miR-22, miR-183 and miR-31, is involved in migration, invasion and viability of ovarian cancer cells. *Oncol Rep*, **27**, 1835-42.
- Liu Y, Yan W, Zhang W, et al (2012). MiR-218 reverses high invasiveness of glioblastoma cells by targeting the oncogenic transcription factor LEF1. *Oncol Rep*, **28**, 1013-21.
- Long XH, Mao JH, Peng AF, et al (2013). Tumor suppressive microRNA-424 inhibits osteosarcoma cell migration and invasion via targeting fatty acid synthase. *Exp Ther Med*, **5**, 1048-52.
- Mao JH, Zhou RP, Peng AF, et al (2012). microRNA-195 suppresses osteosarcoma cell invasion and migration in vitro by targeting FASN. *Oncol Lett*, **4**, 1125-29.
- Nana-Sinkam SP, Croce CM (2013). Clinical applications for microRNAs in cancer. *Clin Pharmacol Ther*, **93**, 98-104.
- Ottaviani G, Jaffe N (2009). The epidemiology of osteosarcoma. *Cancer Treat Res*, **152**, 3-13.
- Osman A (2012). MicroRNAs in health and disease--basic science and clinical applications. *Clin Lab*, **58**, 393-402.
- Qin S, Ai F, Ji WF, et al (2013). miR-19a Promotes Cell Growth and Tumorigenesis through Targeting SOCS1 in Gastric Cancer. *Asian Pac J Cancer Prev*, **14**, 835-40.
- Takahashi M, Cuatrecasas M, Balaguer F, et al (2012). The clinical significance of MiR-148a as a predictive biomarker in patients with advanced colorectal cancer. *PLoS One*, **7**, e46684.
- Venkataraman S, Birks DK, Balakrishnan I, et al (2013). MicroRNA 218 acts as a tumor suppressor by targeting multiple cancer phenotype-associated genes in medulloblastoma. *J Biol Chem*, **288**, 1918-28.
- Wu X, Zhong D, Gao Q, et al (2013). MicroRNA-34a Inhibits Human Osteosarcoma Proliferation by Downregulating Ether à go-go 1 Expression. *Int J Med Sci*, **10**, 676-82.