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Original Article

miR-31 inhibits cell proliferation and invasion through targeting ROCK1 in prostate cancer

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Abstract: Aberrant expression of microRNAs (miRNAs) has been frequently implicated in the development and progression of human malignancies. The objective of the present study was to explore the expression profile and biological function of miR-31 in prostate cancer (PCa). Through quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis, we found that the expression of miR-31 in PCa tissues was markedly lower than that of adjacent normal prostate tissues. Additionally, CCK-8 and transwell assays were conducted to determine the effects of miR-31 on the PCa cell proliferation, migration, and invasion, and we found that miR-31 overexpression inhibited PCa cell proliferation, migration, and invasion. Potential target genes of miR-31 were predicted by bioinformatics tools and confirmed by dual-luciferase reporter assay. The underlying mechanisms of miR-31 in modulating PCa cell behavior was to directly regulate Rho associated coiled-coil containing protein kinase 1 (ROCK1) by binding its 3'-untranslated regions (3'-UTR). In summary, the results of the current study suggest that miR-31 may inhibit proliferation and invasion in PCa cells at least partially by targeting ROCK1. This finding may provide a therapeutic approach for future treatment of ROCK1.

Keywords: Prostate cancer, miR-31, ROCK1, proliferation, invasion

Introduction

Although great achievements have been made in the diagnosis and treatment of malignant tumors, prostate cancer (PCa) remains the fifth leading cause of male mortality due to cancer [1]. Owing to improvements in diagnostic techniques, more than 80% of new cases are organ-confined at the time of diagnosis [2]. However, unfortunately, distant metastasis often occur in patients with advanced stage of PCa, which has been identified a major leading cause of death. Therefore, a deep and comprehensive understanding of the molecular mechanisms in PCa progression and development is urgently required, which will be helpful to exploit novel strategies for diagnosis and treatment of human PCa.

MicroRNAs (miRNAs) are a class of small and non-coding RNA of approximately 19-25 nucleotides that are usually transcribed from genes by polymerase II and eventually processed into single-strand miRNAs [3, 4]. miRNA exert their functions mainly through binding to the 3' untranslated region (3'-UTR) of their targeting mRNAs, which lead to translational repression or degradation of mRNAs [5]. Recently, various publications have shown that miRNAs may act as oncogenic miRNAs, tumor suppressors, or have multiple roles depending on the regulated targets [6]. Lower expression of a specific miRNA may induce increased expression of target genes and reactivation of previously silenced sequences, which can cause all around changes in the epigenome and, eventually, tumor progression [7, 8]. For example, miR-31 is a well-known carcinogenic-miRNA that participates in cancer pathology including enhanced proliferation, migration, and invasion. Rasheed et al. have demonstrated that down-regulation of miR-31 in breast cancer and its reduced expression is linked to the progression of breast cancer [9]. Moreover, miR-31 acts as a tumor promoter in colorectal carcinoma [10], and restrains cell proliferation, migration, and

Table 1. Association between miR-31 expression and clinicopathological characteristics of 69 PCa patients.

Characteristics	Total number	miR-31 expression		χ^2	*P*
		High (n = 23)	Low (n = 46)		
Age (years)				0.469	0.494
≤ 60	38	14	24		
> 60	31	9	22		
Family history of PCa				0.322	0.570
Yes	41	13	28		
No	26	10	16		
Gleason score				8.348	0.004
≤ 8	46	10	36		
> 8	23	13	10		
Preoperative PSA (ng/mL)				0163	0.687
< 10	16	6	10		
≥ 10	53	17	36		
Pathological stage				8.464	0.004
I+II	38	7	31		
III+IV	26	16	15		
Distant metastasis				8.540	0.011
Negative	47	11	36		
Positive	22	12	10		
Lymph node metastasis				4.037	0.045
Negative	47	12	35		
Positive	22	11	11		

invasion of ovarian cancer cells [11]. A recent study has identified that expression of miR-31 was lower than that of control group and it was associated with the biochemical PCa recurrence [12]. Another study showed that low miR-31 expression is correlated with unfavorable overall survival of PCa patients [13]. However, the underlying mechanism of miR-31 in PCa progression remains to be clearly elucidated.

The goal of the present study is to investigate the biological role of miR-31 in PCa and to explore the underlying mechanisms of its function. We examined the expression levels of miR-31 in human PCa tissue samples and cell lines through qRT-PCR analysis. In addition, we demonstrated that overexpression of miR-31 significantly suppresses the proliferation, migration, and invasion of PCa cells. Moreover, 3'-UTR of the human Rho associated coiled-coil containing protein kinase 1 (ROCK1) mRNA was confirmed as a target of miR-31. Collectively, we discovered that miR-31 exerts its tumor suppressive effects on PCa through targeting ROCK1.

Materials and methods

Clinical specimens

Sixty-nine pairs of human PCa tissues and their corresponding adjacent normal prostate tissues were collected from patients who underwent needle biopsy or autopsy at The First Affiliated Hospital of Fujian Medical University (Quanzhou, China). All patients did not receive any local or systemic therapies before surgery. All the tissue samples had been clinically and histopathologically diagnosed by two experienced pathologists. The specimens were rapidly frozen in liquid nitrogen and then stored at −80°C for further analysis. The patients' characteristics are summarized in **Table 1**. Informed consents from every patient were obtained, and the study was approved by the Ethics Committee of The First Affiliated Hospital of Fujian Medical University.

Cell culture and transfection

Human PCa cells (PC3 and DU145) and normal prostate cells (RWPE-1), purchased from Institute of Cell Biology of Chinese Academy of Science (Shanghai, China), were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

The sequence of ROCK1 was synthesized and cloned into the pcDNA3.1 (+) vector (Promega, Madison, WI, USA). miR-31 mimics (miR-31), miR-31 inhibitor (anti-miR-31), negative control miRNA mimics, and miRNA inhibitor (miR-NC and anti-miR-NC) were provided by GenePharma

(Shanghai, China). Cell transfection was performed using Lipofectamine 2000 (Invitrogen). To assess the transfection efficiency, qRT-PCR was performed 24 hours after transfection.

RNA extraction and qRT-PCR

Total RNA was extracted from tissue samples and cells using Trizol Reagent (Invitrogen). RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For miRNA detection, the RNA was reverse transcribed into cDNA using One Step Prime script miRNA cDNA Synthesis Kit (Qiagen, Valencia, CA, USA). miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) was then used to conduct qRT-PCR on the LightCycler 480 II System (Roche, Basel, Switzerland). For mRNA detection, cDNA was synthesized using the Primer Script RT reagent Kit (Takara, Dalian, China). mRNA levels were then quantified using the real-time PCR Mixture Reagent (Takara). The qRT-PCR results were analyzed by the $2^{-\Delta\Delta Ct}$ method and normalized to U6 snRNA or GAPDH mRNA expression.

Cell proliferation assay

Cell proliferation was evaluated every 24 h for 4 days using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were seeded in 96-well plate with 5000 cells/well. Following incubation for 24, 48, 72, or 96 h, 10 μL CCK-8 solution was added to each well of the 96-well plate and incubated for another 2 h. The absorbance values of cells were measured at 450 nm using a microplate reader.

Transwell assay

Cell migration and invasion assays were performed using transwell chambers (Corning, Tewksbury, MA, USA). Cells in serum-free medium were added to the upper chambers, which contained either Matrigel-coated or uncoated membranes, and lower chambers were filled with 500 μL medium with 10% FBS. After 24 h of incubation, the cells migrating or invasion to the lower chambers were fixed with methanol, and then stained with 0.1% crystal violet. Migrated or invaded cells were counted manually under a microscope.

Western blot analysis

Cells were lysed with RIPA buffer supplemented with protease inhibitors. Equal quantities of protein samples were separated using SDS-polyacrylamide gels and transferred to PVDF membranes. After incubation with primary antibodies and secondary antibodies, the blots were visualized by enhanced chemiluminescence (Millipore, Billerica, MA) with β-actin as a loading control.

Bioinformatics methods

The miRNA targets predicted by computer-aided algorithms were obtained from pictar (http://pictar.mdc-berlin.de/cgi-bin/new_PicTar_vertebrate.cgi.), targetscan (http://www.targetscan.org) and mirbase targets (http://microrna.sanger.ac.uk/cgi-bin/targets/v5/search.pl).

Dual-luciferase reporter assay

The 3'-untranslated regions (3'-UTR) of ROCK1 containing predicted miR-31 seed-matching sites and corresponding mutant sites were inserted downstream of the firefly luciferase gene in a pGL3 promoter vector (Promega), named as: WT-ROCK1-3'UTR and MUT-ROCK1-3'UTR, respectively. For dual-luciferase assay, the luciferase reporter gene vectors, together with miR-31 mimics or miR-NC were co-transfected into PC3 cells. At 48 h after transfection, Firefly and Renilla luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega). Luciferase activities were normalized to Renilla luciferase.

Statistical analysis

Each experiment was performed in triplicate, and repeated at least three times. The data are expressed as the mean ± standard deviation (SD). The significance of differences between groups was estimated by Student's t-test or chi-square test. The survival curves were calculated by the Kaplan–Meier method with the log-rank test applied for comparison. All statistical analyses were performed using Graphpad Prism (version 6.01) software (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA). A two-tailed value of $P < 0.05$ was considered statistically significant.

Results

Downregulated miR-31 predicts poor clinical outcome in PCa patients

To reveal the role of miR-31 in PCa, qRT-PCR was performed to examine the expression of

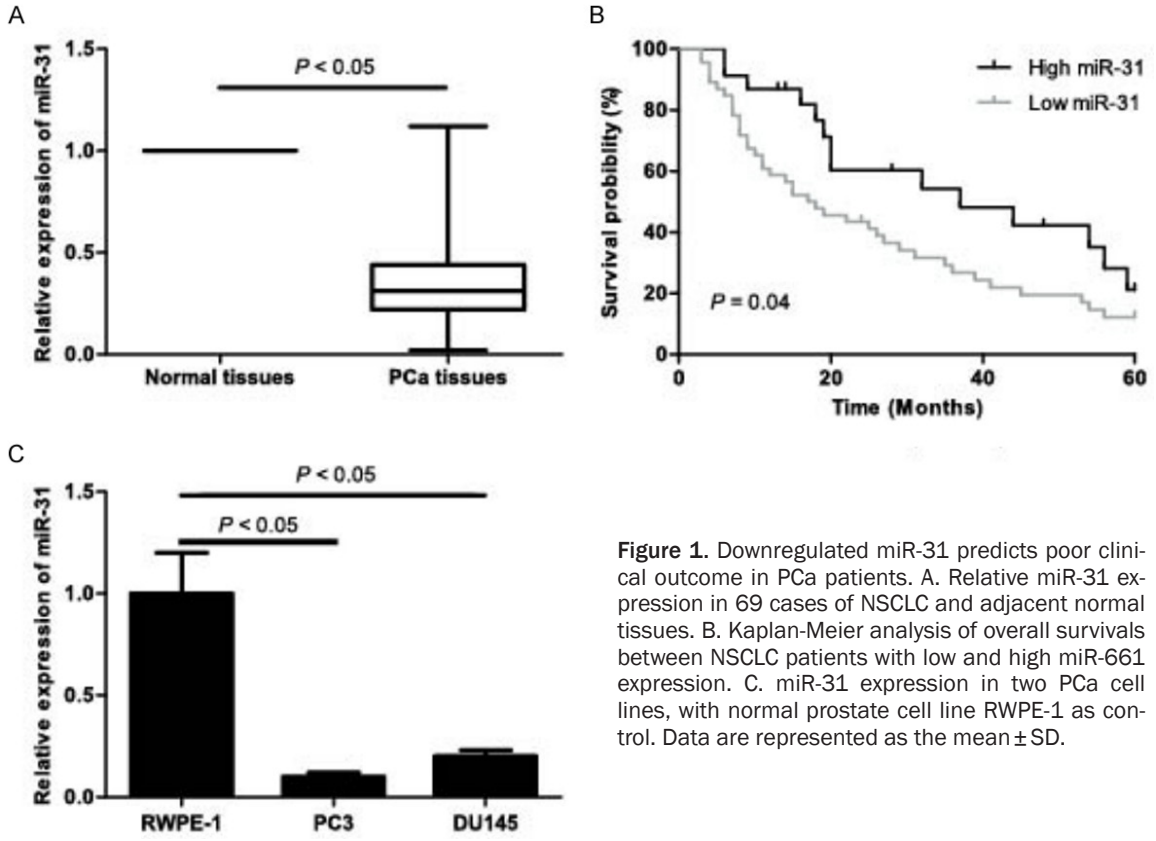

Figure 1. Downregulated miR-31 predicts poor clinical outcome in PCa patients. A. Relative miR-31 expression in 69 cases of NSCLC and adjacent normal tissues. B. Kaplan-Meier analysis of overall survivals between NSCLC patients with low and high miR-661 expression. C. miR-31 expression in two PCa cell lines, with normal prostate cell line RWPE-1 as control. Data are represented as the mean ± SD.

miR-31 in the tissue samples of 69 PCa patients with corresponding adjacent normal prostate tissues used as controls. We observed that, as revealed in **Figure 1A**, miR-31 was frequently downregulated in the PCa tissues compared with adjacent normal tissues. Additionally, expression of miR-31 in tumor tissues was inversely associated with Gleason score (P = 0.004), pathological stage (P = 0.004), distant metastasis (P = 0.011), and lymph node metastasis (P = 0.045) in PCa patients (**Table 1**). Importantly, patients with low miR-31 expression had a significant poor overall survival compared to patients with high miR-31 expression (P = 0.004; **Figure 1B**). Also, we observed that miR-31 was markedly down-regulated in the PCa cells (PC3 and DU145) compared with the normal prostate cells (RWPE-1) (**Figure 1C**).

miR-31 inhibits proliferation, migration and invasion of PCa cells

We further explored the function of miR-31 in proliferation, migration and invasion in PCa cell lines. PC3 cells were transiently transfected with miR-31 mimics, which efficiently promoted endogenous expression of miR-31 (**Figure 2A**). Also, miR-31 expression in DU145 cells transfected with miR-31 inhibitor was markedly decreased. CCK-8 assay were performed to assess the role of miR-31 in PCa cell proliferation. The PCa cells transfected with miR-31 mimics grew obviously slower compared to control cells, and the cell proliferation of cells transfected with miR-31 inhibitor was significantly enhanced (**Figure 2B**). Furthermore, as shown in **Figure 2C**, PCa cells with reduced miR-31 expression showed increased ability of migration and invasion compared with control cells, whereas elevated miR-31 suppressed cell migration and invasion. These findings indicate that miR-31 markedly inhibits PCa cell proliferation, migration and invasion.

ROCK1 is a direct target of miR-31 in PCa cells

To clarify the molecular mechanisms of miR-31 on the biological phenotypes of PCa cells, we searched for the target mRNAs using Pictar, Targetscan and mirbase targets. ROCK1 was

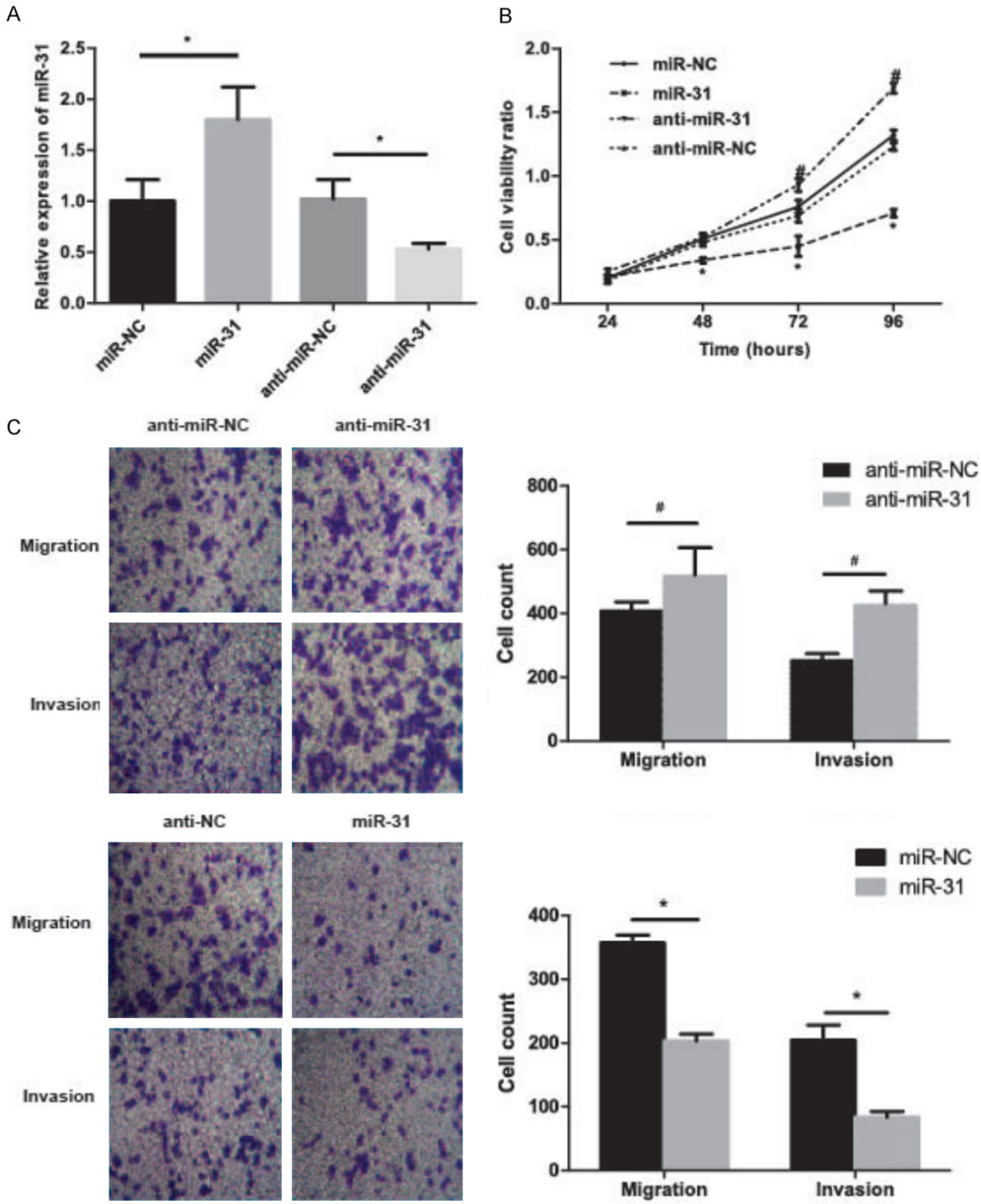

Figure 2. miR-31 inhibits the proliferation, migration and invasion of PCa cells. A. qRT-PCR analysis of transfection efficacy. B. CCK-8 assay of PC3 cells with miR-31 overexpression and DU145 cells with miR-31 inhibition. C. Transwell assay of PC3 cells with miR-31 overexpression and DU145 cells with miR-31 inhibition. Data were represented as the mean ± SD. $^{*}P < 0.05$ versus miR-NC group; $^{\#}P < 0.05$ versus anti-miR-NC group.

selected as one of the candidate targets of miR-31, due to the putative target sequences at the 3'UTR of ROCK1 (**Figure 3A**). To further confirm targeting of ROCK1 by miR-31, luciferase activity assay was performed. As exhibited in **Figure 3B**, we found that PC3 cells transfect-

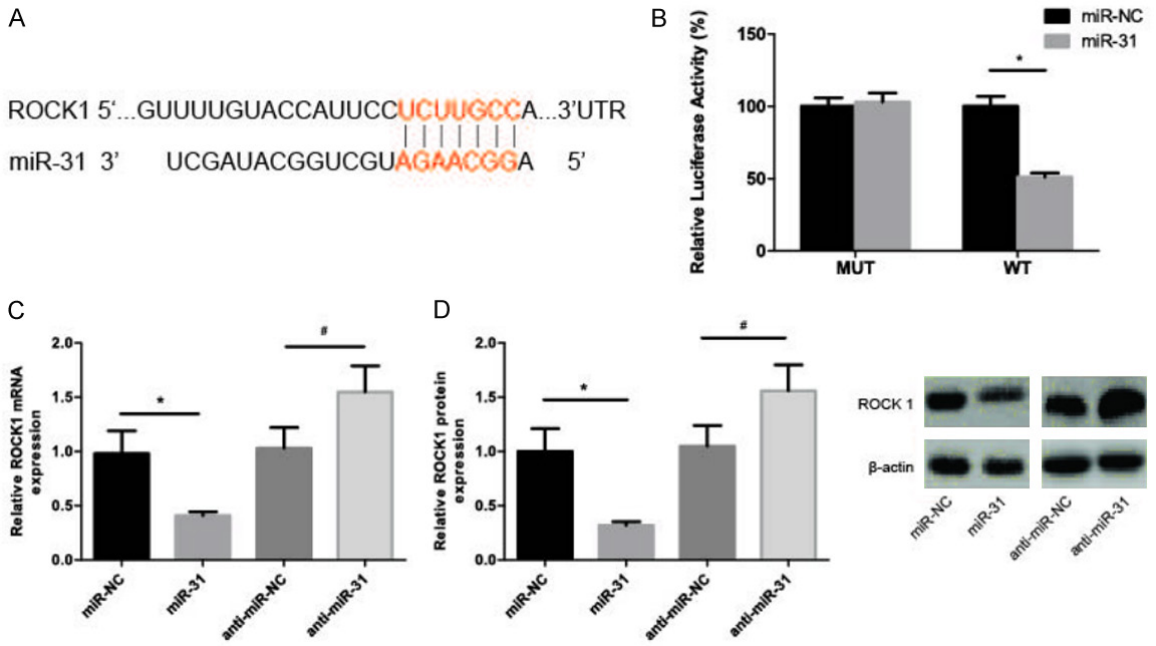

Figure 3. ROCK1 is a direct target of miR-31 in PCa cells. A. Schematic diagram of miR-31 binding sites in the ROCK1 3'UTR. B. Relative luciferase activity was analyzed in PC3 cells. C. qRT-PCR results of ROCK1 mRNA in PC3 cells. (D) Western blot results of ROCK1 protein in PC3 cells. Data are represented as the mean ± SD. $^{*}P < 0.05$ versus miR-NC group; $^{\#}P < 0.05$ versus anti-miR-NC group.

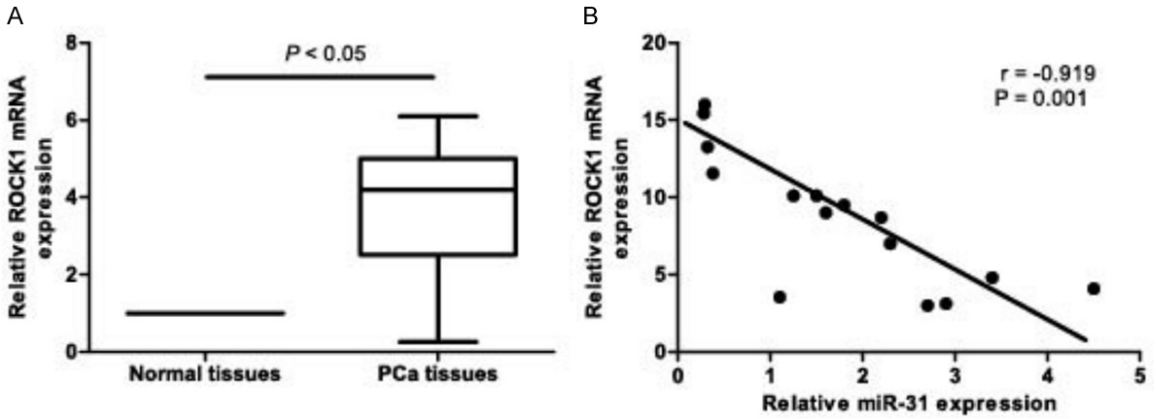

Figure 4. ROCK1 mRNA expression is negatively correlated with that of miR-31 in PCa. A. Relative ROCK1 mRNA expression in 69 cases of NSCLC and adjacent normal tissues. B. Correlation analysis between miR-31 and ROCK1 mRNA expression in 15 NSCLC tissues.

ed with miR-31 mimics significantly inhibited WT-ROCK1-3'UTR reporter activity, while had no obvious inhibitory effect on the MUT-ROCK1-3'UTR reporter activity.

Then we measured the levels of ROCK1 mRNA and protein levels after altering the expression of miR-31. As indicated in **Figure 3C**, the levels of ROCK1 mRNA and protein were markedly decreased after transfection of miR-31 mimics in PCa cells, whereas anti-miR-31 led to a remarkable increase of ROCK1 mRNA and protein in PCa cells. These data reveal that ROCK1 is a potential target of miR-31 and the expression of ROCK1 is regulated by miR-31.

Subsequently, we evaluated relative ROCK1 mRNA levels in 15 patients' PCa tissues and corresponding adjacent normal prostate tissues. We found that ROCK mRNA was signifi-

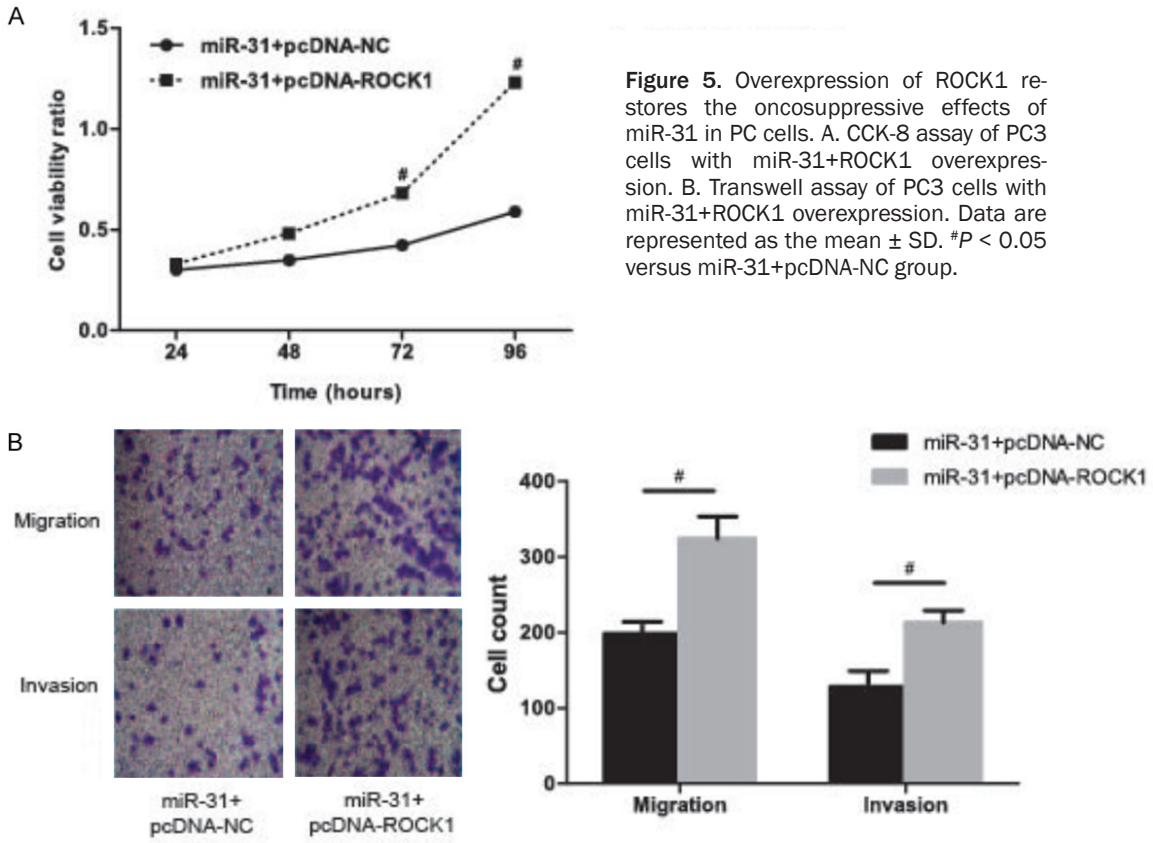

Figure 5. Overexpression of ROCK1 restores the oncosuppressive effects of miR-31 in PC cells. A. CCK-8 assay of PC3 cells with miR-31+ROCK1 overexpression. B. Transwell assay of PC3 cells with miR-31+ROCK1 overexpression. Data are represented as the mean ± SD. $^{\#}P < 0.05$ versus miR-31+pcDNA-NC group.

cantly upregulated in PCa tissues more than the corresponding adjacent normal prostate tissues (**Figure 4A**). Moreover, as exhibited in **Figure 4B**, expression of ROCK1 mRNA was negatively correlated with that of miR-31 in the PCa tissues of these 15 patients (r = -0.919, *P* = 0.001).

Overexpression of ROCK1 restores the oncosuppressive effects of miR-31 in PC cells

A rescue experiment was performed to further verify the roles of miR-31 and ROCK1 in PCa. pcDNA3.1-ROCK1 was constructed to enhance endogenous ROCK1 expression. The results of CCK-8 assay indicate that the proliferation suppressing effect of miR-31 was reversed by ROCK1 in PC3 cells (**Figure 5A**). Besides, the transwell assay indicates that ROCK1 reversed the inhibitory effects of miR-31 on the migration and invasion abilities of PCa cells (**Figure 5B**). These results suggest that overexpression of ROCK1 could significantly reverse the effects of miR-31 on the malignant phenotypes of PCa cells.

Discussion

Previous studies have identified that miRNAs play critical roles in the progression of various human cancers [5]. Furthermore, deregulated expression of miRNAs have been demonstrated in various cancers and associated with formation and progression of tumor cells. The functions of miRNAs usually tend to act as tumor suppressor miRNAs or oncogenic miRNAs in the tumor cells [14]. miR-31 has previously been confirmed as a tumor suppressor and its lower expression has been frequently linked with tumor progression in PCa and other tumor types, via genetic or epigenetic mechanisms [9, 15]. In the present study, we found that expression of miR-31 in a panel of PCa tissues was significantly lower than that of adjacent normal tissues. Furthermore, miR-31 was markedly down expressed in the PCa cells (PC3 and DU145) more so than in the normal prostate cells (RWPE-1). Notably, the reduced miR-31 expression was positively correlated with overall survival of PCa patients. All of these results reveal that loss of miR-31 might be an

important determinant in the progression of PCa.

In previous publications, miR-31 has been reported to be down-regulated in different tumor types, such as breast and ovarian cancer [9, 11]. In Liu's study, engineered repression of miR-31 could markedly promote repression of lung cancer growth by targeting oncogenes LATS2 and PPP2R2A [16]. Importantly, previous studies have demonstrated that enforced expression of miR-31 could significantly inhibited tumor cells invasion and metastasis of tumor cells [17, 18]. At present, when PCa cells were transfected with miR-31 mimics, the cells grew significantly slower than control group. On the other hand, proliferation of PCa cells transfected with miR-31 inhibitor was enhanced. More importantly, when the expression of miR-31 was reduced, we observed that PCa cells showed dramatically enhanced invasion and migration capacities. These results indicate that miR-31 contributes to cancer metastasis and invasion in PCa.

miRNAs regulate cell biological processes through inducing cleavage or inhibiting translation of target mRNAs [4, 19]. The target genes of miR-31 have been confirmed in previous studies, including LATS2, PPP2R2A, and tyrosine kinase MET [11, 16]. The human Rho-associated coiled-coil containing protein kinase 1 (ROCK1) is located on human chromosome 18 and translates RhoA serine/threonine [20, 21]. To date, considerable evidence has revealed that ROCK1 plays an important role in the biological behaviors of various cancers, especially in the motility and invasion of cancer cells [22-24]. Here, we observed that the levels of ROCK mRNA and protein were significantly increased in miR-31-overexpressed PCa cells. On the other hand, WT-ROCK1-3'UTR reporter activity was markedly inhibited in PCa cells transfected with miR-31 mimics, as indicated by luciferase activity assay. Moreover, the inhibitory effects of miR-31 on cell proliferation, migration, and invasion could be significantly restored by ROCK1 overexpression in PCa cells. Therefore, we believe that the expression of ROCK1 expression regulated by miR-31 is the one of the underlying mechanisms in PCa.

In conclusion, the results from the present study reveal that the miR-31-ROCK1 regulatory axis might be related to enhanced proliferation and invasion abilities of PCa cells. Therefore, miR-31 may be a potential diagnostic predictor and therapeutic target for PCa.

Disclosure of conflict of interest

None.

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