

miR-202-5p inhibits the migration and invasion of osteosarcoma cells by targeting ROCK1

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Abstract. Many studies have shown that microRNA regulates the development and treatment of osteosarcoma (OS). In many human cancer studies, the expression of microRNA-202 has been shown to be abnormal. The aim of the study was to examine the role of miR-202-5p in the occurrence and formation of OS. miR-202-5p and Rho-associated coiled-coil containing protein kinase 1 (ROCK1) levels were assessed using RT-qPCR in OS tissues and cell lines. The cell migrating and invasive abilities were detected by the Transwell assay in OS. Moreover, the relationship between miR-202-5p and ROCK1 was verified via luciferase reporter assay. The protein level of ROCK1 was identified by western blot analysis. Downregulation of miR-202-5p was identified in OS tissues and cell lines. In addition, the miR-202-5p overexpression had inhibitory action for cell migration and invasion in OS. Moreover, miR-202-5p directly targeted ROCK1 and negatively regulated its expression. Upregulation of ROCK1 had a carcinogenic effect in OS. Furthermore, the upregulation of ROCK1 restored the suppressive effect of miR-202-5p. miR-202-5p, in turn, weakened the abilities of cell migration and invasion in OS by inhibiting ROCK1 expression. As a result, miR-202-5p may be developed as a potential pathway in the treatment of OS.

Introduction

Osteosarcoma (OS) is a common malignant bone tumor occurring in children or teenagers younger than 20 years (1). More specifically, OS usually occurs in children with bone malignancy, accounting for almost 5% of all pediatric tumors (2). Moreover, incidence of OS in primary malignant tumor ranks first, showing a very high tumor malignant degree and poor prognosis of OS patients (3). In addition, OS can spread to the lungs within a few months, resulting

in a 3- to 5-year survival rate of only 5-20% after amputation (4). Thus, finding effective biomarkers for the diagnosis and therapy of OS is required.

MicroRNAs (miRNAs) have been reported to be unusually upregulated or downregulated in various human cancers. For instance, miR-21 was upregulated in colorectal cancer (5), while miR-126 was downregulated in lung cancer (6). Moreover, the dysregulated expression of miRNAs was also closely-associated with the pathogenesis and development of cancers (7). In OS, downregulation of miR-375, miR-302b and miR-216a was detected in previous studies (8-10). Furthermore, the upregulation of miR-19, miR-92b and miR-146a was found in OS (11-13). Therefore, miRNAs participated in the tumorigenesis of OS, as indicated in previous studies. Specifically, the decrease of miR-202 expression was identified in gastric cancer (14), colorectal carcinoma (15) and endometrial adenocarcinoma (16). Those studies indicated that miR-202 also affects the progression of OS. However, the specific function of miR-202 in OS has yet to be analysed.

Rho-associated coiled-coil containing protein kinase 1 (ROCK1) is considered a direct target gene of many miRNAs in various kinds of human cancers, such as miR-124 (17), miR-144 (18) and miR-148a (19). Moreover, the carcinogenesis of ROCK1 was identified in glioma (20), breast cancer (21) and non-small cell lung cancer (22). In OS, ROCK1 was employed as a potential therapeutic target (23). However, the relationship between ROCK1 and miR-202 in OS still remains unclear.

In the present study, we aimed to investigate the specific effect of miR-202-5p in OS as well as the interaction between miR-202-5p and ROCK1. Finally, we examined whether miR-202-5p weakened the abilities of cell migration and invasion in OS by inhibiting ROCK1 expression.

Materials and methods

Clinical tissues. Thirty-six surgical tumor specimens and adjacent tissue samples were obtained from the People's Hospital of Rizhao (Rizhao, China) after receiving written informed consent. The patients received no treatment prior to surgery. Human tissue was frozen in liquid nitrogen and then stored in a refrigerator at -80°C for further experiments. This experiment was approved by the Institutional Ethics Committee of People's Hospital of Rizhao.

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Cell cultures and cell transfection. The human OC cell lines U2OS, MG-63, HOS and human normal osteoblast cell line hFOB1.19 were used for this experiment. All the cell lines came from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were seeded in DMEM or RPMI-1640 medium containing 10% fetal bovine serum (FBS) and cultured at 37°C with 5% CO₂.

The miR-202-5p mimic and inhibitor, ROCK1 siRNA (si-ROCK1) were obtained from GenePharma Co., Ltd. (Shanghai, China). Then they were transferred into HOS cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) based on the manufacturer's protocols.

RT-qPCR. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was applied to extract total RNA containing miRNA to quantify miR-202-5p expression in OS tissues and cell lines. RT-qPCR was carried out using the SYBR-Green Master Mix (Toyobo Co., Ltd., Osaka, Japan) on Applied Biosystems 7500 Sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). U6 and GAPDH were used as the control for miR-202-5p and ROCK1. Expression was calculated using the 2^{-ΔΔC_q} method.

Dual luciferase reporter assay. 293T cells were incubated in 24-well plates for the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA). The wild or mutant type of 3'-untranslated region (3'-UTR) of ROCK1 was inserted into the pGL3 promoter vector (Invitrogen; Thermo Fisher Scientific, Inc.) for luciferase reporter experiments. Then, wild or mutant type of 3'-UTR of ROCK1 and miR-202-5p mimic was transfected into 293T cells. Subsequently, the Dual Luciferase Reporter Assay System (Promega Corporation) was applied to measure luciferase activities.

Transwell assay. The Transwell chamber (24-well) was employed to perform cell migration and invasion assays. HOS cells (5x10⁴) without serum were placed in the upper chamber on the non-coated membrane, and the lower chamber was filled with 10% FBS to induce HOS cells to migrate or invade through the membrane. In addition, the cells were placed in the upper chamber with the matrigel (BD Biosciences, Franklin Lakes, NJ, USA) for the invasion assay. Then, the cells were incubated for the migration and invasion assay. Finally, the cells were stained with crystal violet. The number of cells was counted using an inverted light microscope (Zeiss, Oberkochen, Germany).

Western blot analysis. The protein samples were obtained using RIPA lysis buffer. Proteins were separated through a 10% SDS-PAGE and incubated with 5% non-fat milk in PVDF membranes at room temperature. Next, we incubated the membranes overnight at 4°C with mouse anti-ROCK1 (1:1,000, rabbit polyclonal antibody, ab97592), anti-GAPDH (1:1,000, rabbit polyclonal antibody, ab9485), which were subsequently incubated with goat anti-mouse secondary antibodies. Then, goat anti-rabbit IgG H&L secondary antibodies (1:1,000, goat polyclonal second antibody, ab150077) protein levels were measured using enhanced chemiluminescence (ECL; Pierce; Thermo Fisher Scientific, Inc.).

Statistical analysis. The obtained data were shown as the mean ± SD. The data were analyzed with GraphPad Prism 6.0. (GraphPad Software Inc., La Jolla, CA, USA). The difference was calculated according to the Chi-square or ANOVA followed by a Tukey's test. P<0.05 was considered statistically significant.

Results

Low expression of miR-202-5p is identified in OS tissues and cell lines. The miR-202-5p level was detected in OS tissues via RT-qPCR (Fig. 1A). The lower expression of miR-202-5p was identified in OS tissues in comparison with the normal tissues. Moreover, downregulation of miR-202-5p was found in OS that had lymph-node metastasis (Fig. 1B). It indicated that the aberrant expression was related to lymph-node metastasis. Similarly, miR-202-5p downregulation was also assessed in U2OS, MG-63 and HOS cell lines, except for the human osteoblast cell line hFOB1.19 (Fig. 1C). In brief, downregulation of miR-202-5p may play a vital role in the metastasis of OS.

Overexpression of miR-202-5p exerts an inhibitory effect on cell migration and invasion in OS. miR-202-5p mimic or inhibitor was transfected into HOS cells to investigate its function in OS. The miR-202-5p levels were measured in these transfected cells using RT-qPCR (Fig. 2A). Then the migrating and invasive abilities in these transfected cells were detected by the Transwell assay. As expected, the migrating and invasive abilities were reduced by miR-202-5p mimics but enhanced by miR-202-5p inhibitor in OS cells (Fig. 2B and C). These findings showed that miR-202-5p as a suppressive miRNA exerts an inhibitory effect on cell migration and invasion in OS.

miR-202-5p directly targets ROCK1 and negatively regulates its expression. Then we performed luciferase reporter assays to confirm the prediction that miR-202-5p binds to the 3'-UTR of ROCK1 (Fig. 3A). As predicted, co-transfection of wild-type of ROCK1 and miR-202-5p mimic in 293T cells reduced the luciferase activity and no change was found in cells containing the mutant type of ROCK1 and miR-202-5p mimic compared to the negative control (Fig. 3B). In addition, the ROCK1 levels in U2OS, MG-63, HOS and hFOB1.19 cell lines were identified via RT-qPCR. Upregulation of ROCK1 was found in U2OS, MG-63, and HOS cells apart from hFOB1.19 (Fig. 3C). Furthermore, we observed the ROCK1 level in cells with miR-202-5p mimic or inhibitor to further explore their relationship. We found that ROCK1 level was declined by the miR-202-5p mimic and enhanced by the miR-202-5p inhibitor by RT-qPCR and western blot analysis (Fig. 3D). Collectively, miR-202-5p directly targeted ROCK1 and negatively regulated its expression.

ROCK1 has a carcinogenic effect on OS cells. Next, ROCK1 siRNA was transfected into HOS cells to block its expression. Moreover, the mRNA and protein level of ROCK1 were declined by ROCK1 siRNA in HOS cells (Fig. 4A and B). Similarly, the ROCK1 siRNA also impaired the migrating and invasive abilities in HOS cells, which was the same as the inhibitory action of miR-202-5p overexpression (Fig. 4C and D). In brief, ROCK1 had a carcinogenic effect on OS.

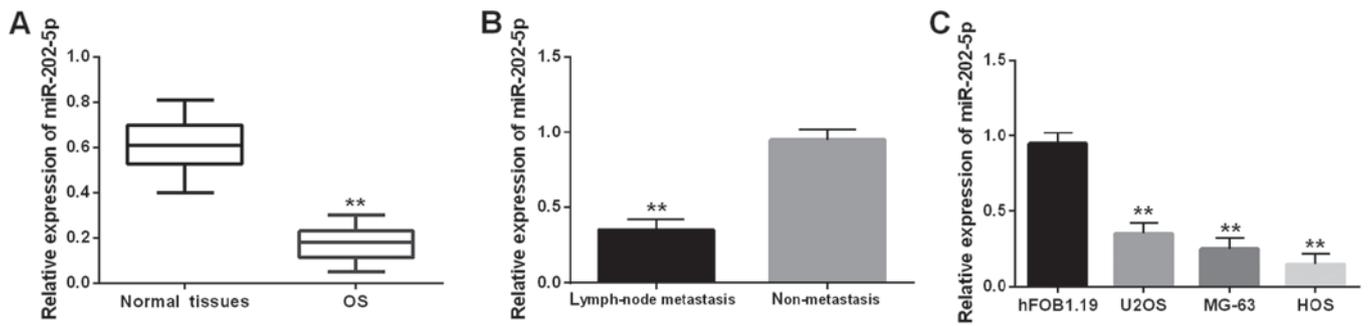


Figure 1. Downregulation of miR-202-5p was identified in OS tissues and cell lines. (A) The miR-202-5p level in OS tissues and normal tissues was detected via RT-qPCR. (B) miR-202-5p level in OS cells with lymph-node metastasis and non-metastasis. (C) miR-202-5p expression in U2OS, MG-63, HOS and human normal osteoblast cell line hFOB1.19. **P<0.01. OS, osteosarcoma.

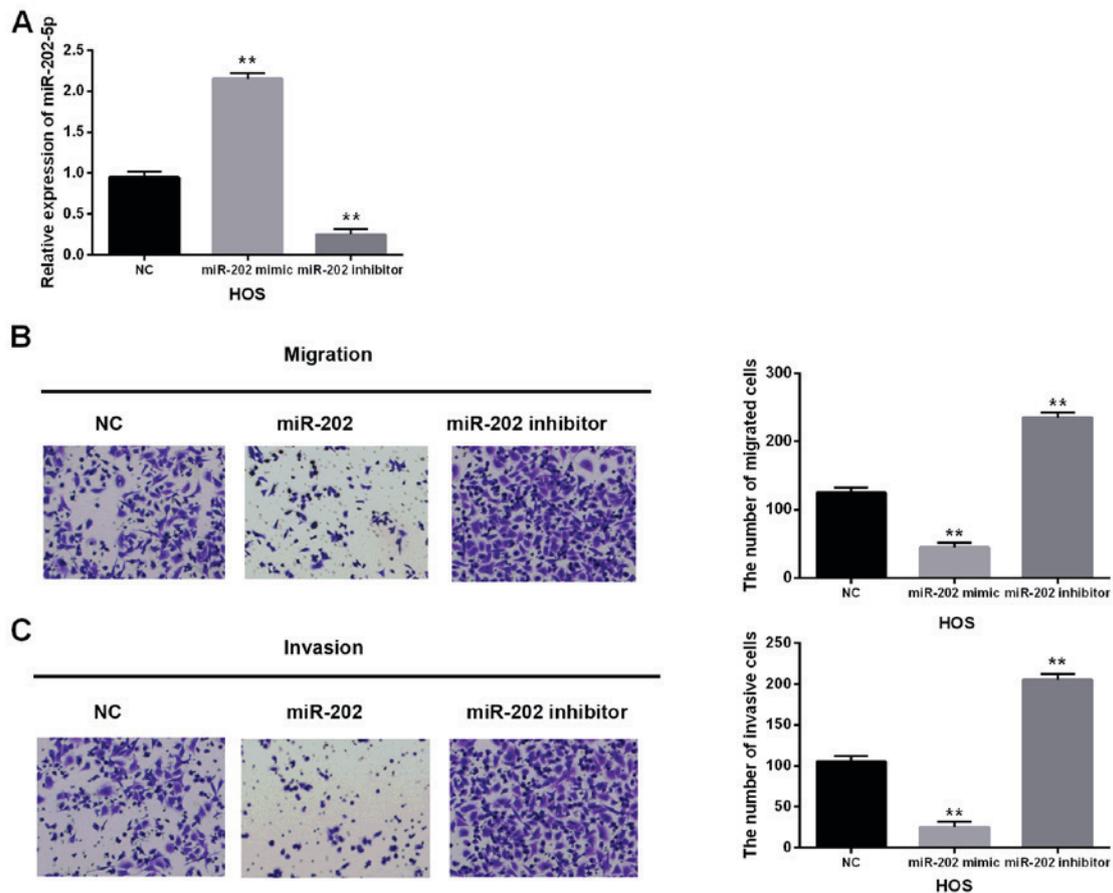


Figure 2. Overexpression of miR-202-5p had an inhibitory effect on cell migration and invasion in OS. (A) miR-202-5p expression was examined in HOS cells containing miR-202-5p mimic or inhibitor via RT-qPCR. (B and C) The cell migration and invasion were measured in cells containing miR-202-5p mimic or inhibitor via the Transwell analysis. **P<0.01. OS, osteosarcoma.

Upregulation of *ROCK1* restores the suppressive effect of miR-202-5p in OS. miR-202-5p mimic and *ROCK1* vector were transfected into HOS cells to further investigate their suppressive function. Overexpression of *ROCK1* recovered the decrease of *ROCK1* mRNA and protein levels induced by miR-202-5p mimic (Fig. 5A and B). Functionally, the abilities of migration and invasion in transfected HOS cells containing miR-202-5p mimic and *ROCK1* vector were regained in comparison with the cells only miR-202-5p mimic (Fig. 5C and D). Generally speaking, upregulation of

ROCK1 restored the suppressive effect of miR-202-5p in OS cells, which further indicated that miR-202-5p repressed cell migration and invasion in OS by regulating *ROCK1*.

Discussion

Previous studies have demonstrated that identification of miRNAs can be used as a biomarker for the diagnosis and prognosis of OS (24). In our study, downregulation of miR-202-5p and the upregulation of *ROCK1* were identified

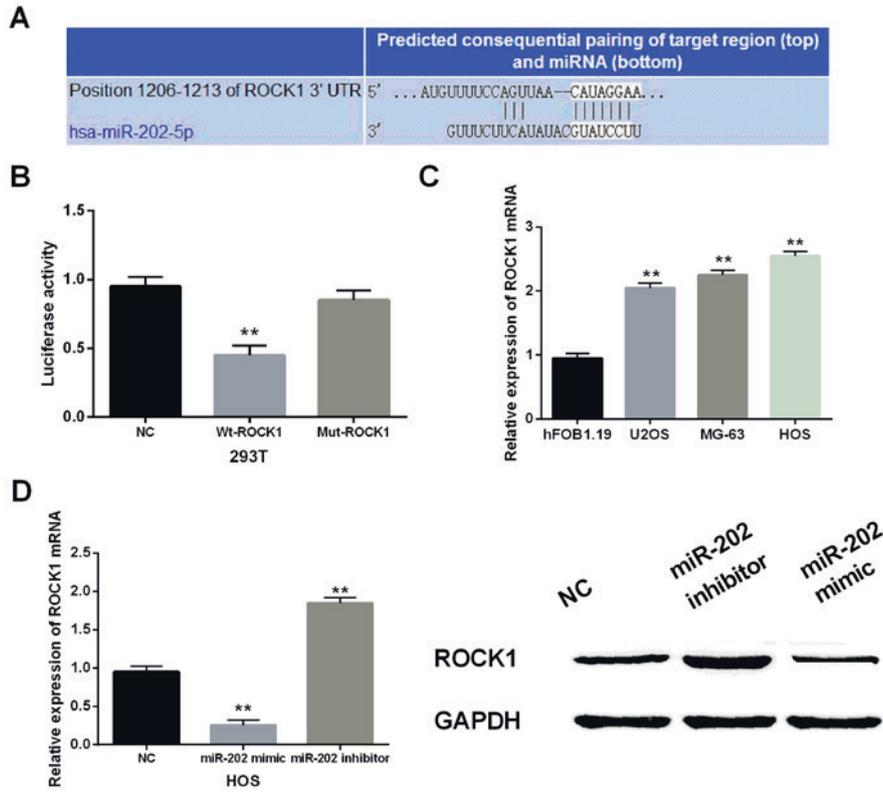


Figure 3. miR-202-5p directly targeted ROCK1 and negatively regulated its expression. (A) The binding sites of miR-202-5p on the 3'-UTR of ROCK1. (B) Luciferase reporter assay. (C) ROCK1 expression in U2OS, MG-63, HOS and hFOB1.19 cells (control). (D) mRNA and protein expressions of ROCK1 were assessed in cells containing miR-202-5p mimic or inhibitor. **P<0.01. ROCK1, Rho-associated coiled-coil containing protein kinase 1; 3'-UTR, 3'-untranslated region.

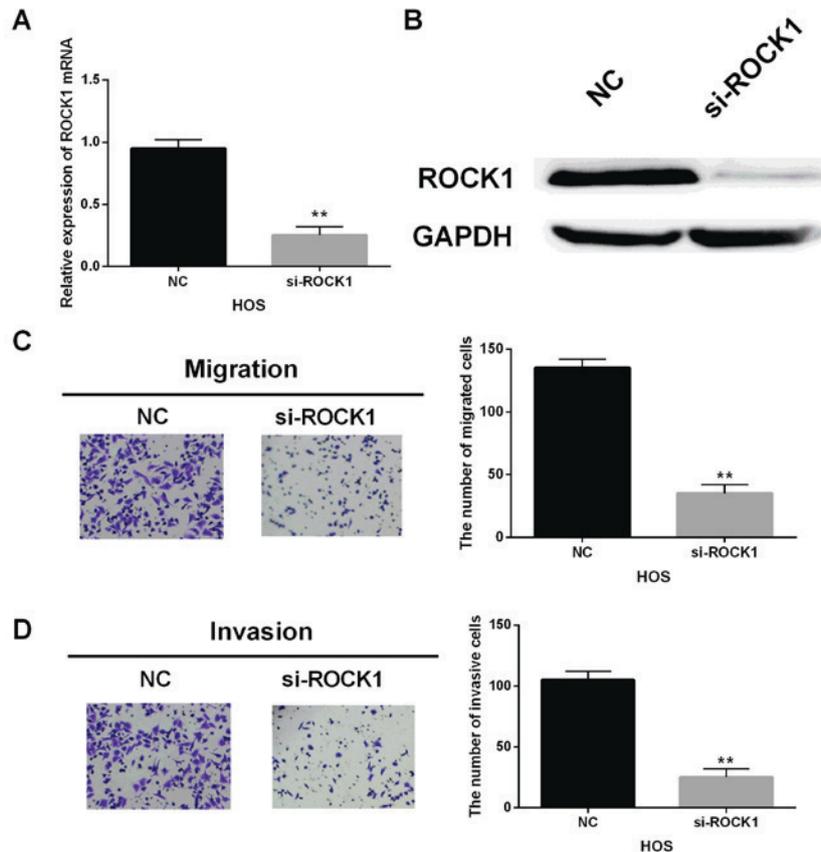


Figure 4. ROCK1 had a carcinogenic effect in OS cells. (A and B) The mRNA and protein expressions of ROCK1 were measured in cells containing ROCK1 siRNA. (C and D) Cell migration and invasion analysis of HOS cells with si-ROCK1 was detected by the Transwell assay. **P<0.01. OS, osteosarcoma; ROCK1, Rho-associated coiled-coil containing protein kinase 1; si-ROCK1, ROCK1 siRNA.

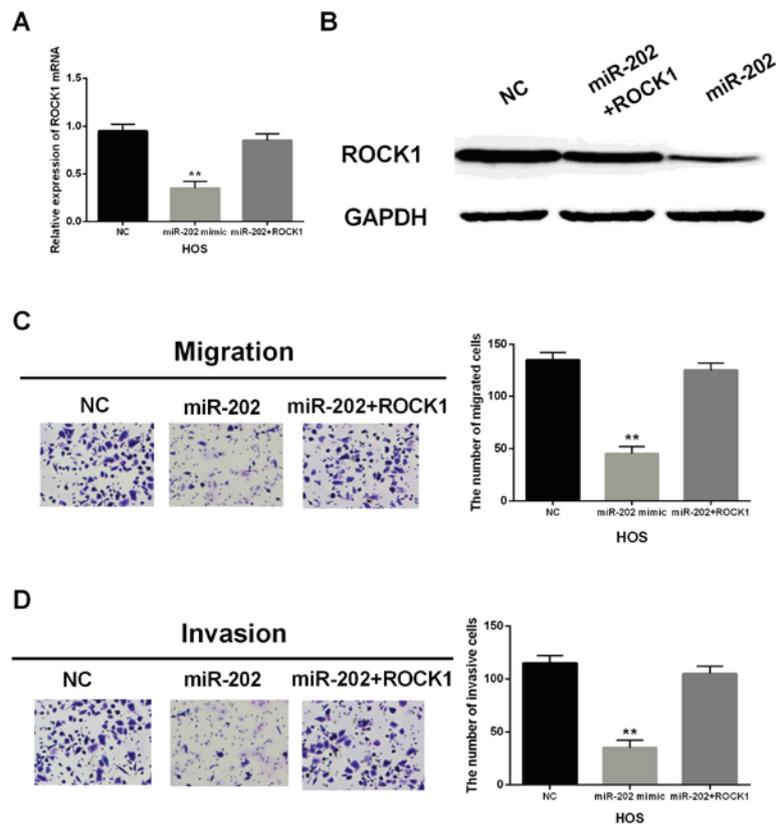


Figure 5. Upregulation of ROCK1 restored the inhibitory effect of miR-202-5p in OS. (A and B) The mRNA and protein expressions of ROCK1 were measured in cells containing miR-202-5p and ROCK1 vector. (C and D) The cell migration and invasion in cells containing ROCK1 vector and miR-202-5p were detected by the Transwell assay. ** $P < 0.01$. OS, osteosarcoma; ROCK1, Rho-associated coiled-coil containing protein kinase 1.

in OS. Overexpression of miR-202-5p impaired the migrating and invasive abilities in OS, which was similar to the knockout of ROCK1. Furthermore, the upregulation of ROCK1 restored the inhibitory effect of miR-202-5p in OS.

Many studies have shown that miR-202 was usually downregulated and participated in the formation of many human cancers. For instance, the miR-202 level was reduced and suppressed tumor progression in esophageal squamous cell carcinoma (25). In hepatocellular carcinoma, the miR-202 overexpression suppressed cell proliferation (26). Additionally, miR-202 was found to function as a suppressive miRNA in non-small cell lung cancer (27). The miR-202 downregulation was frequently identified in human cancers which was in agreement with our results in OS. More importantly, miR-202 was reported to be significantly declined. Moreover, it repressed cell growth and promoted cell apoptosis in OS (28). This study also confirmed our findings of miR-202 in OS. However, to the best of our knowledge, there was no study about the role of miR-202 for migration and invasion in OS cells. In addition, we demonstrated that the miR-202-5p overexpression had an inhibitory effect on cell migration and invasion in OS. Therefore, miR-202 may be used as an indicator for the diagnosis and prediction of OS, which was helpful in the treatment of OS patients.

To further explore the function of miR-202, ROCK1 was verified as a direct target of miR-202 in OS. To date, ROCK1 as a direct target gene has been reported to bind to miR-300 (29), miR-340 (30), miR-584 (31) and miR-1280 (32). However, the relationship between miR-202 and ROCK1 has

not been analysed thus far. In the present study, upregulation and carcinogenic effects of ROCK1 were observed in OS. The same findings of ROCK1 were also reported in OS induced by miR-145 (33), miR-198 (34) and miR-335 (35). Findings of those studies were in agreement with our results. In addition, miR-202-5p was negatively associated with ROCK1 expression. Upregulation of ROCK1 restored the suppressive effect of miR-202-5p in OS. Collectively, miR-202-5p impaired the migrating and invasive abilities in OS partly by inhibiting ROCK1. Therefore, understanding the role of miR-202 is significant for the treatment of OS.

In conclusion, downregulation of miR-202-5p and upregulation of ROCK1 were found in OS. miR-202-5p was verified to directly target ROCK1. More importantly, miR-202-5p was identified to inhibit the migrating and invasive abilities in OS cells by inhibiting ROCK1.

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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

CL as the first author contributed significantly in the analysis of and wrote the manuscript. DM as the second author performed the data analyses and wrote the manuscript. XL as the fourth author helped perform the analysis with constructive discussions. BC as the fifth author sorted out experimental data. JY as the corresponding author contributed to the conception of the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the People's Hospital of Rizhao (Rizhao, China). Signed written informed consents were obtained from the patients and/or guardians.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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