miR-454 is down-regulated in osteosarcomas and suppresses cell proliferation and invasion by directly targeting c-Met

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Abstract

Objectives: Osteosarcoma is the most common primary bone malignancy of children and young adults. Increasing evidence has shown that microRNAs (miRNAs) are associated with cancer development, but, little is known concerning the role of miR-454 in osteosarcoma.

Materials and methods: qRT-PCR was performed to detect expression of miR-454 in osteosarcoma cell lines and tissues. To understand its role in osteosarcoma, we reintroduced expression of miR-454 in the MG-63 cell line by transfection with miR-454 mimics or inhibitors. CCK-8 assay and an invasion assay were used to detect the functional role of miR-454. Luciferase assay and western blot analysis were performed to detect the target gene of miR-454.

Results: miR-454 was found to be down-regulated in osteosarcoma tissues and cell lines. Its over-expression inhibited tumour growth and invasion and its down-regulation promoted cell proliferation and invasion. Subsequent investigation revealed that c-Met was a direct and functional target of miR-454 in osteosarcoma. Overexpression of miR-454 impaired c-Met-induced cell proliferation and invasion. Finally, miR-454 was found to be inversely correlated to c-Met expression in human osteosarcoma tissues.

Conclusions: Reduced-expression of miR-454 in osteosarcoma cells promoted tumour growth by targeting c-Met, thus miR-454 may be a potential therapy target for this tumour.

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Introduction

Osteosarcoma is one of the most common primary malignant bone tumours of adolescents and young adults (1,2). Current advances in osteosarcoma therapy have enhanced patient outcomes and survival, with most effective regimens including neoadjuvant and adjuvant chemotherapy coupled with local control (3,4). However, for patients with metastatic or recurrent osteosarcoma, survival data remain poor (5–7). Thus, it is crucial to develop novel prognostic biomarkers and targeted therapies for treatment of this disease.

MicroRNAs (miRNAs) are a class of 18–25-nucleotide-long, non-coding RNAs, which regulate messenger RNA (mRNA) translation and stability, by binding to the 3′-untranslated region (UTR) of target genes (8–10). Numerous studies have demonstrated that miRNAs act as key regulators in a wide variety of biological processes, including cell proliferation, differentiation, apoptosis, metabolism, signal transduction and response to infection (10,11,12–15). In addition, it has been well established that miRNAs are abnormally expressed in many malignancies such as gastric cancer, oesophageal cancer, lung cancer and hepatocellular carcinoma (16–19). Deregulated miRNAs are associated with tumour initiation, promotion and progression by regulating target gene expression. Thus, further exploration of expression and function of miRNAs will provide insight into pathogenesis and progression of osteosarcoma.

In our study, miR-454 was down-regulated in osteosarcoma cells and tissues. Its ectopic expression inhibited osteosarcoma cell proliferation and invasion. Moreover, c-Met was identified to be the potential target of miR-454, and it seems that miR-454 may suppress tumour growth and invasion by repressing expression of c-Met. Our findings suggest that miR-454 functions as a novel tumour suppressor in osteosarcoma and may be a potential therapy target for osteosarcoma.

Materials and methods

Ethics statement

All patients (patients' parents on behalf of children) involved in this study, agreed to participate and provided their written informed consent. The investigation and the consents were approved by the Ethical Board of the Institute of the Shandong Provincial Hospital, affiliated to Shandong University, and complied with the Declaration of the Helsinki.

Tissue specimens

In total, osteosarcoma and adjacent non-tumour tissues were obtained from patients with primary osteosarcoma undergoing surgery at the Shandong Provincial Hospital affiliated to Shandong University. Clinicopathological characteristics of the patients are summarized in Table S1. After surgical removal, tissues were frozen immediately in liquid nitrogen and stored at −80 °C.

Cell lines and culture

Four osteosarcoma cell lines, MG-63, U2OS, SOSP-9607 and SAOS-2, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human osteoblast cell line, hFOB, was purchased from the Cell Resource Center of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, and Peking Union Medical College (Beijing, China). MG-63, U2OS, SOSP-9607 and SAOS-2 were propagated in Dulbecco's modified Eagle's medium (Gibco; Logan, UT, USA), supplemented with 10% foetal bovine serum and streptomycin (100 µg/ml), penicillin (100 U/ml) while hFOB cells were incubated in osteoblast growth medium (Promo Cell, Heidelberg, Germany).

Cell transfection

miR-454 mimics and negative control (scramble), and miR-454 inhibitor and its negative control (control) were synthesized by GenePharma (Shanghai, China) and transfected into the cells to a final oligonucleotide concentration of 20 nmol/l. All cell transfections were introduced by DharmaFECT1 reagent (Dharmacon, Austin, TX, USA) according to the manufacturer's instructions.

RNA extraction and qRT-PCR analysis

Total RNA was isolated from cells and tissues using miRNeasy Mini Kit (Qiagen, Wolfenbuettel, Germany)

and miRNA Q-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA) was used for quantification of miRNA levels, according to the manufacturer's protocol. For quantification of c-Met mRNA levels, RT reactions were conducted with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, USA). qRT-PCR was performed using an ABI 7500 System (Bio-Rad, Berkeley, CA, USA). U6 and GAPDH were used as normalizing controls for miRNA and mRNA quantification respectively. The 2^{-DDCt} method was employed to calculate relative expression levels.

Cell proliferation assay

Approximately, 4×10^3 MG-63 cells were plated in each well of a 96-well plate and were incubated in 10% CCK-8 (Dojindo, Kumamoto, Japan), diluted in normal culture medium, for 2 h at 37 °C. Viable cells were counted by absorbance measurements at 450 nm using auto microplate reader (infinite M200, Tecan, Salzburg, Austria).

Cell invasion assay

Transwell chambers (8 µm pore size; Costar, Switzerland) were used to determine tumour cell invasion. Transwells were prepared for initial equilibrium by addition of 0.6 ml of RPMI-1640 medium with 10% foetal bovine serum in the lower compartment, as chemoattractant; inserts were coated with 1 mg/ml BD Matrigel Matrix (BD Biosciences, San Jose, CA, USA). Cells were suspended in 0.2 ml fresh medium without foetal bovine serum, added to the inserts, and cultured for 24 h. Subsequently, cells on upper surfaces of membranes were removed using cotton buds, and cells on lower surfaces of inserts were fixed and stained with 0.1% crystal violet. Five visual fields, ×200 magnification, of each insert were randomly selected, and numbers of cells were counted using light microscopy.

Western blot analysis

Total proteins were extracted from cells or tissues using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 7.5) plus 1% complete protease inhibitor. Lysates were clarified by centrifugation at 13,000 rpm for 5 min at 4 °C, and supernatant fractionated using SDS-PAGE before being subsequently transferred to nitrocellulose membranes. Proteins were detected with anti-c-Met and anti-GAPDH antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then washed and incubated in HRP-conjugated secondary antibody. Intensity of bands was visualized

using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Tokyo, Japan), and exposed.

Luciferase reporter assay

Luciferase reporter assays were performed as previously described. MG-63 cells were plated at 4000 cells per well in a 96-well plate. At 24 h after plating, cells were transfected with 20 nM miR-454 mimics or scramble, 10 ng pGL3 and pGL3–c-Met-3′UTR or pGL3–c-Met-3′UTR-MUT plasmid per well, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Relative luciferase activity was calculated after 48 h by normalizing Firefly luminescence to that of Renilla using the Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA).

Statistical analysis

Data are presented as mean ± standard deviation (SD) from three separate experiments. Differences between groups were analysed using Student's *t*-test when only two groups were compared, or one-way analysis of variance (ANOVA) when more than two groups were compared. Differences between groups of metastasis *in vivo* were analysed using χ^2 analysis. All statistical analyses were performed using SPSS 16.0 (SPSS Inc., IBM, Chicago, IL, USA) and differences were considered to be statistically significant at $P < 0.05$.

Results

miR-454 is frequently down-regulated in both osteosarcoma clinical samples and cell lines; here it was reduced to different degrees in all four osteosarcoma cell lines (MG-63, U2OS, SOSP-9607 and SAOS-2) compared to the human osteoblast cell line (hFOB) (Fig. 1a). We further compared expression levels of miR-454 between osteosarcoma samples and adjacent non-tumour tissues, from 30 cases. miR-454 was lower in osteosarcomas compared to adjacent tissues (Fig. 1b) and was down-regulated in 24 cases (24/30; 80%) of osteosarcoma compared to their adjacent areas (Fig. 1c). When correlated to disease outcome, loss of miR-454 levels in patients with osteosarcoma was associated with reduced disease-free survival (hazards ratio = 0.25, Fig. 1d).

Cell proliferation and invasion is inhibited by miR-454 in human osteosarcomas

miR-454 increased when transfected with miR-454 mimics and reduced when transfected with miR-454 inhibitor

(Fig. 2a). CCK-8 proliferation assay showed that cell population growth rate was reduced in MG-63 cells transfected with miR-454 mimics compared to cells transfected with scramble mimics (Fig. 2b). Meanwhile, miR-454 inhibitor promoted MG-63 cell proliferation (Fig. 2b). In addition, invasiveness of cells transfected with miR-454 mimics was lower than in scramble and control group cells, and invasiveness of cells transfected with miR-454 inhibitor was higher than scramble and control group cells (Fig. 2c).

miR-454 directly targeted c-Met 3′-UTR

Database TargetScan showed that *c-Met* is a putative target gene of miR-454 (Fig. 3a). Thus, the effect of miR-454 on translation of c-Met mRNA into protein was then assessed using a luciferase reporter assay (Fig. 3b). Enforced expression of miR-454 reduced luciferase activity of the reporter gene with the wild-type construct but not with mutant c-Met 3′UTR construct, indicating miR-454 directly targeted the c-Met 3′UTR. Overexpression of miR-454 in MG-63 cells reduced expression of *c-Met*, and miR-454 inhibitor enhanced its expression (Fig. 3c, d). *c-Met* is involved in miR-454-induced repression of osteosarcomas cell proliferation and invasion.

Western blot analysis showed that pCDNA–c-Met enhanced c-Met protein expression (Fig. 4a) and CCK-8 proliferation and invasion assays showed that overexpression of c-Met promoted osteosarcoma cell proliferation and invasion. When miR-454 mimic and pCDNA–c-Met was cotransfected into MG-63 cells, miR-454 mimic inhibited pCDNA–c-Met-induced cell proliferation and invasion (Fig. 4b, c).

miR-454 expression was inversely correlated with c-Met expression in osteosarcoma tissues

The osteosarcoma cells lines exhibited higher c-Met mRNA expression compared to hFOB cells (Fig. 5a) and expression of c-Met was higher in osteosarcomas compared to adjacent tissues (24/30; 80%) (Fig. 5b). In general, expression of c-Met in osteosarcomas was higher than in adjacent tissues (Fig. 5c, $P < 0.001$). As shown in Fig. 5d, when c-Met levels were plotted against miR-454 expression, significant inverse correlation was obtained (two-tailed Pearson's correlation analysis, $r^2 = -0.688$; $P < 0.001$).

Discussion

A great deal of work has been performed to identify diagnostic and prognostic markers of human osteosarcoma (20,21) and a large body of evidence has indicated
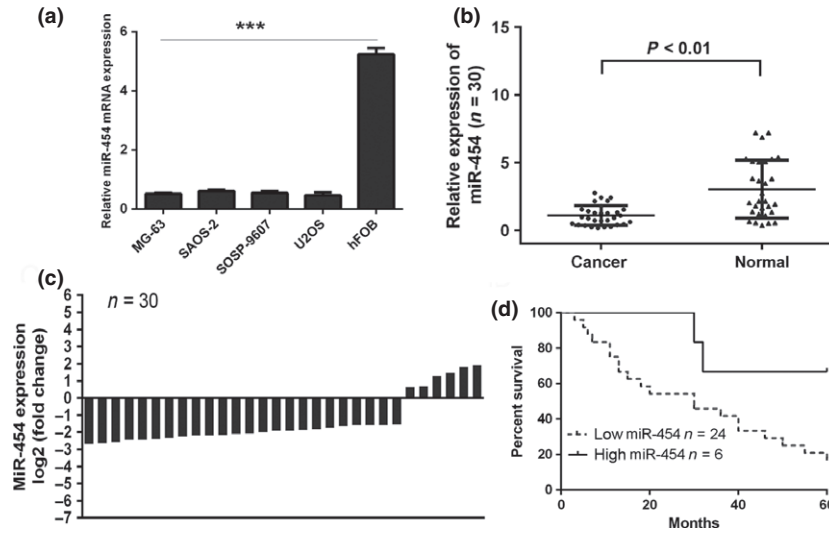
Figure 1. miR-454 is frequently down-regulated in both osteosarcoma clinical samples and cell lines. (a) qRT-PCR analysis of miR-454 expression in human osteosarcoma cell lines (MG-63, U2OS, SOSP-9607 and SAOS-2) and one human osteoblast cell line (hFOB). Expression of miR-454 was normalized to U6 snRNA. (b) Relative miR-454 expression levels in osteosarcoma tissues and their corresponding adjacent normal tissues. Expression of miR-454 was normalized to U6 snRNA.(c) qRT-PCR analysis of miR-454 expression in 30 pairs of osteosarcomas and their corresponding adjacent normal tissues. Data are presented as log 2-fold change of osteosarcomas relative to non-tumour adjacent tissues.(c) Relative miR-454 expression levels in osteosarcomas and their corresponding adjacent normal tissues. (d) Loss of miR-454 levels in patients with osteosarcoma was associated with considerably shortened disease-free survival. ***$P < 0.001$.
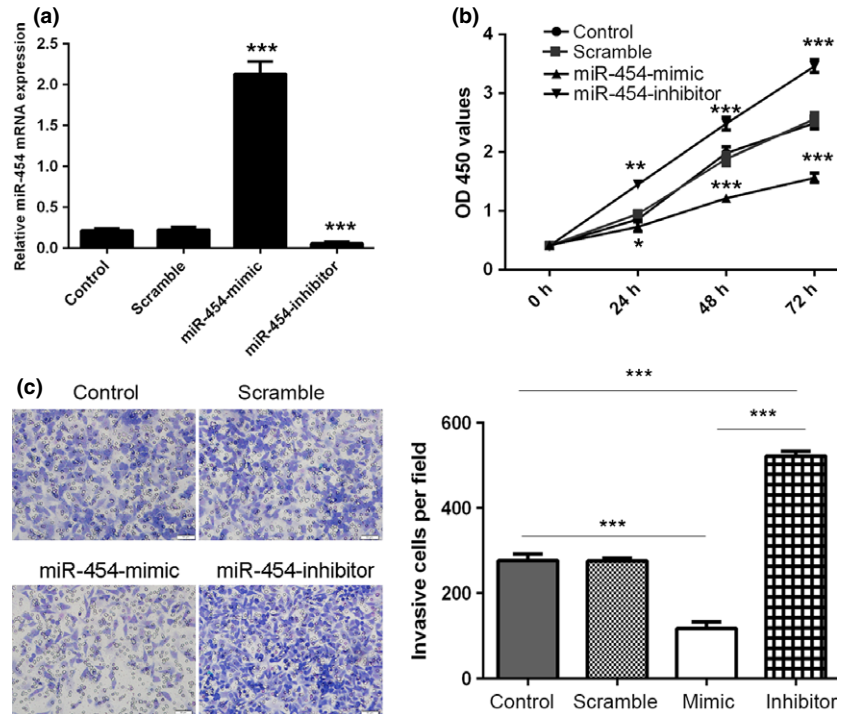

Figure 2. Cell proliferation and invasion was inhibited by miR-454 in human osteosarcomas. (a) qRT-PCR analysis of miR-454 expression after transfection of miR-454 mimics or scramble or control or inhibitor. (b) CCK8 assay used to evaluate proliferation of MG-63 cells after transfection with miR-454 mimics or scramble or control or inhibitor. (c) Invasion analysis of MG-63 cells after treatment with miRNA mimics, inhibitors or scramble or control; relative ratio of invasive cells per field is shown on the right, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

that miRNAs may function as oncogenes or tumour suppressors in human cancers (22–24). A direct link has been proven between miRNA and oncogenesis (25,26). In the current study, miR-454 was down-regulated in osteosarcoma tissues. Forced overexpression of miR-454 inhibited cell proliferation and invasion in MG-63 cells, while miR-454 inhibition promoted cell proliferation and invasion. Moreover, c-Met was also identified as a novel and direct target of miR-454. Importantly, c-Met rescued miR-454-mediated inhibition of cell invasion
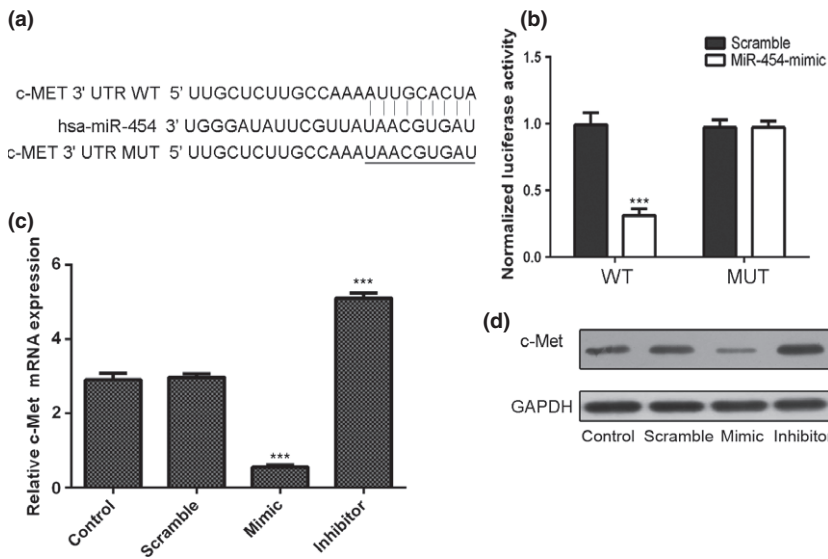
(a)

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c-MET 3' UTR WT   5' UUGCUCUUGCCAAAAUUGCACUA
                                  | | | | | | |
hsa-miR-454       3' UGGGAUAUUCGUUAUAACGUGAU
c-MET 3' UTR MUT  5' UUGCUCUUGCCAAAUAACGUGAU
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(b)

(c)

(d)

Figure 3. **miR-454 directly targeted c-Met 3′-UTR**. (a) Sequences of miR-454 binding sites within the human c-Met 3′UTRs and schematic reporter constructs; in this panel, c-Met-WT represents reporter constructs containing the entire 3′UTR sequences of c-Met, and c-Met-MUT represents reporter constructs containing mutated nucleotides. (b) Analysis of relative luciferase activities of c-Met-WT and c-Met-MUT. Error bars are derived from triplicate experiments. (c) qRT-PCR analysis of c-Met mRNA expression in MG-63 cells after treatment with miRNA mimics, inhibitors, scramble or control. Expression of c-Met was normalized to GAPDH. (d) Western blot analysis of c-Met expression in MG-63 cells transfected with miR-454 mimics, inhibitors, scramble or control. GAPDH was also detected as loading control. ***$P < 0.001$.
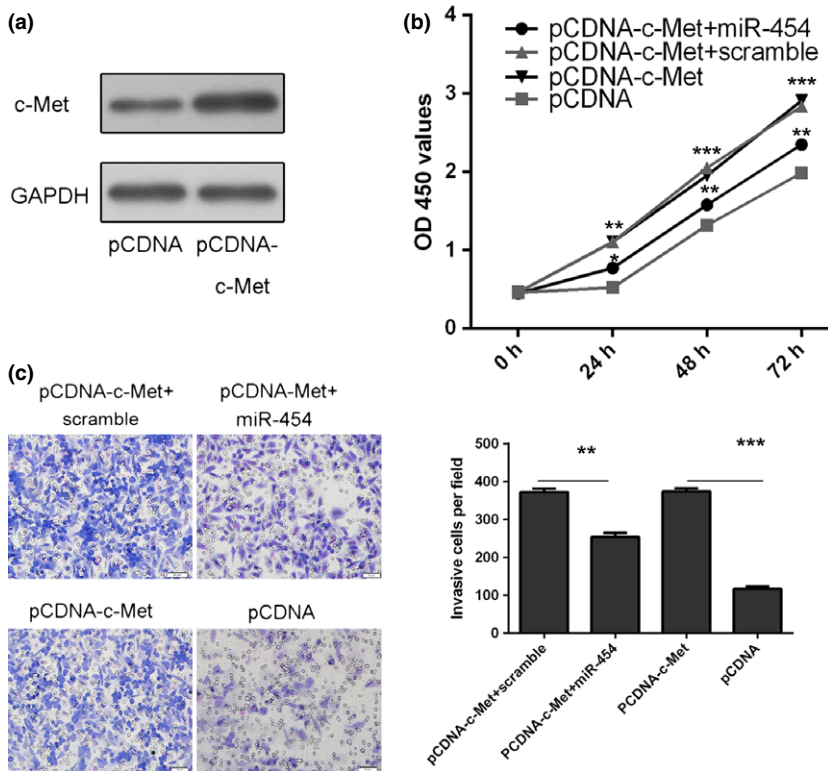
(a)

(b)

(c)

Figure 4. **c-Met was involved in miR-454-induced repression of osteosarcoma cell proliferation and invasion**. (a) Western blot analysis of c-Met in MG-63 cells transfected with either pCDNA–c-Met or pCDNA empty vector. GAPDH was also detected as loading control. (b) Cell growth curves of MG-63 cells transfected with different combinations, using CCK-8. (c) Invasion analysis of MG-63 cells treated with different combinations. Relative ratio of invasive cells per field is shown right. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

and proliferation. Our findings suggest that miR-454 has a suppressor role in osteosarcoma tumourigenesis and cancer cell invasion.

Recently, increased expression of miR-454 has been reported in human colorectal cancers and cell lines (27). Moreover, expression of miR-454 was found to be depressed in activated rat hepatic stellate cells (HSCs) and expression of miR-454 was down-regulated in LX-2 cells treated with TGF-β1 (28). In addition, Perfetti

et al. (29) reported that expression of miR-454 increased in blood of myotonic dystrophy type 1 patients. However, there are no prior studies on expression and function of miR-454 in osteosarcoma. In our study, expression of miR-454 was found to be down-regulated in human osteosarcoma cell lines and tissues. Moreover, introduction of miR-454 reduced osteosarcoma cell proliferation and invasion. These results suggest that miR-454 seemed to act as a tumour suppressor whose
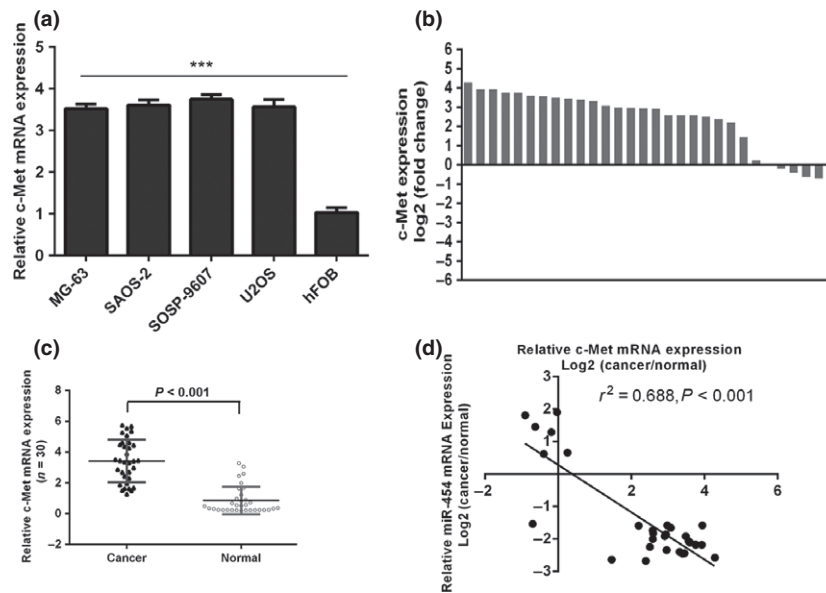
Figure 5. miR-454 expression was inversely correlated to c-Met expression in osteosarcomas. (a) qRT-PCR analysis of c-Met expression in human osteosarcoma cell lines (MG-63, U2OS, SOSP-9607 and SAOS-2) and one human osteoblast cell line (hFOB). Expression of c-Met was normalized to GAPDH. (b) qRT-PCR analysis of c-Met expression in 30 pairs of osteosarcomas and their corresponding adjacent normal tissues. Data presented as log 2-fold change of osteosarcomas relative to non-tumour adjacent tissues. (c) Relative c-Met expression levels in osteosarcomas and their corresponding adjacent normal tissues. Expression of c-Met was normalized to GAPDH. (d) Analysis of correlation of miR-454 and c-Met expression in osteosarcomas. (Two-tailed Pearson's correlation analysis, $r^2 = -0.688$; $P < 0.001$, $n = 30$). Data presented as log 2-fold change of osteosarcomas relative to non-tumour adjacent tissues. ***$P < 0.001$

down-regulation would contribute to progression and metastasis of osteosarcoma.

We further searched for the possible target of miR-454 in osteosarcoma cells. Among the candidate target genes, we focused on *c-Met* due to its role as a regulator of tumour growth and invasion. Using a dual-luciferase reporter assay, we showed that miR-454 directly bound to the 3′-UTR of c-Met, which contains a miR-454-binding site. Overexpression of miR-454 inhibited c-Met expression at both mRNA and protein levels. We also observed substantial up-regulation of c-Met in osteosarcoma tissues, while miR-454 levels negatively correlated with c-Met levels. Collectively, these results demonstrate that miR-454 might function as a tumour suppressor by repressing c-Met expression during osteosarcoma development.

The *MET* oncogene encodes the tyrosine kinase receptor for hepatocyte growth factor (HGF), which elicits a unique physiological program leading to invasive tumour growth (30,31). Activated MET oncoprotein, also known as c-Met, appears to play a crucial role in tumourigenesis in a wide variety of tumours such as gastric, colon and breast cancers (32–34). Numerous studies have linked inappropriate expression of c-Met to most types of human solid tumour and its activation *via* autocrine, paracrine or mutational mechanisms can lead to tumourigenesis and metastasis (35,36). c-Met also

plays an important role in development of osteosarcoma, and expression of c-Met is up-regulated in it (37,38); its activation contributes to osteosarcoma cell proliferation, migration and invasion (39,40). In our study, c-Met expression was up-regulated in osteosarcoma cells and tissues compared to paired adjacent non-tumour bone tissues; expression of c-Met negatively correlated with miR-454 levels. Down-regulation of miR-454 in osteosarcoma may facilitate expression of c-Met, leading to enhanced tumour metastasis.

In conclusion, the present study demonstrates that miR-454 was down-regulated in osteosarcoma cell lines and tissues examined. Ectopic miR-454 expression resulted in inhibition of osteosarcoma cell proliferation and invasion. Further investigation revealed c-Met to be a potential target of miR-454. Expression of c-Met negatively correlated with miR-454 levels. Therefore, miR-454 may serve as a predictor for prognosis and as a therapeutic target in osteosarcoma patients.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of clinicopathological parameters of patients with osteosarcoma.

Table S2. Primer sequence.