

## Original Article

# Down-regulation of miR-451 promotes invasion and proliferation of human osteosarcoma U2OS cells via RAB14-mediated AMPK-Akt-mTOR signaling pathway

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**Abstract:** MicroRNAs (miRNAs) have been reported function as tumor suppressors or oncogenes. Ectopic expression of miRNA may lead to dysfunction of cell biological behaviors. MiR-451 is down-regulated and functions as a tumor suppressor mediated by different signal pathways. However, the expression pattern and the regulation mechanism of miR-451 remain to be elucidated. Our results showed that miR-451 was frequently down-regulated in osteosarcoma tumor and cell lines. Over-expression of miR-451 in osteosarcoma cell line significantly inhibited cell proliferation and invasion, and promoted cell adhere to matrix by down-regulating Ras-related protein 14 (RAB14) expression. SiRNA mediated silencing of RAB14 phenocopied the over-expression of miR-451. Together, these findings demonstrated that miR-451 functions as a tumor suppressor via RAB14-mediated AMPK-AKT-mTOR signal pathway.

**Keywords:** miR-451, RAB14, AMPK-AKT-mTOR pathway, tumor suppressor

## Introduction

Osteosarcoma is one of the most common primary malignant bone tumor in China, which accounts for about 34% of malignant bone tumor [1]. Osteosarcoma possesses the characteristics of aggressiveness, recurrence, metastasis, etc [2]. The 5-year cumulative survival rate of osteosarcoma has been significantly improved due to the application of neoadjuvant chemotherapy, while some patients are not sensitive to chemotherapy. Tumor recurrence/metastasis is also a difficult problem for the treatment [3]. Invasion/metastasis is the main reason for poor curative effect and death of patients [4]. However, the molecular mechanism of invasion and metastasis remains to be elucidated. Further researches on the molecular mechanism will provide possible gene therapy targets and prognosis factors for osteosarcoma invasion and metastasis.

Tumor invasion and metastasis is a complex process of dynamic variation with multiple factors and every process is regulated by multiple genes or proteins accurately [5]. Although fundamental researches on osteosarcoma have

been emerging in endlessly, the role of microRNA in the occurrence and development of osteosarcoma remains to be elucidated. Small RNA (microRNAs, miRNAs) is a kind of endogenous non encoding RNA with a length of about 22 bp, which plays an important role in the occurrence and development of osteosarcoma. MiRNAs can regulate the proliferation and metastasis of tumor cells by targeting certain oncogenes or tumor suppressor genes to suppress or promote tumor [6]. For example, miR-21 promoted cell invasion and migration of osteosarcoma cells by inhibiting the expression of tumor suppressor gene RECK [7]. MiR-93 inhibited the expression of p21 by regulating the expression of E2F1 [8]. MiR-145 was found to inhibit the invasion and metastasis of lung cancer cells [9]. These miRNAs have different roles in cancer cells and the specific mechanism of miRNAs affecting the invasion and migration is important for our understanding about tumorigenesis [10].

MicroRNA-451 is a multifunctional miRNA which has been widely confirmed to play a significant role in differentiation of hematopoietic system, blood system diseases, neurological

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development and cardiovascular diseases [11]. At present, related studies indicated that miR-451 is a regulator of cancer proliferation, which can not only inhibit the proliferation of different tumor cells, but also inhibit the differentiation of tumor cells and promote the apoptosis of tumor cells. Related studies indicated that the expression of miRNA-451 has significant differences in gastric cancer, colon cancer, glioma, bladder cancer, pancreatic cancer, breast cancer and myeloma multiple when compared with normal control group, respectively [12-16]. Report about the mechanism of microRNA-451 regulation on osteosarcoma was rare. Y Tian et al. reported miR-451 could inhibit the CAB39/AMPK signaling pathway by inhibiting the expression of CAB39 gene [17].

One microRNA usually regulates multiple target genes while a target gene can be regulated by multiple microRNAs. MicroRNA may act as different target genes in different cells. Tian Zhang et al. showed that miR-451 could target RAB14 to enhance the sensitivity of radiotherapy for the treatment of rhinitis cancer. Zhiyong Li et al. reported that liver could inhibit the growth of osteosarcoma cells by inhibiting receptor homolog-1 miR-451. The study of miR-451 target genes will broaden our understanding of its function in different tumors.

### Materials and methods

#### General clinical data

The study included 20 patients with osteogenic sarcoma in the first affiliated Hospital of Xinxiang Medical University, among them, 14 were male and 6 were female. Samples of osteogenic sarcoma and peripheral non malignant tissue were collected before treatment. The study was approved by the Ethics Committee of the first affiliated Hospital of Xinxiang Medical University. All the patients provided written informed consent for the procedure.

#### Cell culture

Human osteosarcoma cell lines 143B, MG63, hFOB, Saos-2 and U2OS were bought from China Center for Type Culture Collection (CCTCC), cells were all cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were incubated in a humidified atmosphere with 5% CO<sub>2</sub> in 37°C.

#### Cell transfection

MiR-451 mimics was synthesized according to its mature sequence searched on miRBase website (miRBase accession Number: MI00-00148): 5'-AAACCGUUACC AUUAC UGAGUU-3', 3'-CUCAGUAAUGGUAACGGUUUUU-5'. MiRNA independent sequence control: 5'-UUCUCCGAA-CGUGUCACGUTT-3', 3'-TTAAG AGGCUUGCACAGUGCA-5' (Guangzhou RiboBiotechnology Company). Transfection procedures were performed according to manufacturer's instructions. Four groups were set in this experiment: A. Blank control group (with none transfection); B. Negative control group (transfected with arbitrary nucleotide sequence); C. MiR-451 mimics group (transfected with 100 nmol/L miR-451 mimics); D. MiR-451 inhibitor group (transfected with miR-451 inhibitor). After successfully transfection, cells were incubated in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a 5% CO<sub>2</sub> humidified atmosphere, then collected for various detection. SiRNA-RAB14 (Guangzhou RiboBiotechnology Company) was used for specific silencing of RAB14 gene expression. The oligonucleotide (50 nM) and siRNA (100 nM) transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

#### Quantitative PCR

Total RNA was isolated using Trizol reagent. cDNA was synthesized by reverse transcription using PrimeScripts® RT reagent Kit. The expression level of miR-451 in bladder cancer cell lines was detected by quantitative real-time PCR. The PCR primers for RAB14 were as follows: forward 5'-CGCTCGAGATGGCA AC TGCAC CATAACAAC-3' and reverse 5'-CGGAATTC-CTAGCAGCC ACAGCCTT CTC-3'. The PCR primers for GAPDH were as follows: forward 5'-AG AA GGT GGGGCTCATTG-3' and reverse 5'-AGGGGCCATCCACAGTCTTC-3'. Experiments were repeated 3 times. GAPDH was used as internal control. The amount of target gene miR-451 relative to the internal control was calculated using the cycle threshold ( $\Delta\Delta CT$ ) method as follows: relative expression =  $2^{-\Delta\Delta CT}$ ,  $\Delta\Delta CT = \Delta CT$  (test)  $\Delta CT$  (calibrator).

#### MTT assay

U2SO cells were seeded in 96-well plates ( $1 \times 10^4$ /well) with 200  $\mu$ L culture medium per

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well, 24 hours later, the culture medium was replaced, 20  $\mu$ L MTT solution was added to each well (final concentration of 5 mg/mL). After incubation in MTT solution at 37°C for 4 h, remove the medium and gently rinse cells with PBS for twice, and add 150  $\mu$ L DMSO to dissolve purple crystals by low-speed shock on shaking table for 15~20 min. Optical density (OD) values were detected at 570 nm wavelength on a microplate reader. All experiments in each group set were done with 6 replicates per experiment and repeated 3 times. MTT assay was conducted after transfection for 1 d, 2 d, 3 d, 4 d and 5 d, respectively.

### *Plate clone formation assay*

Cells in logarithmic growth phase (pretreated with siRNA or oligonucleotide transfection for 48 h) were seeded in six-well plates, and then exposed to radiation at a dose rate of 3 Gy/min using 6-MV X-rays from linear accelerators (Varian 2300EX, Varian, Palo Alto, CA). After incubation at 37°C for 9 to 14 days, the plates were fixed with 100% methanol and then stained with 1% crystal violet. Colonies containing over 50 cells were counted by microscopic inspection. All experiments were done with at least 3 replicates per experiment and repeated 3 times.

### *Transwell invasion assay*

Cell invasion ability was assessed using a Transwell chamber (Milipore, US) with an 8.0  $\mu$ L pore polycarbonate membrane filter. 50  $\mu$ L serum-free RPMI-1640 medium supplemented with 10 g/L BSA was added into the chamber, and incubated for 30 min at 37°C to hydrate the basement membrane. Cells were seeded in the upper chamber at a density of  $2 \times 10^4/200$   $\mu$ L/well with serum-free RPMI-1640 medium, and the lower chambers were filled with 500  $\mu$ L RPMI-1640 supplemented with 10% fetal bovine serum. After culturing at 37°C in a 5% CO<sub>2</sub> atmosphere for 24 h, the medium on the upper chamber was removed, cells were washed carefully with PBS for 3 times, fixed with 4% paraformaldehyde at 4°C for 1 h and stained with 0.25% crystal violet. Then cells on the upper basement membrane were wiped clean using cotton swab. The cell invasion ability was assessed by counting the number of cells that had migrated to the lower side of the membrane. Cells in five visual fields (magnification,  $\times 400$ ) selected randomly were counted in each Transwell chamber.

### *Cell adhesion assay*

Adhesion assays were performed in 96-well culture plates. Wells were pre-coated with 2  $\mu$ g matrix. The transfected cells were seeded 96-well culture plates to at  $4 \times 10^5$ /well and incubated for 2 h at 37°C. Non-adhesion cells were removed by gentle washing with PBS, adhesion cells were reacted with 5 mg/mL MTT solution for 4 h at 37°C. Remove culture medium, add 150  $\mu$ L DMSO and incubate in shaking incubator for 10 min. Optical density (OD) values were measured at 490 nm wavelength. The adhesion rate of control cells was assumed to be 100%. Experiments were repeated 3 times.

### *Western blot*

Cells were collected after transfection for 72 h, total protein was prepared and quantitated with BCA protein assay. Equal amounts of protein were separated on SDS-PAGE gels and transferred to PVDF membranes. The membrane was incubated with rabbit polyclonal anti-RAB14 antibody (Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-GAPDH antibody (CWBiotech, Beijing, China) overnight at 4°C. After washing with PBST, membranes were incubated with horseradish peroxidase HRP-conjugated secondary antibodies for 2 h at room temperature and visualized by the enhanced chemiluminescence (ECL) assay kit. GAPDH was used as internal control. Experiments were repeated at least 3 times.

### *Statistical analysis*

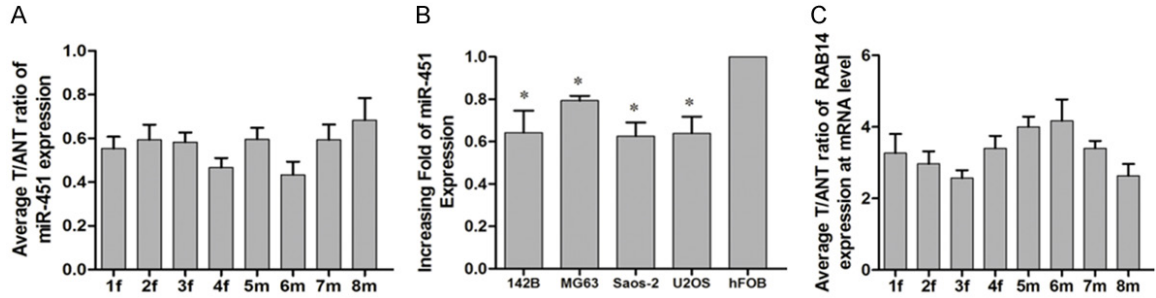
All the data were shown as mean  $\pm$  SD. The SPSS 19.0 was applied to complete data processing. Comparison among groups was analyzed by single factor analysis of variance (ANOVA), while differences between two mean were determined by two-tailed Student's t-test.  $P < 0.05$  was considered as statistically significant.

## **Results**

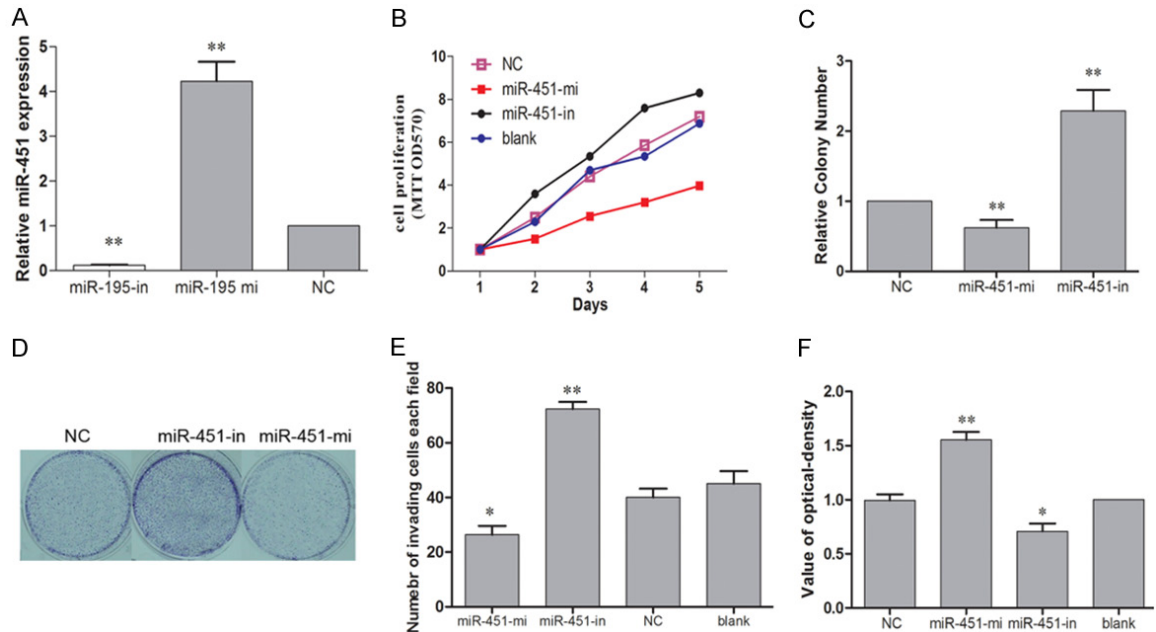
### *Expression levels of miR-451 are low in the osteosarcoma tissues and cell lines*

4 women and 4 men were randomly selected from the 40 patients with osteosarcoma, for measurement of the expression of miR-451 in osteosarcoma tissues versus in adjacent normal tissues, and the expression in 4 common osteosarcoma cells versus in normal osteoblast cell lines by qPCR methods. As shown in

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**Figure 1.** Relative expression of miR-451 in osteosarcoma tissues and cell lines. (A) Relative expression of miR-451 in osteosarcoma tissues (T) versus in adjacent normal tissues (ANT) in 8 patients measured by qPCR. F, female; m, male. (B) Relative expression of miR-451 in 4 osteosarcoma cell lines (142B, MG63, Saos-2 & U2OS) versus in normal osteoblast cell line hFOB measured by qPCR, which was done in parallel and repeated at least 3 times. \* $P < 0.05$ . (C) Relative expression of RAB14 in osteosarcoma tissues versus in adjacent normal tissues in the 8 sets of samples as shown in (A).



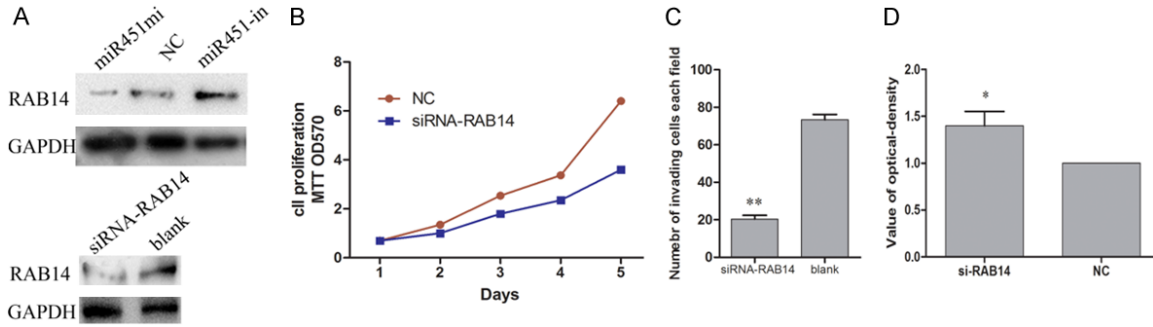
**Figure 2.** Influence of over-expression or down-regulation of miR-451 on U2SO cell proliferation, invasion and adhesion. (A) Measurement of the relative expression of miR-451 by qPCR after transfection. (B) Measurement of the influence of miR-451 on cell proliferation by MTT assay. Measurement of the viability of the cells was conducted at 1 d, 2 d, 3 d, 4 d & 5 d after the transfection. NC, nucleotide of any sequence; blank, blank control. (C) Measurement of cell proliferation in the miR-451-mi and miR-451-in transfection group versus in the negative control group by colony-forming assay. (D) Representative results of the colony-forming assay as shown in (C). (E) Measurement of changes in cell invasion by Transwell assay. \* $P < 0.05$ , \*\* $P < 0.01$ . (F) Measurement of the influence of over-expressed miR-451 on cell adhesion by cell adhesion assay. Measurement of absorbance was conducted at 490 nm. \* $P < 0.05$ , \*\* $P < 0.01$ .

**Figure 1A**, the expression level of miR-451 is lower in osteosarcomas than in adjacent normal tissues. Similarly, the expression levels of miR-451 are significantly lower in 142B, MG63, Saos-2 and U2OS cells than in normal osteoblast hFOB ( $P < 0.05$ ). The expression of RAB14 gene at the mRNA level in osteosarcoma tis-

sues versus in adjacent normal tissues was also measured for the 8 patients. As shown in **Figure 1C**, the expression level of RAB14 is higher in osteosarcomas than in normal tissues. Our data indicated that miR-451 is expressed at low levels in osteosarcoma tissues and cell lines; and RAB14 is expressed at



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**Figure 3.** Influence of down-regulated RAB14 on the proliferation, invasion and adhesion of osteosarcoma cells. A. Influence of the transfection of miR-451-mi and miR-451-in into U2SO cells on RAB14 protein expression measured by Western blot, which is done in parallel and repeated at least three times. The results showed significantly down-regulated RAB14 protein expression by siRNA-RAB14. B. Changes in cell viability in siRNA transfection group versus in NC control group measured by MTT assay. C. Influence of siRNA-RAB14 on cell invasion measured by Transwell assay. D. Influence of siRNA-RAB14 on cell adhesion measured by cell adhesion assay. \* $P < 0.05$ , \*\* $P < 0.01$ .

high levels in osteosarcoma tissues, which is consistent with the results of the previous study on high expression level of RAB14 in osteosarcoma cells.

### *Over-expression of miR-451 in U2SO cells significantly improves cell proliferation, invasion and adhesion*

To explore the influence of abnormal expression of miR-451 on osteosarcoma, the relative expression of miR-451 was measured 48 hours after the transfection of miR-451 mimic and inhibitor into U2SO cells. The efficiencies of miR-451-mi and miR-451-in transfection are shown in **Figure 2A**. MTT and colony-forming assays were conducted to assess the influence of miR-451 on the proliferation of U2SO cells; and the results showed that over-expressed miR-451 can inhibit the U2SO cell proliferation while the miR-451 transfection group had opposite results (**Figure 2C** and **2D**). The Transwell assay showed that the over-expression of miR-451 can significantly inhibit the U2SO cell invasion (**Figure 2E**). The cell adhesion assay indicated significantly increased cell adhesion in the over-expressed miR-451 group than in other groups.

### *MiR-451 regulates through targeted regulation of RAB14 expression*

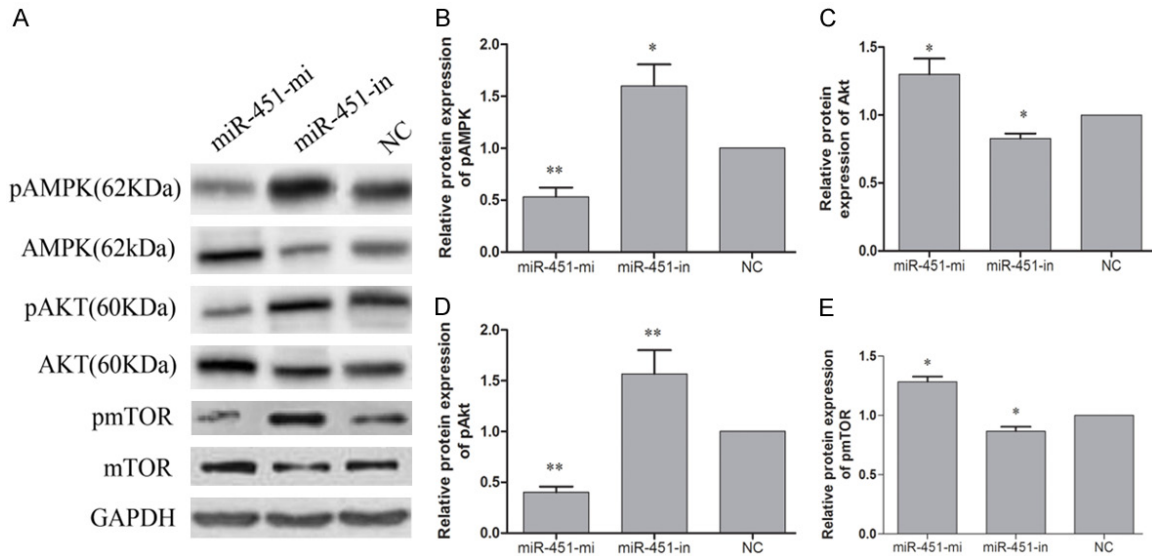
The study of Tian Zhang et al. reported that miR-451 can enhance the radio sensitivity of nasopharyngeal carcinoma through targeted regulation of RAB14. However, whether and how miR-451 regulates the expression of

RAB14 protein in osteosarcoma cells are yet to know. MiR-451 is expressed at low levels, and RAB14 at high levels in the osteosarcoma tissues (**Figure 1C**). According to the experimental results of Western blot, the miR-451-mi transfection group showed significantly down-regulated RAB14 protein expression compared to the NC control group, while miR-451-in transfection group has an opposite results. According to the MTT, colony-forming and cell adhesion assays conducted after the transfection of siRNA, which specifically down-regulates the expression of RAB14 protein, into U2SO cells (**Figure 3A**), siRNA-RAB14 influences the cell phenotype the same way as miR-451 over-expression does (**Figure 3B-D**). In conclusion, miR-451 can influence the cell proliferation, invasion and adhesion by regulating the expression of RAB14 gene.

### *RAB14 regulation is correlated with AMPK-AKT-mTOR pathway*

Changes in the protein expression of RAB14-related pathways after the miR-451-mi and miR-451-in transfection are measured. As shown in **Figure 4A**, there is a significant decrease in the pAMPK expression level, and substantially increased AMPK level in the miR-451-mi transfection group, as well as significantly decreased expression levels of corresponding downstream proteins pAKT and pmTOR. The miR-451-in transfection group sees an opposite trend. In conclusion, the influence of miR-451 on the cell proliferation, invasion and adhesion through targeted gene

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**Figure 4.** Method of Western blot used to measure the expression of downstream proteins of AMPK. A. Changes in the expression level of pAMPK, AMPK, pAKT, AKT, pmTOR & mTOR protein with the over-expression or down-regulation of miR-451. B-E. Gray values of pAMPK, pAKT, AKT and mTOR protein expression in the miR-451-mi transfection group versus in the miR-451-in group and NC control group. \*P<0.05, \*\*P<0.01.

RAB14 correlates with RAB14-mediated AMPK-AKT-mTOR pathway.

### Discussion

miR-451 has been found in many studies to play a part in the pathology of several kinds of tumors, though the role of miR-451 and the mechanism of regulation vary. It inhibits the proliferation of osteosarcoma cells by inhibiting the expression of liver receptor homolog-1 via Wnt/b-catenin pathway, and enhances the radiosensitivity of nasopharyngeal carcinoma through targeted regulation of RAB14. In addition, down-regulation of miR-451 can inhibit the fatty acid-induced production of proinflammatory cytokine via AMPK/AKT pathway in non-alcoholic fatty liver [18]. Also, miR-451 regulates multiple myeloma by influencing the stem cell of the side population via PI3K/Akt/mTOR signaling pathway [11]. MiR-451 regulates a range of tumor cells via various signaling pathways. This study proved, for the first time, that miR-451 is also expressed at a low level in osteosarcoma tissues, and is capable of targeted regulation of RAB14. The MTT, colony-forming, cell invasion and cell adhesion assays indicated that the over-expression of miR-451 can significantly inhibit the proliferation and invasion of osteosarcoma cells, and enhance cell adhesion to some extent, which is closely

correlated with AMPK-Akt-mTOR signaling pathway.

Rab14, a member of the Ras oncogene family, is involved in the proliferation and apoptosis of a wide range of cells [19]. Ras, a member of the small G proteins superfamily, is an important factor for regulating the transport pathways in the endomembrane system of eukaryotic cells, and acts as a molecular switch during the regulation of intercellular vesicular transport, which, once blocked, will fail the protein targeting, causing a number of diseases, including cancer [20]. Rab small GTPase and their related regulatory protein and effectors (such as mitochondria, Golgi apparatus & endoplasmic reticulum, etc.) malfunction in many human diseases, including cancer [21]. Rab14 promotes the invasion and metastasis of tumor cells during the development of cancer. Zhang et al. inhibited the Rab14 expression through RNA interference, which is found to have significantly reduced the proliferation of tumor cells and promoted the apoptosis of cysteine protease. Wiesner C et al. proved that Rab14 is the key regulatory factor for membrane type-1 matrix metalloproteinase (MT1-MMP) signaling pathway and plays an essential role in tumor invasion and metastasis using gene silencing technology, which further proved that Rab14 is closely related to the proliferation, apoptosis,

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invasion and metastasis of tumor cells [22]. AMPK signaling pathway, a kinase chain in the cells, whose main function is the conduction of biological information from the surface to the nucleus of the cells, is a key factor to the regulation of gene expression of oncogene [11]; It has been found by some studies to be involved in the regulation of the growth, adhesion, migration, proliferation, apoptosis, invasion and angiogenesis in several types of tumors, including osteosarcoma. It has been definitely proved that mTOR can regulate the proliferation, cell cycle, apoptosis, and is involved in multiple cell signaling pathways [23].

In conclusion, our study about regulatory effect of miR-451 in osteosarcoma and its signaling pathways will provide a new approach to the diagnosis and treatment of osteosarcoma.

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### Disclosure of conflict of interest

None.

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