MicroRNA-432 is downregulated in osteosarcoma and inhibits cell proliferation and invasion by directly targeting metastasis-associated in colon cancer 1

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Received July 3, 2018; Accepted October 12, 2018

DOI: 10.3892/etm.2018.7029

Abstract. The aberrant expression of microRNAs (miRNAs/miRs) in osteosarcoma (OS) has been demonstrated in previous studies, and deregulation of miRNA expression has been associated with several types of cancer, including OS development and progression. Therefore, identifying the functional role of miRNAs in OS onset and development may facilitate the identification of novel and effective therapeutic targets for the treatment of patients with OS. Previous studies have demonstrated that miR-432 is involved in tumor formation and progression in several types of cancer. However, the expression pattern, functional role and underlying mechanism of miR-432 in OS remain unknown. In the current study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to measure miR-432 expression levels in OS tissue samples and cell lines. The effect of miR-432 overexpression on OS cell proliferation and invasion was detected using Cell Counting Kit-8 and Transwell invasion assays, respectively. Bioinformatics analysis was used to predict metastasis-associated in colon cancer 1 (MACC1) as a putative target of miR-432 and this was confirmed using a dual-luciferase reporter assay, RT-qPCR and western blot analysis. The current study demonstrated that miR-432 expression levels were significantly reduced in OS tissue samples and cell lines. In addition, functional assays revealed that overexpression of miR-432 significantly decreased OS cell proliferation and invasion. Furthermore, MACC1 was identified as a direct target gene of miR-432 in OS. MACC1 expression levels were significantly increased in OS tissue samples and an inverse correlation was observed between miR-432 and MACC1 expression in OS tissue samples. In addition, rescue experiments demonstrated that overexpression of MACC1 partially reversed the anti-proliferative and anti-invasive effects of miR-432 in OS cells. In conclusion, the present study demonstrated that miR-432 inhibited OS cell proliferation and invasion in vitro through direct targeting of MACC1, and miR-432 may be a potential therapeutic target for the treatment of OS.

Introduction

Osteosarcoma (OS) originates from primitive transformed cells of mesenchymal origin (1) OS is the most common aggressive primary bone malignancy in children and young adults and accounts for ~2.4% of all pediatric malignancies worldwide (2). The estimated morbidity rate for OS is 4.4 individuals per million worldwide, with a peak incidence at 15-19 years of age (3). Currently, the standard treatment for patients with OS is a combination of therapies consisting of preoperative chemotherapy, surgical resection and adjuvant postoperative chemotherapy (4). Although significant progress in diagnostic and surgical techniques has been made, the clinical outcome of patients with OS remains unsatisfactory due to the high incidence of metastasis and disease recurrence (5). Following combined modality therapy, ~80% of patients with OS will develop a secondary metastasis, which is a leading contributor of cancer-associated mortality (6). It is therefore important to gain a greater understanding of the mechanisms underlying OS development and progression, as well as identify potentially novel therapeutic approaches that may improve the prognosis of patients with OS.

MicroRNAs (miRNAs/miRs) are a class of small non-coding RNA molecules that are recognized as crucial gene modulators (7) miRNAs regulate gene expression by base pairing with complementary target sites in the 3'-untranslated region (3'-UTR) of target genes, which results in translational repression and/or mRNA degradation (8). Previous studies have revealed that miRNAs can regulate multiple cellular processes, including cell proliferation, cell cycle, apoptosis, metastasis, epithelial-mesenchymal transition and angiogenesis (9,10). In addition, miRNAs are involved

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Key words: osteosarcoma, microRNA-432, proliferation, invasion, metastasis-associated in colon cancer 1
Reverse transcription -quantitative polymerase chain reaction (RT-qPCR) and Transwell invasion assays were performed 48 h following transfection. The cell proliferation assay and western blotting were performed at 24 and 72 h post-transfection, respectively.

RT-qPCR. Total RNA was extracted from tissue samples or cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according on the manufacturer’s protocol. To examine miR-432 expression, total RNA was reverse transcribed into cDNA using the TaqMan™ miRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions for reverse transcription were as follows: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. qPCR was subsequently performed using the TaqMan™ MicroRNA assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling conditions for qPCR were as follows: 50°C for 2 min, 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 sec; and annealing/extension at 60°C for 60 sec. To examine MACC1 mRNA expression, total RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The thermocycling conditions for reverse transcription were as follows: 37°C for 15 min and 85°C for 5 second. qPCR was subsequently performed using the SYBR® Premix Ex Taq™ kit (Takara Biotechnology Co. Ltd.). The thermocycling conditions for reverse transcription were as follows: 5 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec. The following primer pairs were designed and used for the qPCR: miR-432 forward, 5’-AAC GAG ACG ACA GACA GAC-3’, and reverse, 5’-CTT GGAGA TGG GGT TCC-3’; U6 forward, 5’-GCT TCG GCA GCA CAT ATA CTA AAA T-3’, and reverse, 5’-CTT GGA GTA GGT CAT TGG GT-3’. The following primer pairs were designed and used for the qPCR: miR-432 forward, 5’-AAC GAG ACG ACA GACA GAC-3’, and reverse, 5’-CTT GGAGA TGG GGT TCC-3’; U6 forward, 5’-GCT TCG GCA GCA CAT ATA CTA AAA T-3’, and reverse, 5’-CTT GGA GTA GGT CAT TGG GT-3’. The following primer pairs were designed and used for the qPCR: miR-432 forward, 5’-AAC GAG ACG ACA GACA GAC-3’, and reverse, 5’-CTT GGAGA TGG GGT TCC-3’. The following primer pairs were designed and used for the qPCR: miR-432 forward, 5’-AAC GAG ACG ACA GACA GAC-3’, and reverse, 5’-CTT GGAGA TGG GGT TCC-3’; MACC1 forward, 5’-CAACATGCGAGGTACTAC-3’, and reverse, 5’-AAC GAG ACG ACA GACA GAC-3’. The following primer pairs were designed and used for the qPCR: miR-432 forward, 5’-AAC GAG ACG ACA GACA GAC-3’, and reverse, 5’-CTT GGAGA TGG GGT TCC-3’; MACC1 forward, 5’-CAACATGCGAGGTACTAC-3’, and reverse, 5’-AAC GAG ACG ACA GACA GAC-3’. The following primer pairs were designed and used for the qPCR: miR-432 forward, 5’-AAC GAG ACG ACA GACA GAC-3’, and reverse, 5’-CTT GGAGA TGG GGT TCC-3’; MACC1 forward, 5’-CAACATGCGAGGTACTAC-3’, and reverse, 5’-AAC GAG ACG ACA GACA GAC-3’.

Materials and methods

Human tissue collection. The present study analyzed tissue samples from patients with OS. In total, 21 pairs of OS tissue and adjacent non-tumorous tissue samples were collected from patients (15 males, 6 females; age range, 14-42 years) who had undergone surgery at Jining No. 1 People’s Hospital (Jinan, China) from June 2014 to April 2017. All patients enrolled in the present study did not undergo chemotherapy, radiotherapy or any other treatment prior to surgical resection. All tissue samples were frozen in liquid nitrogen and stored at -80°C until further use. The present study was approved by the Medical Ethics Committee of Jining No. 1 People’s Hospital (ref. no. 20140712) and written informed consent was obtained from each patient enrolled in the study.

Cell culture and transfection. OS cell lines, HOS, U2OS and MG-63, as well as the normal human osteoblast cell line, hFOB1.19 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (both Thermo Fisher Scientific, Inc.) and maintained at 37°C in a 5% CO2-humidified incubator. miR-432 mimics (5’-UCU UGGAGUAGCAUUUGGGUG-3’) and negative control miRNA mimics (miR-NC; 5’-UUCUCCGAACUGUGUCACGU TT-3’) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The MACC1 expression plasmid pcDNA3.1-MACC1 and the empty pcDNA3.1 plasmid were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Cells were plated in 6-well plates at a density of 6x10^5 cells/well and cultured at 37°C until 60-70% confluence was reached. Cells were subsequently transfected with miR-432 mimics (100 pmol), miR-NC (100 pmol), pcDNA3.1-MACC1 (4 µg) or empty pcDNA3.1 (4 µg) vectors using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Transwell invasion assays were performed following transfection. The cell proliferation assay and western blotting were performed at 24 and 72 h post-transfection, respectively.

Cell proliferation assay. The Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to examine OS cell proliferation. At 24 h following transfection, cells were harvested and seeded into 96-well plates at a density of 3x10^3 cells/well. The CCK-8 assay was performed following 0, 24, 48 and 72 h of culture. A total of 10 µl CCK-8 solution was added to each well and cells were incubated at 37°C for an additional 2 h. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Transwell invasion assay. Following 48 h transfection, OS cells were harvested and resuspended in serum-free DMEM at a density of 2x10^5 cells/ml. For the invasion assay, a 200-µl cell suspension was added to the upper chamber of a 24-well Transwell permeable support with 8 µm pores (Corning Life Sciences, Acton, MA, USA) were coated with Matrigel® (BD Biosciences, San Jose, CA, USA) at room temperature overnight. DMEM supplemented with 20% FBS was added to the lower chamber to serve as a chemoattractant. Following
miR-432, microRNA-432; OS, osteosarcoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

24-h incubation, the culture medium in the upper chamber was discarded and the non-invasive cells were removed using a cotton swab. Cells in the lower chamber were fixed in 95% ethanol at room temperature for 15 min and stained with 0.5% crystal violet at room temperature for 15 min. The IX71 inverted microscope (magnification, x200; Olympus Corporation, Tokyo, Japan) was used to image cells in the lower chamber. The number of invasive cells was calculated by counting the number of cells in five randomly selected visual fields of view.

Bioinformatics analysis. Bioinformatics analysis was used to predict the putative target genes of miR-432. Two publicly available algorithms, TargetScan (www.targetscan.org/) and miRDB (mirdb.org/miRDB/index.html) identified a conserved miR-432 binding site in the 3'-UTR of MACC1.

Luciferase reporter assay. The 3'-UTR of MACC1 containing the wild-type miR-432 binding site sequence and the corresponding mutant construct, generated by mutating the seed sequences of the miR-432 binding site, were designed and synthesized by Shanghai GenePharma Co., Ltd. These fragments were cloned into the pMIR-REPORT™ luciferase vector (Promega Corporation, Madison, WI, USA) to generate pMIR-MACC1-3'-UTR wild-type and pMIR-MACC1-3'-UTR mutant constructs, respectively. Cells were transfected in triplicate in 24-well plates at a density of 1.0x10⁵ cells per well and maintained at 37°C in a 5% CO₂-humidified incubator. Cells were co-transfected with pMIR-MACC1-3'-UTR wild-type or pMIR-MACC1-3'-UTR mutant or miR-432 mimics or miR-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following incubation for 48 h, cells were collected and luciferase activity was detected using a Dual-Luciferase® Reporter assay system (Promega Corporation), according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

Western blot analysis. Total protein was extracted from cells and tissue samples using radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Total protein was quantified using a bicinchoninic acid assay and equal quantities of protein (30 μg) were separated via 10% SDS-PAGE. The separated proteins were transferred onto polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology) and blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween® 20 (TBST) at room temperature for 2 h. Membranes were incubated with primary antibodies against MACC1 (dilution, 1:1,000; cat. no. ab106579) and GAPDH (dilution, 1:1,000; cat. no. ab181603; both Abcam, Cambridge, UK) overnight at 4°C. Membranes were washed three times with TBST and then incubated with goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (dilution, 1:2,500; cat. no. ab6721; Abcam) for 2 h at room temperature. Membranes were subsequently washed with TBST and protein bands were visualized using an enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc.). Protein expression was quantified using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc.) with GAPDH as the loading control.

Statistical analysis. Data are presented as the mean ± standard deviation. All statistical analyses were performed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA) and Graph Pad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). The Student’s t-test (paired or unpaired) was used to analyze differences between two groups. One-way analysis of variance followed by Tukey’s post hoc test was used to analyze differences among multiple groups. Spearman’s correlation analysis was used to analyze the association between miR-432 and MACC1 expression. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-432 is downregulated in OS tissue samples and cell lines. The expression level of miR-432 OS tissues and normal adjacent control tissues was first detected by RT-qPCR analysis. The results demonstrated that miR-432 expression was significantly decreased in OS tissues when compared with adjacent non-tumorous tissue samples from patients with OS (P<0.05; Fig. 1A). In addition, the expression level of miR-432 was significantly decreased in all three OS cell lines when compared with the normal human osteoblast cell line, hFOB1.19 (P<0.05; Fig. 1B). These results suggest that miR-432 may serve an important role in OS progression.
miR-432 suppresses OS cell proliferation and invasion. To investigate the role of miR-432 in the development of OS, cell proliferation and invasion were analyzed in OS cell lines, HOS and U2OS, following transfection with miR-432 mimics or miR-NC. The results presented in Fig. 1 suggest that HOS and U2OS cell lines exhibit a relatively low miR-432 expression profile, and they were therefore used in all functional experiments. The expression level of miR-432 significantly increased in OS cells transfected with miR-432 mimics compared with the miR-NC-transfected group (P<0.05; Fig. 2A). The CCK-8 assay was used to examine the effect of miR-432 on OS cell proliferation. HOS and U2OS cell proliferation significantly decreased following transfection with miR-432 mimics when compared with the miR-NC-transfected group (P<0.05; Fig. 2B). Transwell invasion assays were used to examine the effect of miR-432 on OS cell invasion. The invasive ability of HOS and U2OS cells was significantly decreased following transfection with miR-432 mimics compared with miR-NC-transfected cells (P<0.05; Fig. 2C). Taken together, these results suggest that miR-432 may function as a tumor suppressive miRNA in OS progression via inhibition of OS cell proliferation and invasion.
MACC1 is a direct target of miR-432 in OS cells. To investigate the underlying mechanism of miR-432 in OS cell proliferation and invasion, bioinformatics analysis was used to predict the putative targets of miR-432. Among these candidates (213 genes) MACC1 was identified as a putative target gene of miR-432 (Fig. 3A). Previous studies have demonstrated that MACC1 is involved in OS development; therefore, the role of MACC1 in OS was selected for further investigation in the current study (23,24). Luciferase reporter plasmids were generated and used in luciferase reporter assays to validate the direct interaction between miR-432 and the 3'‑UTR of MACC1. In both cell lines, miR-432 overexpression was associated with a significant decrease in MACC1-3'-UTR wild-type luciferase activity when compared with MACC1-3'-UTR mutant, which did not significantly alter the luciferase activity (P<0.05; Fig. 3B). To investigate whether miR-432 regulates endogenous MACC1 expression in OS cells, endogenous MACC1 expression was analyzed in OS cell lines HOS and U2OS following transfection with miR-432 mimics or miR-NC. The mRNA and protein expression levels of MACC1 were significantly decreased in OS cells transfected with miR-432 mimics compared with miR-NC (P<0.05; Fig. 3C and D). These results confirm MACC1 as a direct target gene of miR-432 in OS cells.

MACC1 expression is upregulated in OS tissue samples. To further investigate the association between miR-432 and MACC1 in OS, MACC1 expression was analyzed in OS and adjacent non-tumorous tissue samples from patients with OS. The mRNA expression level of MACC1 was significantly increased in OS tissues when compared with adjacent non-tumorous tissue samples (P<0.05; Fig. 4A). Similarly, the protein expression level of MACC1 was significantly increased in OS tissue samples compared with adjacent non-tumorous tissue samples (P<0.05; Fig. 4B and C). Furthermore, Spearman's correlation analysis was used to examine the association between miR-432 and MACC1 expression in OS tissue samples, which revealed an inverse correlation (r=-0.5536; P=0.0092; Fig. 4D). These results provide additional evidence that MACC1 is a direct target gene of miR-432 in OS.

Overexpression of MACC1 reduces the suppressive effects of miR-432 in OS cell proliferation and invasion. The results presented thus far suggest that MACC1 is a direct target of miR-432 in OS cells. In addition, the observed tumor suppressive effects of miR-432 in OS progression in vitro may be mediated by MACC1. To investigate this hypothesis, MACC1 expression was analyzed in OS cell lines HOS and U2OS following co-transfection with miR-432 mimics or miR-NC and pcDNA3.1-MACC1 or pcDNA3.1. The protein expression levels of MACC1 significantly decreased in HOS and U2OS cells transfected with miR-432 mimics compared with miR-NC (P<0.05; Fig. 5A). In addition, rescue experiments demonstrated that overexpression of MACC1 partially reversed the suppressive effect of miR-432 on OC cell proliferation and invasion (P<0.05; Fig. 5B and C). These results demonstrated that MACC1, as
a target gene of miR-432, may mediate the inhibitory role of miR-432 in OS cell proliferation and invasion.

Discussion

The aberrant expression of miRNAs with oncogenic and tumor suppressive roles in OS has been demonstrated in several studies (25-27), and deregulation of miRNA expression has been associated with several types of cancer, including OS (28). Therefore, identifying the functional role of miRNAs in OS onset and development may facilitate the identification of novel and effective therapeutic targets for the treatment of patients with OS. In the present study, the expression pattern, functional role and underlying mechanism of miR-432 was examined in OS. The current study demonstrated that miR-432 expression levels were significantly reduced in OS tissue samples and cell lines. In addition, functional assays revealed that overexpression of miR-432 significantly decreased OS cell proliferation and invasion. Furthermore, MACC1 was identified as a direct target of miR-432 in OS cells. MACC1 expression levels were significantly increased in OS tissue samples and an inverse correlation was observed between miR-432 and MACC1 expression in OS tissue samples. In addition, rescue experiments demonstrated that overexpression of MACC1 partially reversed the suppressive effects of miR-432 in OS cells. The results from the present study suggest that miR-432 may function as a tumor suppressive miRNA in OS.

Deregulated miR-432 expression has been identified in several types of cancer. In lung cancer, downregulation of miR-432 in lung adenocarcinoma cell lines and tissues was associated with a higher clinical stage in patients with lung adenocarcinoma (18). In human neuroblastoma cells, miR-432 decreases cell proliferation, promotes G0-G1 cell cycle arrest and induces neurite projections (19). In hepatocellular carcinoma, miR-432 overexpression inhibits cell proliferation and colony formation, promotes G0-G1 cell cycle arrest in vitro and reduces tumor growth in vivo via deactivation of the Wnt/β-catenin signaling pathway (20). In prostate cancer, ectopic expression of miR-432 inhibits activation of the Wnt/β-catenin signaling pathway and participates in the regulation of cell proliferation and apoptosis (21). These findings suggest that miR-432 may be a potential therapeutic target for patients with these specific types of cancer.

Previous studies have identified a number of direct target genes of miR-432, including E2F transcription factor 3 and anexelektos in lung adenocarcinoma (18), nestin and REST corepressor 1 in neuroblastoma (19), and tripartite motif-containing protein 29 and pygopus homolog 2 in prostate cancer (21). Therefore, miR-432 may be a potential biomarker for the diagnosis of patients with these specific types of cancer.
in patients with OS (23). MACC1 was previously identified as an independent prognostic factor predicting the overall survival of patients with OS (23). Furthermore, MACC1 serves important roles in the development and progression of OS by regulating cell proliferation, cycle, apoptosis, invasion, colony formation and tumorigenicity in vitro and in vivo (24). Therefore, inhibition of MACC1 via miR-432 target therapy may be a suitable therapeutic approach for the treatment of patients with OS.

In conclusion, the current study demonstrated that miR-432 was downregulated in OS tissues and cell lines. miR-432 over-expression suppressed OS proliferation and invasion through direct targeting of MACC1. Identification and characterization of the expression patterns and cellular function of miR-432 in OS may be important to understand the mechanisms underlying OS development and progression, as well as to identify novel therapeutic targets for the treatment of OS.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

DH designed the study. DL and ZZ performed the experiments. DH carried out the statistical analysis. All authors read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Jining No. 1 People’s Hospital (Shandong, China). The present study was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of Jining No. 1 People’s Hospital. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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