

LncRNA-zinc finger protein 281 downregulates rho-associated coiled-coil containing protein kinase 1 by upregulating miR-144 in osteosarcoma

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Received May 27, 2019; Accepted December 12, 2019

DOI: 10.3892/ol.2020.11940

Abstract. Zinc finger protein 281 (ZNF281) has been characterized as a tumor suppressive lncRNA in glioma. The present study aimed to analyze the functionality of ZNF281 in osteosarcoma (OS). It was demonstrated that ZNF281 was downregulated in OS tissue specimens and predicted the survival of patients with OS. In tissues from patients with OS, ZNF281 was negatively associated with rho-associated coiled-coil containing protein kinase 1 (ROCK1), but positively associated with miR-144. In the U2OS cell line, ZNF281 overexpression mediated the upregulation of miR-144 and downregulation of ROCK1. miR-144 overexpression led to the downregulation of ROCK1, but failed to affect ZNF281. Expression of ZNF281 and miR-144 resulted in decreased cell migration and invasion, while ROCK1 overexpression resulted in increased invasion and migration of OS cells. In addition, ROCK1 overexpression attenuated the effects of ZNF281 and miR-144 overexpression. Thus, ZNF281 may downregulate ROCK1 by upregulating miR-144 and inhibit cancer cell invasion and migration in OS.

Introduction

Osteosarcoma (OS) is a common type of primary bone cancer that affects ~3.4/1,000,000 people per year in the past decades worldwide (1). Despite the low incidence rate, osteosarcoma is a heavy burden on public health; as it mainly affects teenagers and young adults, early diagnosis is rare and curative therapies are lacking (2,3). With the advances in OS treatment, the overall 5-year survival rate has increased from 20 to 50% during the

20th century (4). However, there is still a significant population of patients with OS diagnosed with metastatic tumors for which there are no radical treatment regimens available (5). Thus, novel therapeutic approaches are still needed.

OS occurrence is associated with a number of physical factors, such as age and height (6). However, genetic factors are the most critical causative factors of OS (7). Rho-associated coiled-coil containing protein kinase 1 (ROCK1) functions downstream of GTPase RhoA and regulates the generation of contractile force, motility and metastasis of OS cells (8). ROCK1 is upregulated in a number of cancers including OS and can promote tumor metastasis by enhancing the mobility of cancer cell (9). A previous study has demonstrated that ROCK1 expression can be downregulated by certain microRNAs (miRNAs), such as microRNA (miR)-144 (10). Therefore, regulating the expression of certain tumor-suppressive miRNAs may directly suppress cancer metastasis through the downregulation of ROCK1.

Zinc finger protein 281 (ZNF281) is a recently identified tumor-suppressive long non-coding (lncRNA) in glioma (11). Our preliminary RNA-seq data showed that ZNF281 was inversely associated with ROCK1 expression in OS cells (data not shown). However, the mechanisms of interaction among ZNF281, miR-144 and ROCK1 have not been explored. The present study aimed to explore the role of ZNF281 in OS and possible interactions with ROCK1 and miR-144.

Materials and methods

Collection of tissue specimens from patients with OS. A total of 60 patients with OS [36 male and 24 female; aged 12-31 years; mean \pm standard deviation (SD): 21.1 \pm 3.4 years] were enrolled in the present study from a total of 108 patients with OS admitted to Honghui Hospital affiliated to Xi'an Jiaotong University between April 2011 and April 2014. This study was approved by the Ethics Committee of Honghui Hospital Affiliated to Xi'an Jiaotong University (Xi'an, China). All patients or their guardians if the patient was <18 years old signed an informed consent form. Inclusion criteria for the patients with OS were: i) No prior treatment; ii) newly diagnosed; and iii) completion of treatment and a 5-year follow-up at Honghui Hospital. Exclusion criteria included: i) Prior

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Key words: osteosarcoma, miR-144, rho associated coiled-coil containing protein kinase 1, survival, zinc finger 281

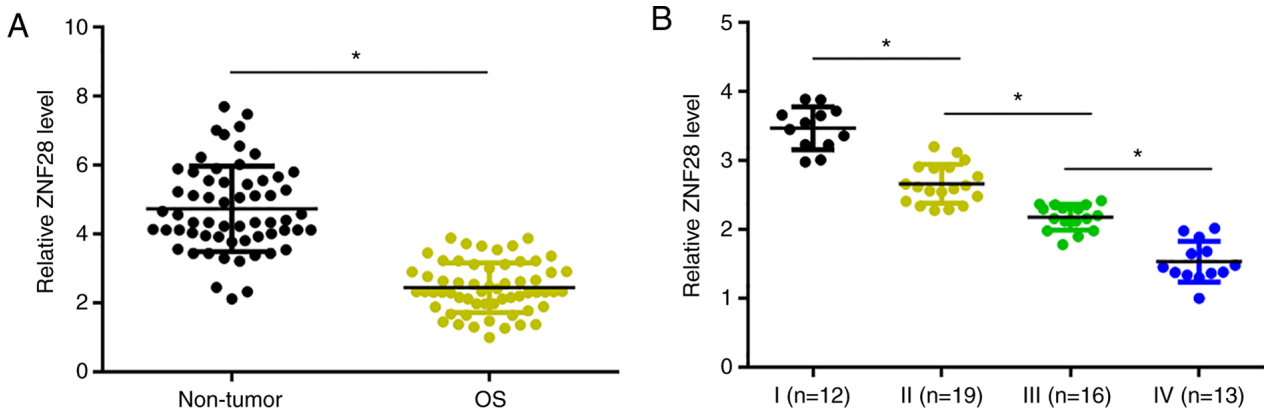


Figure 1. ZNF281 is downregulated in tumor specimens from patients with OS and affected by clinical stages. mRNA expression levels of ZNF281 in OS and non-tumor tissues were measured by quantitative PCR. (A) Expression levels of ZNF281 in OS tissues compared with non-tumor tissues and compared by paired Student's t-test. (B) Expression levels of ZNF281 in OS tissues were compared among different clinical stages by one-way ANOVA and Tukey's post-hoc test. Mean values of three biological replicates are presented. * $P < 0.05$. OS, osteosarcoma; ZNF281, zinc finger protein 281.

treatment; ii) recurrent OS; iii) patient transfer from another institute; and iv) the presence of other severe diseases. The American Joint Committee on Cancer staging system (12) was used to stage the 60 patients with OS. A total of 12, 19, 16 and 13 cases were clinical stage I-IV, respectively.

Patients with OS were subjected to MRI-guided fine needle biopsy. During biopsy, OS and adjacent (within 2 cm of the tumor) non-tumor tissue samples were collected from each patient. All OS samples contained $>98\%$ cancer cells and all non-tumor samples contained $<1\%$ cancer cells.

OS cells and transient transfections. A human OS cell line U2OS (ATCC) was obtained and used for subsequent experiments. Cells were cultured in a mixture of 90% Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich; Merck KGaG) and 10% FBS. Cell culture conditions were 95% humidity, 37°C and 5% CO₂. Cells were harvested when they were 70-80% confluent for subsequent transfections. ZNF281 and ROCK1 overexpression vectors were constructed using the pcDNA3.1 vector (Sangon Biotech Co., Ltd). Negative control (NC) miRNA (5'-CACGUACGGUAGUACCCGUAUU-3') and the miR-144 mimic (5'-GGUAUCAUCAUAUACUGUAA G-3') were obtained from Guangzhou RiboBio Co., Ltd. Cells were counted, and 3×10^6 cells were transfected with 45 nM miRNA (NC miRNA as NC group) or 10 nM vector (empty vector as NC group) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were harvested at 24 h post-transfection to perform subsequent experiments. Control cells were untransfected cells.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. RNAzol (Merck KGaA) was used to extract the RNA from tissue specimens from patients with OS (0.1 g tissue ground in liquid nitrogen) and U2OS cells (6×10^5) according to the manufacturer's instructions. RNA precipitation for harvesting miRNAs was performed using 85% ethanol. RNA samples were first digested with DNase I (2 h at 37°C, Sigma-Aldrich; Merck KGaG) and then reverse transcribed into cDNA using AMV Reverse Transcriptase kit (Promega Corporation) by incubating at 55°C for 10 min, followed by 53°C for 20 min and 80°C for 10 min. Subsequently, QuantiTect

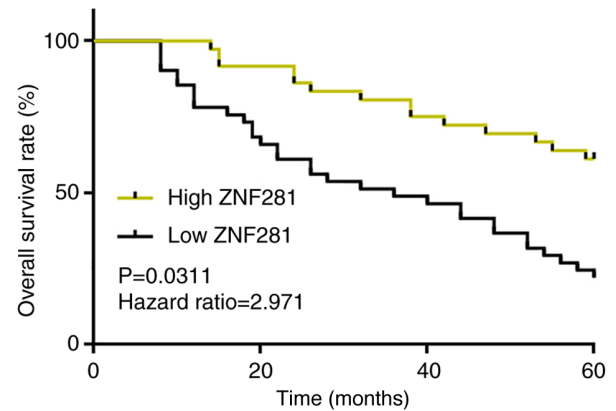


Figure 2. Low levels of ZNF281 mRNA expression predict poor survival of patients with OS. Patients were grouped into high and low ZNF281 mRNA expression levels according to the median expression level in OS tissues. Survival curves were plotted and compared using the GraphPad Prism 6 software and log-rank test. OS, osteosarcoma; ZNF281, zinc finger protein 281.

SYBR[®] Green PCR kit (Qiagen China Co., Ltd.) was used to prepare the qPCR to assay the expression of ZNF281 and ROCK1 mRNA. GAPDH was used as the internal reference gene. Expression levels of miR-144 were measured using the All-in-One[™] miRNA qRT-PCR Detection kit (GeneCopoeia, Inc.), which was used to perform 3'-polyadenylation, reverse transcriptions and preparation of qPCR mixtures. U6 was used as the endogenous control. PCR conditions were: 95°C for 1 min, followed by 40 cycles of 95°C for 12 sec and 58.5°C for 45 sec. Primer sequences were: ZNF281, forward 5'-CAG GGTATACAAATATGATG-3' and reverse 5'-GCATTGAAA GGGCATCACATTA-3'; ROCK1, forward 5'-AGTATTTCT CCCATATGGATA-3' and reverse 5'-ACCAATGGATTGTTC ACCTGAA-3'; GAPDH, forward 5'-GTCTCCTCTGACTTC AACAGCG-3' and reverse 5'-ACCACCCTGTTGCTGTAG CCAA-3'. Forward primer of miR-144 was 5'-GGATATCAT CATATACTG-3'. Universal reverse primers and U6 primers were from the kit (sequences unavailable). Each experiment included three replicates and data were normalized using the $2^{-\Delta\Delta C_q}$ method (13).

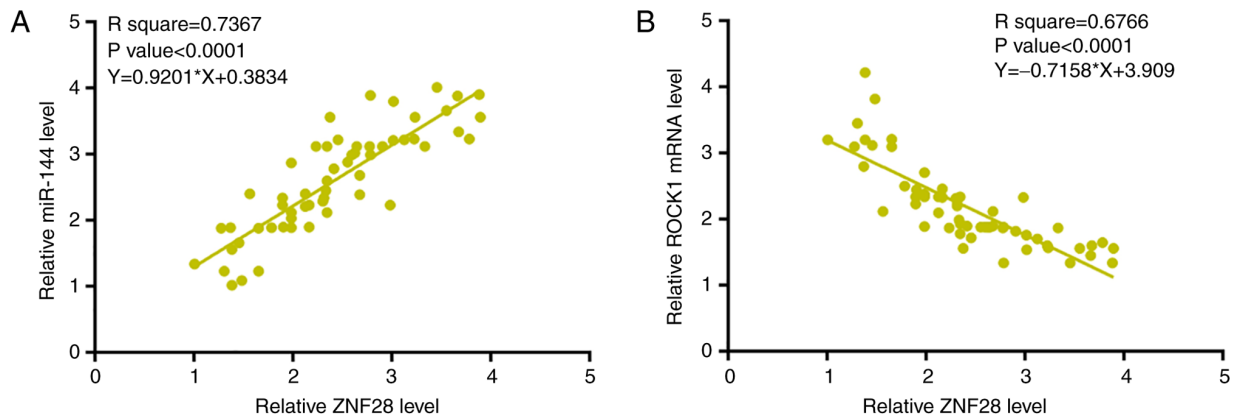


Figure 3. ZNF281 mRNA expression is significantly associated with ROCK1 mRNA and miR-144 expression in OS tissues. (A and B) Association between ZNF281 and (A) miR-144 and (B) ROCK1 mRNA were analyzed by linear regression. OS, osteosarcoma; ZNF281, zinc finger protein 281; ROCK1, rho-associated coiled-coil containing protein kinase 1.

Western blotting. RIPA buffer (Sangon Biotech Co., Ltd) was used to extract total proteins from U2OS cells. Protein concentrations were measured using a bicinchoninic acid assay kit. Protein samples were boiled for 5 min for denaturation. Subsequently, 10% SDS-PAGE was used to separate the proteins with 30 μ g protein per lane. The proteins were transferred to PVDF membranes, followed by blocking for 2 h in PBS containing 5% FBS (Sigma-Aldrich; Merck KGaA) at room temperature. The membranes were probed with rabbit anti-ROCK1 (1:800; cat. no. ab97592; Abcam) and anti-GAPDH (1:800; cat. no. ab37168; Abcam) for 12 h at 4°C, followed by incubation with horseradish peroxidase-conjugated goat secondary antibody (IgG; 1:1,000; cat. no. ab6721; Abcam) for 2 h at 24°C. Signal development was performed using the ECL Western Blotting Substrate kit (cat. no. ab65623; Abcam), and the data were processed using Image J v.1.48 software (National Institute of Health).

Transwell invasion and migration assays. The effects of the various transfections on the invasion and migration of U2OS cells were determined by Transwell invasion and migration assays. Transwell membranes were precoated with Matrigel at 37°C for 6 h for the invasion assay. Cells were harvested, counted and mixed with serum-free EMEM at 3×10^4 cells/ml to prepare single-cell suspensions. Cells were added into the upper chamber (0.1 ml/well), and the lower chamber was filled with EMEM containing 20% FBS. Transwell chambers were incubated at 37°C for 12 h. Subsequently, non-invasive and non-migrated cells were removed using cotton swabs and the lower surface of membranes was stained with 0.5% crystal violet (Merck KGaA) for 20 min at room temperature and the cells were observed under an optical microscope in five random visual fields (magnification, x40). The number of cells in the control group was set to 100% and all other groups were normalized to this group.

Statistical analyses. All data are expressed as mean \pm SD values of three biological replicates. Differences between OS and non-tumor tissues were analyzed by paired Student's t-test. Differences among multiple cell transfection groups were analyzed by one-way ANOVA followed by the Tukey's

post-hoc test. Correlations were analyzed by linear regression. To perform survival analysis, 60 patients with OS were grouped into high and low ZNF281 groups according to its median expression level in OS tissues. Survival curves were plotted and compared by GraphPad Prism v6 software (GraphPad Software, Inc.) using the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ZNF281 is downregulated in tissue specimens from patients with OS and affected by the clinical stages. Expression levels of ZNF281 in OS and non-tumor tissues from the patients were measured by qPCR and compared by the paired Student's t-test. The resulted revealed significantly higher expression levels of ZNF281 in OS tissues compared with non-tumor tissues (Fig. 1A). Expression levels of ZNF281 in OS tissues were compared among different clinical stages. Significantly decreased expression levels of ZNF281 were observed with the increase of clinical stages (Fig. 1B). Of note, expression levels of ZNF281 significantly decreased with the increase in clinical stages.

Low levels of ZNF281 mRNA expression predict the poor survival of patients with OS. Survival curves of the high and low ZNF281 expression groups were plotted. Compared with the low ZNF281 expression group, the 5-year overall survival rate of the high ZNF281 expression group was significantly higher (Fig. 2).

ZNF281 expression levels are significantly associated with ROCK1 mRNA and miR-144 expression in OS tissues. Expression levels of ROCK1 mRNA and miR-144 in OS tissues were also measured by qPCR (data not shown). Associations between ZNF281 and ROCK1 mRNA/miR-144 were analyzed by linear regression. The mRNA expression levels of ZNF281 and miR-144 were significantly positively associated (Fig. 3A). However, the mRNA expression level of ZNF281 was significantly inversely associated with that of ROCK1 (Fig. 3B).

ZNF281 upregulates miR-144, which downregulates ROCK1 in U2OS cells. Transfections of ZNF281 and ROCK1

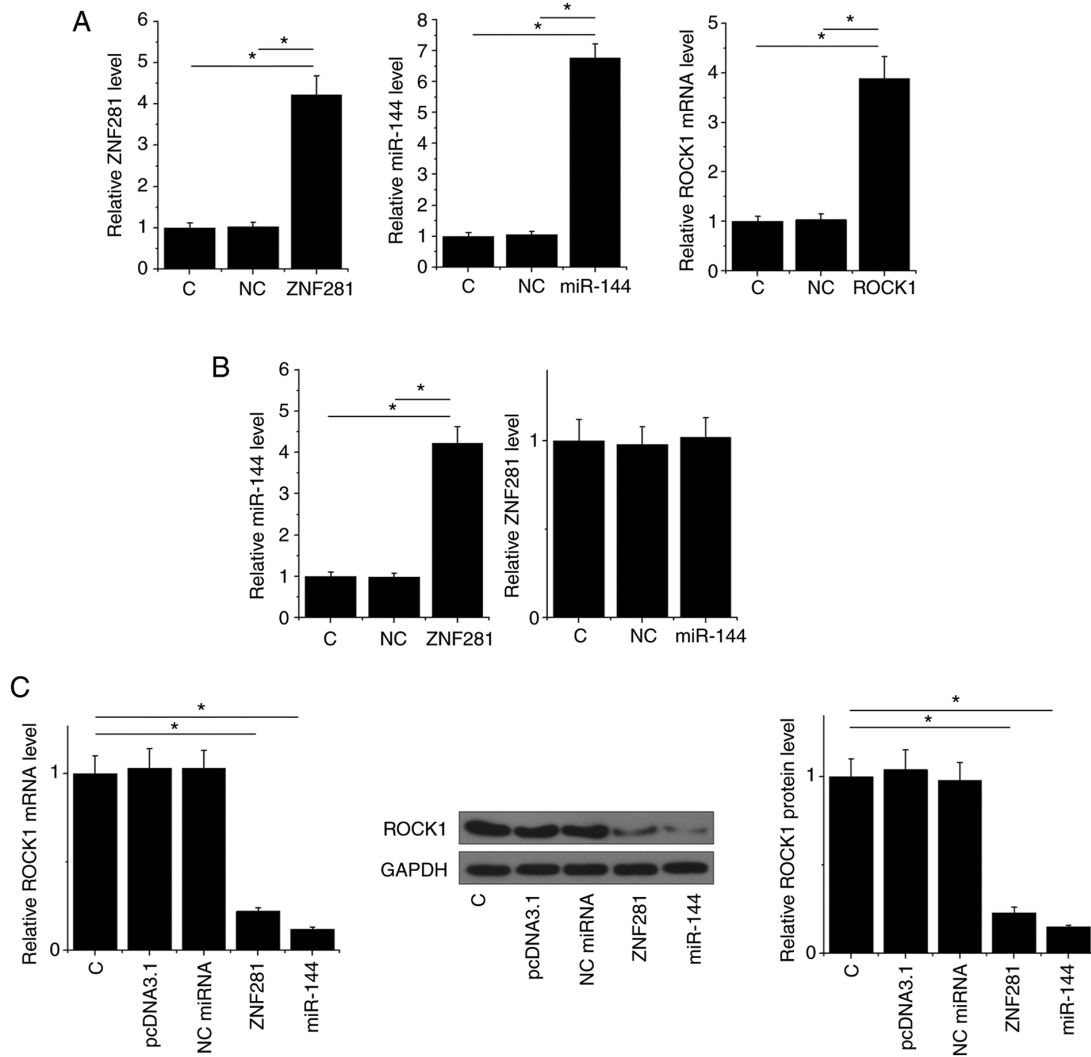


Figure 4. ZNF281 upregulation of miR-144 downregulates ROCK1 in U2OS cells. Transfections were performed to analyze the interactions among ZNF281, miR-144 and ROCK1. (A) Overexpression of ZNF281, miR-144 and ROCK1 was confirmed by qPCR at 24 h post-transfection. (B) The interaction between ZNF281 and miR-144 analyzed by quantitative PCR. (C) The effects of ZNF281 and miR-144 overexpression on ROCK1 analyzed by western blot and qPCR. Mean values of three biological replicates are presented. *P<0.05. OS, osteosarcoma; ZNF281, zinc finger protein 281; ROCK1, rho-associated coiled-coil containing protein kinase 1.

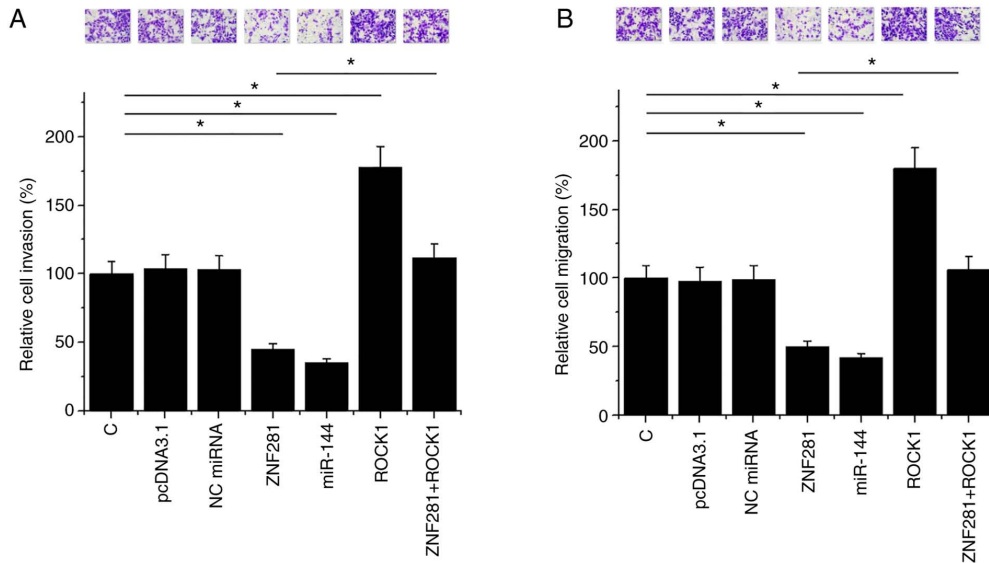


Figure 5. ZNF281 suppresses U2OS cell invasion and migration through miR-144 and ROCK1. (A and B) The effects of transfections on U2OS cell invasion and migration were analyzed by the Transwell (A) invasion and (B) migration assays. Mean values of three biological replicates are presented. *P<0.05. OS, osteosarcoma; ZNF281, zinc finger protein 281; ROCK1, rho-associated coiled-coil containing protein kinase 1.

overexpression vector, as well as a miR-144 mimic, were performed to analyze the interactions among ZNF281, miR-144 and ROCK1. Compared to C (untransfected cells) and NC groups, expression levels of ZNF281, miR-144 and ROCK1 were increased at 24 h post-transfection, indicating that the transfections were successful (Fig. 4A). Compared with the two control groups, ZNF281 overexpression mediated the upregulation of miR-144, whereas miR-144 over-expression failed to affect ZNF281 (Fig. 4B). Moreover, ZNF281 and miR-144 overexpression mediated the downregulation of ROCK1 (Fig. 4C).

ZNF281 suppresses U2OS cell invasion and migration through miR-144 and ROCK1. Compared with the untransfected and NC groups, cell invasion and migration analysis demonstrated that ZNF281 and miR-144 resulted in decreased U2OS cell invasion (Fig. 5A). ROCK1 overexpression resulted in increased invasion (Fig. 5A) and migration (Fig. 5B) of OS cells. In addition, ROCK1 overexpression attenuated the effects of ZNF281 overexpression in U2OS cells (Fig. 5B).

Discussion

The present study, investigated the functions of ZNF281 in OS. In U2OS cells, ZNF281 overexpression upregulated miR-144 which downregulated ROCK1, thus inhibiting the invasion and migration of OS cells.

In a recent study, Li *et al* (11) identified a novel lncRNA termed ZNF281 in glioma with a tumor-suppressive role in regulating cancer cell stemness, proliferation and invasion. ZNF281 was downregulated in glioma, and the multiple functions of ZNF281 were associated with numerous cancer-related molecular markers, such as tCD133, Nestin, OCT4, Nanog and the NF- κ B1 signaling pathways (11). The involvement of ZNF281 in other human diseases is unknown. The present study demonstrated that ZNF281 was downregulated in OS tissue samples from patients and overexpression of ZNF281 led to the suppressed invasion and migration of OS cells. Therefore, ZNF281 may be a tumor suppressive lncRNA in OS.

ROCK1 serves a critical role in the invasion and migration of OS cells (14,15). Increased rates of OS invasion and migration were observed after ROCK1 overexpression (14,15). A number of tumor suppressive miRNAs in OS target ROCK1 to inhibit cancer progression. For instance, miR-150 directly targets ROCK1 in OS to inhibit the invasion, proliferation and migration of cancer cells (16). Wang *et al* (10), have demonstrated that miR-144 suppresses the proliferation and metastasis of OS cells by targeting ROCK1. The present study demonstrated the downregulation of ROCK1 after miR-144 overexpression in OS cells, further confirming the targeting of ROCK1 by miR-144.

The NF- κ B signaling pathway serves oncogenic roles in the majority of types of cancers (17). NF- κ B exerts its roles in cancer biology by interacting with numerous oncogenic or tumor-suppressive factors, such as miRNAs (18,19). A recent study has demonstrated that NF- κ B may interact with miR-144 to affect cell development (20). The findings of the present study demonstrated that ZNF281 upregulated miR-144 to downregulate ROCK1. ROCK1 can inactivate NF- κ B to

suppress OS (8). Therefore, NF- κ B may mediate the interaction between ZNF281 and miR-144.

The present study had several limitations. Firstly, the interaction mechanism of ROCK1, ZNF281 and miR-144 was only investigated *in vitro*. *In vivo* studies are required to verify and elucidate this mechanism. Secondly, only one cell line was included in the present study. Future studies with other cell lines are needed to verify the findings of the present study. Thirdly, the effects of chemotherapy on ZNF281 were not investigated.

In conclusion, ZNF281 overexpression in a U2OS cell line may serve a tumor suppressive role by downregulating ROCK1 through the upregulation of miR-144 to suppress cancer cell invasion and migration.

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

YS and TY designed the experiments. YS and ZT performed experiments. JW analyzed data. TY drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Honghui Hospital Affiliated to Xi'an Jiaotong University (Xi'an, China; approval no. 32556HHXU20110322). All patients signed an informed consent form.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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