

miR-150 is downregulated in osteosarcoma and suppresses cell proliferation, migration and invasion by targeting ROCK1

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Abstract. Osteosarcoma (OS) is the most common form of bone malignancy in children and adolescents. A class of molecules known as microRNAs (miRNAs) have been routinely associated in the development and progression of OS. The present study was centered on the less well-known miRNA, miRNA (miR)-150, and its role in OS was investigated. The levels of miR-150 were examined in 40 tissue specimens from patients with OS and adjacent normal tissues using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. In addition the expression levels of miR-150 were examined in three OS cell lines and a normal osteoblast cell line. Cell proliferation, migration and invasion assays were performed to establish the correlation between miR-150 and metastasis. The potential targets of miR-150 were theoretically predicted and one high-scoring target, Rho-associated kinase 1 (ROCK1), was established to be a direct target using RT-qPCR and western blot analyses and Pearson's correlation analysis. The results indicated that miR-150 was downregulated in tissues from patients with OS and cell lines. Secondly, it was shown that the overexpression of miR-150 was inversely correlated with OS cell proliferation, migration and invasion. It was also shown that miR-150 negatively regulated the gene expression of ROCK1 in the OS cell lines. Finally, the interaction between miR-150 and ROCK1 was established and it was shown that miR-150 directly targeted ROCK1. In conclusion, miR-150 was found to be a tumor suppressor, and the suppression of miR-150 resulted in elevation in the levels of ROCK1. This interaction between miR-150 and ROCK1 may be key in the progression of OS. Furthermore, miR-150 or ROCK1 may be potential therapeutic targets for the treatment of OS.

Introduction

Osteosarcoma (OS) is a tumor, which normally affects the long bones of legs and arms, and commonly affects children and adolescents. OS is the most common type of primary bone malignancy with an incidence of 4-5/1,000,000 cases in the United States in 2009 (1). Owing to advances in therapeutic strategies, including radiotherapy and combinatorial chemotherapy, the survival rates of patients have improved significantly over the past decade (1). However, the underlying mechanisms of tumorigenesis, chemotherapy resistance, disease progression and metastasis remain to be fully elucidated (2). A major bottleneck in OS therapy lies in its poor prognosis, therefore, the identification of novel approaches to systematically combat OS is an urgent requirement.

MicroRNAs (miRNA) are one of the most abundant classes of regulatory molecules, consisting of ~22 nucleotides and having important regulatory roles in multicellular organisms (3). A number of studies have reiterated the crucial roles of miRNAs in the regulation of various types of cancer, including OS (4-7). Several studies have shown that miRNAs are differentially expressed in OS cell lines and tissues, often functioning as oncogenes or tumor suppressors with important roles in the pathogenesis of OS. A previous study attributing miRNA dysregulation to OS was performed in the human OS cell line, MG-63, and a total of 268 miRNAs were identified, which were either upregulated or downregulated (8). In this study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was used to profile selected miRNAs, which validated miRNA (miR)-9, miR-99, miR-195, miR-148a and miR-181a as upregulated, and miR-143, miR-145, miR-335 and miR-539, as downregulated (8).

Similar studies based on high throughput RT-PCR analysis have identified 22 differentially expressed miRNAs in OS cell lines and tumor samples, with miR-135b, miR-150, miR-542-5p and miR-652 being validated in different groups of tumors (9). Of these, miR-150 has been implicated in stomach (10), colorectal (11), lung (12), squamous cell (13), blood (14) and pancreatic cancer (15). However, to the best of our knowledge, the specific function and precise mechanistic role of miR-150 in OS remain to be elucidated.

In an attempt to bridge this gap, the present study aimed to investigate the potential role of miR-150 in the development of OS and elucidate its underlying mechanism. It was shown

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that miR-150 was downregulated in tissues of patients with OS and OS cell lines, and the ectopic expression of miR-150 in OS cell lines suppressed cell proliferation, migration and invasion. Furthermore, a set of target genes regulated by miR-150 were identified, and it was established that miR-150 functioned as a tumor suppressor by downregulating Rho-associated protein kinase 1 (ROCK1). These findings suggested that ROCK1 is a potential metastasis-promoter, and may be a valuable therapeutic target in the prognosis and diagnosis of OS.

Materials and methods

Patients and tissue samples. OS tissue samples and their adjacent normal tissue counterparts were collected from 40 patients with OS from the Department of Orthopedics, Affiliated Hospital of Qingdao University (Qingdao, China) with written informed consent obtained by the subjects or their guardians. All the tissue samples were obtained at the time of surgery and were immediately preserved in liquid nitrogen until further use. All samples used in the current study were collected between February and June 2014. The Institute Research Medical Ethics Committee of Qingdao University granted approval for the present study. The clinical characteristics of the 40 patients involved in the present study are summarized in Table I.

Cell culture and transfection. The SaOS2, U2OS and MG63 human OS cell lines and the hFOB1.19 normal human osteoblast cell line were obtained from the American Type Culture Collection (Rockville, MD, USA). The cell lines were maintained and cultured according to the manufacturer's protocols. All the cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and incubated at 37°C in a 5% CO₂ humidified incubator. All transfections were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using the RNeasy kit (Qiagen AG, Hombrechtikon, Switzerland) according to manufacturer's protocol. The RT protocol was as follows: 18°C for 30 min, 42°C for 30 min and 90°C for 5 min. Quantification of specific RNA transcripts was performed using SYBR Green qPCR with the ABI Prism 7700 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Specific amplification was confirmed using dissociation curve analysis. β -actin mRNA (Applied Biosystems; Thermo Fisher Scientific, Inc.) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were used as internal controls. Sequences of the primers used in the present study are shown in Table II. The qPCR was performed on a 7900HT Fast Real-Time system (Thermo Fisher Scientific, Inc.). The reaction conditions were: 95°C for 10 min and 35 cycles of 95°C for 20 sec and 58°C for 1 min.

Cell proliferation, migration and invasion assays. To assess the proliferation rates, $\sim 5 \times 10^6$ cells were seeded in 24-well plates and were transfected with miRNA mimics (Thermo Fisher Scientific, Inc.) at a final concentration of 20 nM for 24 h at 37°C. Cell proliferation was determined using a CyQUANT cell proliferation assay kit (Invitrogen; Thermo Fisher Scientific,

Table I. Clinical characteristics of patients involved in the present study.

Patient ID	Gender	Age	Site	M/NM
1	Male	11	Tibia	M
2	Male	13	Femur	M
3	Male	9	Femur	M
4	Female	18	Femur	NM
5	Female	25	Tibia	M
6	Female	12	Humerus	M
7	Male	12	Femur	M
8	Female	20	Tibia	NM
9	Male	40	Ulna	M
10	Female	35	Femur	M
11	Male	12	Tibia	M
12	Female	15	Femur	NM
13	Male	17	Tibia	NM
14	Female	15	Humerus	NM
15	Female	16	Tibia	M
16	Male	18	Tibia	M
17	Male	14	Humerus	M
18	Female	34	Tibia	M
19	Male	21	Femur	M
20	Female	20	Tibia	M
21	Female	8	Humerus	NM
22	Female	7	Tibia	M
23	Male	9	Femur	M
24	Male	13	Tibia	M
25	Female	16	Humerus	M
26	Male	18	Tibia	M
27	Male	16	Femur	NM
28	Female	26	Femur	NM
29	Male	55	Tibia	NM
30	Female	50	Femur	NM
31	Male	25	Ulna	M
32	Female	27	Tibia	M
33	Female	14	Tibia	M
34	Male	16	Femur	M
35	Male	12	Tibia	M
36	Female	19	Femur	NM
37	Male	8	Ulna	NM
38	Female	10	Tibia	M
39	Male	12	Femur	NM
40	Male	11	Tibia	M

M, metastasis; NM, no metastasis.

Inc.) according to the manufacturer's protocol. The fluorescence intensity was measured using a fluorescence microplate reader at 450 nm (Molecular Devices, LLC, Sunnyvale, CA, USA). Cell migration was assessed in two 24-well plates (3,500 cells/well) of 8 μ m Transwells (BD Biosciences; Franklin Lakes, NJ, USA) according to the manufacturer's protocol, followed by incubation for 16 h at 37°C in 5% CO₂. For the invasion

Table II. Specific primer sequences used in the present study.

Gene	Sequence
miR-150	Forward: 5'-ACACTCCAGCTGGGTCTCCCAACCCTTGTA-3' Reverse: 5'-TGTCGTGGAGTCGGCAATTCAGTTGAGCACTGG-3'
ROCK1	Forward: 5'-ACCTGTAACCCAAGGAGATGTG-3' Reverse: 5'-CACAATTGGCAGGAAAGTGG-3'
β -actin	Forward: 5'-GGGACCTGACTGACTACCTCA-3' Reverse: 5'-TGACTCGTCATACTCCTGCTTG-3'
GAPDH	Forward: 5'-TGCACCACCAACTGCTTAGC-3' Reverse: 5'-GGCATGGACTGTGGTTCATGAG-3'

miR, microRNA; ROCK1, Rho-associated kinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

assay, $\sim 1 \times 10^6$ cells were added to Matrigel invasion chambers in two 24-well plates of 8 μ m (BD Biosciences) according to the manufacturer's protocol. Following addition, the plates were incubated at 37°C for 40 h in 5% CO₂. Following incubation, the cells on the upper surface of the membrane were scraped off, and the migrated cells on the bottom of the membrane were fixed with formaldehyde (3.7% in PBS) for 2 min and stained with 0.1% crystal violet staining solution. The cells were counted using a light microscope in 4 randomly selected microscopic fields and averages were calculated.

Western blot analysis. The proteins were extracted by lysing the cells in sample loading buffer, which contained 1.5% SDS, 10% glycerol, 5 mM β -mercaptoethanol, bromphenolblue and 75 mM Tris (pH 7). 15% SDS-PAGE was used to separate the whole cell lysates. Following SDS-PAGE, the proteins were transferred onto PVDF membranes. Subsequently, the membranes were incubated with appropriate primary antibodies (ROCK1, catalog no., 21850-1-AP; dilution, 1:500; ProteinTech, Inc., Wuhan, China; β -actin, catalog no., 20536-1-AP; dilution, 1:1,000) at 4°C overnight. Following incubation, specific secondary antibodies coupled to horseradish peroxidase (catalog., 15134-1-AP; dilution, 1:1,000; ProteinTech, Inc.) were used to detect the bands at room temperature. Evaluation of the expression of proteins was performed using ImageJ version 1.38 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All statistical analysis was performed using GraphPad Prism software version 5.0 (GraphPad, Software, Inc., La Jolla, CA, USA) or SigmaPlot version 12.6 (SysStat Software Inc., San Jose, CA, USA). A paired or unpaired *t*-test was applied to compare samples with a parametric distribution. $P < 0.05$ was considered to indicate a statistically significant difference. Two-tailed Pearson's correlation was applied for analysis of the correlation between the expression levels of miR-150 and ROCK1. In all statistical analyses, two-sided tests were applied. All data are shown as the mean \pm standard deviation.

Results

miR-150 is downregulated in OS tissues and cell lines. The present study first aimed to determine whether miR-150 was

differentially expressed in human OS. To investigate this, 40 pairs of human OS tissues and paired adjacent matched non-cancerous tissues were analyzed to determine the expression levels of miR-150 using RT-qPCR analysis. On analyzing the results, significantly lower expression levels of miR-150 were observed in 87% (35/40) of the OS patient tissues, compared with the adjacent normal non-cancerous tissues (Fig. 1A; $P < 0.05$). Similarly, the expression levels of miR-150 were investigated using RT-qPCR analysis in the three OS cell lines (SaOS2, U2OS and MG63) and compared with that of the normal hFOB1.19 human OS cell line. The results of the RT-qPCR analysis revealed significantly lower expression levels of miR-150 in all cell lines, particularly in the U2OS and MG63 cell lines (Fig. 1B; $P < 0.05$). Together with the clinical data derived from the patient OS tissues, these results indicated that the downregulation of miR-150 was associated with OS. In addition, the results suggested that miR-150 may act as a tumor suppressor in OS.

miR-150 overexpression is inversely correlated with OS cell proliferation, migration and invasion. To determine the significance of miR-150 in cell growth, the U2OS, MG63 and SaOS2 OS cell lines were transfected with miR-150. Analysis of the results revealed that the overexpression of miR-150 significantly suppressed U2OS, MG63 and SaOS2 cell proliferation, compared with their corresponding negative controls (Fig. 2A-C). As migration and invasion are the two key stages in malignant progression and metastasis, the present study further assessed the effects of miR-150 on cell migration and invasion in the U2OS, MG63 and SaOS2 OS cell lines. The results of the Transwell assay indicated that the overexpression of miR-150 significantly suppressed the migration and invasion of all OS cell lines, compared with the corresponding negative controls (Fig. 3A-C). Collectively, these results suggested that miR-150 acted as a tumor suppressor miRNA, and that the overexpression of miR-150 *in vitro* contributed to the suppression of cell proliferation, migration and invasion.

miR-150 negatively regulates the gene expression of ROCK1 in OS cell lines. In order to further investigate the underlying regulatory mechanisms of miR-150, the present study aimed

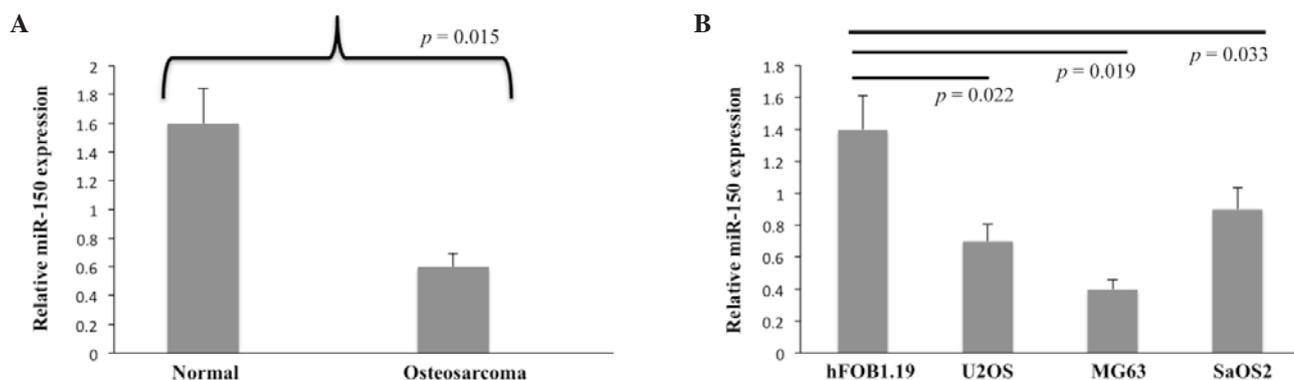


Figure 1. miR-150 is downregulated in OS patient tissues and cell lines. (A) Relative expression levels of miR-150 in 40 OS patient tissue samples and adjacent normal tissue sample counterparts. The relative expression levels of miR-150 were determined using RT-qPCR analysis. (B) Relative expression levels of miR-150, determined using RT-qPCR analysis in U2OS, MG63 and SaOS2 OS cell lines and the normal hFOB1.19 human OS cell line. Student's *t*-test was used to analyze significant differences. * $P < 0.05$. Data are presented as the mean + standard deviation. OS, osteosarcoma; miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

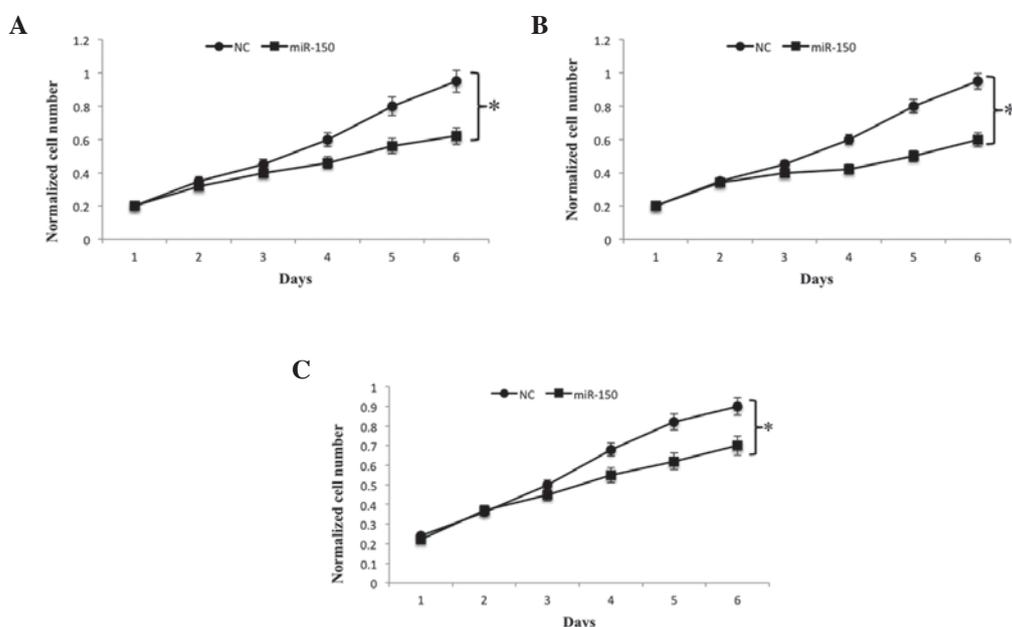


Figure 2. miR-150 overexpression is inversely correlated with OS cell growth. (A) U2OS, (B) MG63 and (C) SaOS2 OS cell lines were transfected with miR-150 and the numbers of cells were determined at different time points using a fluorescence-based CyQUANT cell proliferation assay kit. In all cases, Student's *t*-test was used to analyze significant differences, * $P < 0.05$. Data are presented as the mean + standard deviation. OS, osteosarcoma; miR, microRNA; NC, negative control.

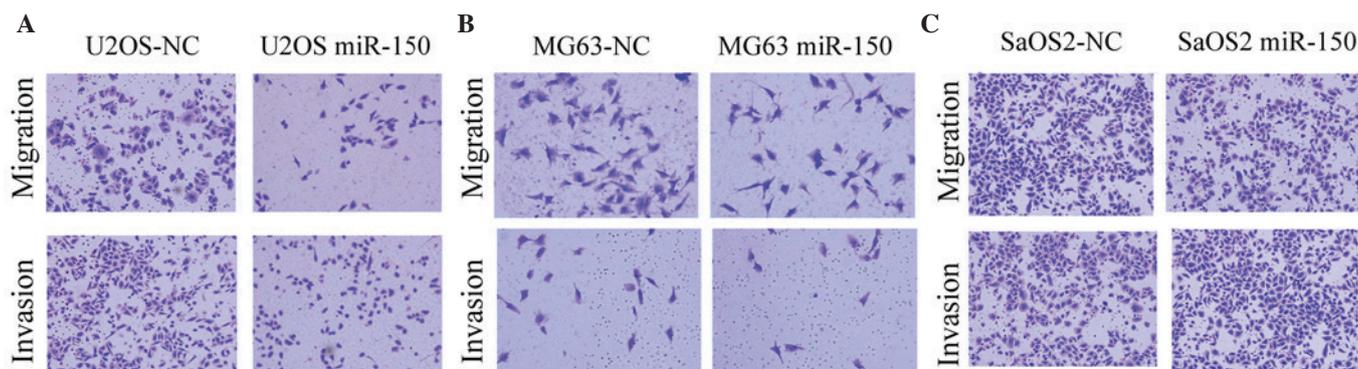


Figure 3. miR-150 overexpression is inversely correlated with OS cell migration and invasion. Transwell migration and invasion assays of (A) U2OS, (B) MG63 and (C) SaOS2 OS cell lines. The assays were performed following transfection of the cell lines with miR-150. Representative images of migrated and invaded cells on the membrane (magnification, $\times 100$). OS, osteosarcoma; miR, microRNA; NC, negative control.

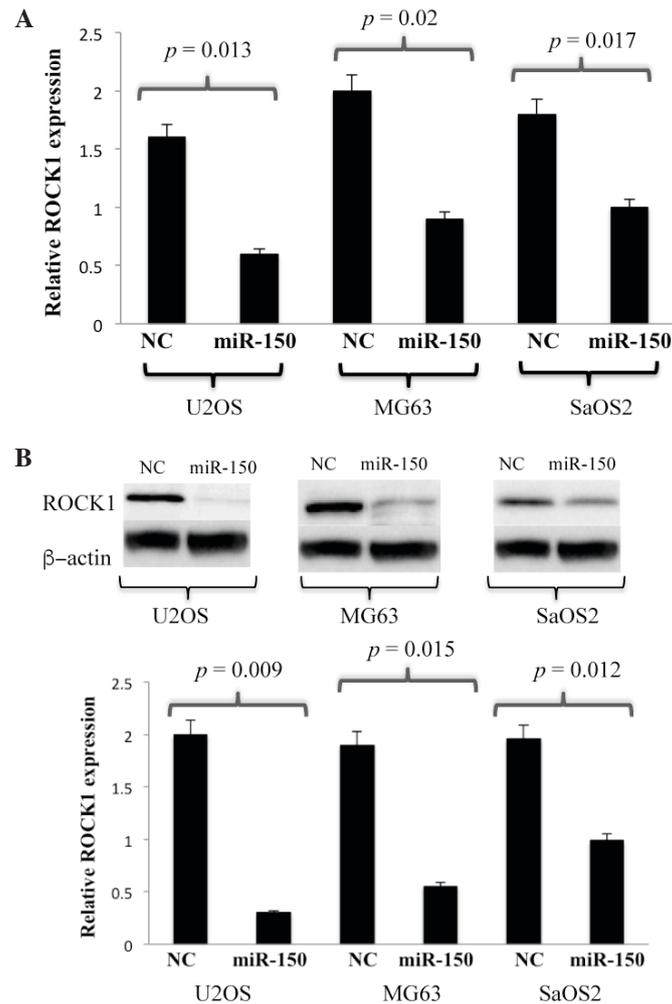


Figure 4. miR-150 negatively regulates gene expression of ROCK1 in OS cell lines. (A) Reverse transcription-quantitative polymerase chain reaction analysis of mRNA expression of ROCK1 in U2OS, MG63 and SaOS2 OS cell lines overexpressing miR150. Student's *t*-test was used to analyze significant differences. **P*<0.05. Data are presented as the mean + standard deviation. (B) Western blot analysis of protein expression of ROCK1 in U2OS, MG63 and SaOS2 OS cell lines overexpressing miR150. A corresponding NC was used in the absence of miR-150. β -actin protein was used as an additional control. miR, microRNA; ROCK1, Rho-associated kinase 1; NC, negative control.

to predict the biological targets of miR-150. A routinely used bioinformatics program for such investigations, TargetScan (www.targetscan.org/vert_71/), was used to identify the potential targets of miR-150. The algorithm listed potential targets regulated by miR-150 in human OS, and the targets were further shortlisted by incorporating more specific and stringent filtering elements in the algorithm. USP13, ZNF229 and ROCK1 were found to be amongst the highest scoring targets. Taking into account previous reports on the regulation of ROCK1 by other microRNAs in OS, the present study focussed on ROCK1 and aimed to experimentally validate whether ROCK1 was a direct target of miR-150. Using a combination of RT-qPCR analysis followed by western blot analysis, the expression levels of ROCK1 in the U2OS, MG63 and SaOS2 cell lines were probed. The results of the RT-qPCR analysis indicated low mRNA levels of ROCK1 in all the cell lines overexpressing miR150, compared with the corresponding controls (Fig. 4A). Similarly, western blot analysis revealed that the ROCK1 protein was under-expressed in the cells overexpressing miR150, compared with its control (Fig. 4B). These results indicated that the

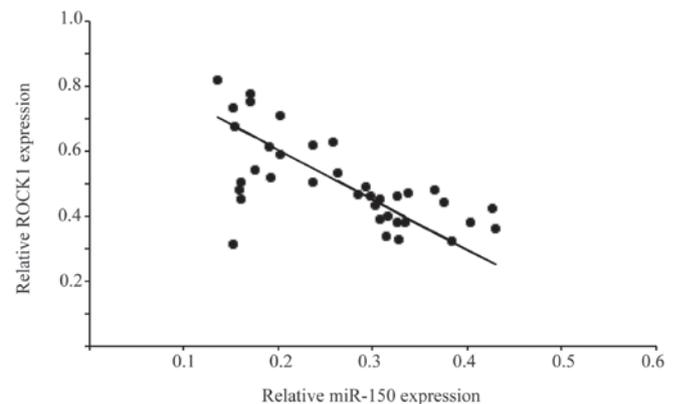


Figure 5. Correlation between expression levels of ROCK1 and miR-150 in 40 OS tissue samples using Pearson's correlation analysis, ($r=-0.78$; *P*<0.05). miR, microRNA; ROCK1, Rho-associated kinase 1.

overexpression of miR-150 in the OS cell lines significantly downregulated the expression of ROCK1 at the mRNA and protein levels.

Association between ROCK1 and OS. The present study also examined the relative expression levels of ROCK1 and miR150 in 40 OS patient tissue samples and compared them with the normal non-cancerous adjacent tissue samples. Examining the correlation between ROCK1 and miR-150 in the OS and adjacent normal tissue samples can determine the presence of an interaction between these two molecules in the progression of OS. The RT-qPCR analysis of the tissue specimens revealed a significant difference in the mRNA levels of ROCK1 in the OS tissues, compared with the adjacent normal tissues. A significant inverse correlation was observed when the expression levels of ROCK1 were plotted against the levels of miR-150. The strength of the association between the two molecules was measured using Pearson's correlation analysis ($r=-0.78$; $P<0.05$; Fig. 5). The results of the Pearson's inverse correlation analysis from the OS tissues, together with the results from the OS cell lines demonstrated that miR-150 directly targeted ROCK1.

Discussion

OS is a mesenchymally-derived bone tumor, primarily affecting children and adolescents (16). As with other types of cancer, the treatment of OS requires a rigorous multidisciplinary approach involving clinicians and researchers. The advent of high-throughput technologies has provided a wealth of information pertaining to the miRNA class of molecules, and their role in the development of cancer, including OS. However, the molecular mechanisms underlying the involvement of specific miRNAs in OS development remain to be fully elucidated. The present study attempted to bridge this gap in understanding by delineating the molecular mechanism of miR-150 and its role in OS.

The present study first investigated whether miR-150 was differentially expressed in OS tissues. By comparing the levels of miR-150 with adjacent normal tissue counterparts, it was established that miR-150 was significantly downregulated in OS tissues. The downregulation of miR-150 was also shown in three OS cell lines, compared with a normal human osteoblast cell line. The present study also investigated the correlation between the overexpression of miR-150 and OS cell proliferation, migration and invasion. The results indicated an inverse correlation, which suggested that miR-150 acted as a tumor suppressing miRNA. Several other independent studies have revealed that miR-150 functions as a tumor suppressor. A previous study on malignant lymphoma revealed that the aberrant downregulation of miR-150 results in continuous activation of the phosphoinositide 3-kinase-AKT pathway resulting in cancer (17).

In the present study, the potential targets of miR-150 were identified using bioinformatics analysis. Although this analysis revealed few high scoring targets, the present study focussed on one of the highest scoring targets, ROCK1, a serine/threonine kinase, which has been implicated in cancer, particularly metastasis (18). The association between miRNAs and ROCK1 and their involvement in cancer has been well documented. miRNA148a, miRNA-584 and miRNA-340 have been shown to downregulate ROCK1 in gastric cancer, human clear cell renal cell carcinoma and OS, respectively (19-21). To conclusively establish whether ROCK1 is a target of miR-150, the present study quantified the levels of ROCK1 at the gene and protein

levels following transfection of the OS cell lines with miR-150. The results revealed that miR-150 negatively regulated the gene expression of ROCK1. Furthermore, quantification of the relative levels of ROCK1 with respect to miR-150 in the OS tissues revealed a significant inverse correlation between miR-150 and ROCK1. The results indicated that the suppression of miR-150 resulted in elevation of the levels of ROCK1, and this interaction between miR-150 and ROCK1 may be key in the progression of OS. miR-150 or ROCK1 may also act as potential therapeutic targets for the treatment of OS.

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