LncRNA LINK-A regulates ROCK1 expression in early-stage pancreatic adenocarcinoma

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Abstract. Long intergenic non-coding RNA for kinase activation (LINK-A) has been characterized as an oncogenic long non-coding (lnc)RNA in triple-negative breast cancer and ovarian carcinoma, but its involvement in other malignancies remains elusive. In the present study, it was determined that the plasma levels of LINK-A lncRNA and Rho-associated protein kinase 1 (ROCK1) were significantly increased in patients with pancreatic adenocarcinoma compared with those in healthy controls. The plasma levels of LINK-A lncRNA were positively correlated with the plasma levels of ROCK1 in pancreatic adenocarcinoma patients, but not in healthy controls. Silencing of LINK-A led to inhibition of pancreatic adenocarcinoma cell proliferation, migration and invasion. Simultaneous overexpression of ROCK1 attenuated the inhibitory effect of LINK-A silencing on cancer cell proliferation, migration and invasion. Overexpression of LINK-A lncRNA led to upregulation of ROCK1 expression, while overexpression of ROCK1 had no significant effect on LINK-A lncRNA expression. It may therefore be concluded that LINK-A lncRNA may have a role in pancreatic adenocarcinoma, at least in part, by promoting ROCK1 expression.

Introduction

Pancreatic cancer is a highly aggressive malignancy with an unacceptably high mortality rate. The overall 5-year survival rate of patients with pancreatic cancer is generally <5% in China and is even <1% in certain regions of the world (1). It has been reported that pancreatic cancer causes more deaths than breast cancer in the European Union, in spite of having a much lower incidence rate (2). The 5-year survival rate of patients after proper surgical resection may be up to 25% (3). However, the application of surgical operation is limited by the high prevalence of tumor metastasis by the time of diagnosis (4). Therefore, early diagnosis and treatment still has pivotal roles in the survival of patients with pancreatic cancer.

Rho-associated protein kinase 1 (ROCK1) is a serine/threonine kinase protein that widely participates in numerous aspects of cancer biology (5,6). ROCK kinases regulate associated gene expression to participate in cell proliferation, differentiation and apoptosis, so as to affect oncogenic transformation (7). A growing body of evidence has indicated that inhibition of ROCK1 may serve as a potential therapeutic target for cancer treatment (5-8). ROCK1 participates in cancer biology through interaction with various functional molecules, including long non-coding RNAs (lncRNAs) (9-11). Long intergenic non-coding RNA for kinase activation (LINK-A) is an lncRNA with characterized functionality only in triple-negative breast cancer (12) and ovarian carcinoma (13). The interaction between LINK-A and ROCK1 is unknown. Preliminary microarray data (unpublished; 24 pancreatic adenocarcinoma tissues, 24 control tissues) revealed the close correlation between them, indicating a possible interaction. The present study indicated that LINK-A may have a role in pancreatic cancer by upregulating ROCK1.

Materials and methods

Patients and specimens. A total of 42 patients with pancreatic adenocarcinoma were enrolled in the present study (Table I). All of these patients were diagnosed and treated at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) between March 2016 and March 2018. The inclusion criteria were as follows: i) Pancreatic adenocarcinoma patients confirmed by pathological examination; ii) patients at stage IA-IIA prior to development of lymph node metastasis; iii) patients received surgical resection. The exclusion criteria were as follows: i) Any treatments within 3 months prior to admission; ii) complication with other malignancies. During the same time period, 36 healthy controls were also enrolled from a population of healthy people undergoing physical examination. One day after admission, fasting blood was

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extracted from the patients and controls in the morning to prepare plasma. The patient group was composed of 24 males and 18 females with an age range of 24-66 years and a mean age of 45.4±7.1 years. The control group was composed of 19 males and 17 females with an age range of 26-67 years and a mean age of 46.9±6.4 years. The two groups had similar age and gender distributions. The ethics committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) approved the present study. All participants provided written informed consent. All specimens were stored in liquid nitrogen prior to use.

Reverse transcription-quantitative (RT-q)PCR. Plasma samples were obtained by venipuncture of the patients (n=42) and controls (n=36). Venous blood (3-5 ml) was collected into tubes containing EDTA, and a 2 step centrifugation protocol was followed: samples were first centrifuged at 1,500 x g for 15 min at 4˚C, before the supernatant was centrifuged again at 14,000 x g for 15 min at 4˚C. The supernatant plasma was removed, split into aliquots and frozen at -80˚C until use. Total RNA was extracted from plasma samples of the patients and controls using an RNeasy Mini Spin kit (Qiagen China Co., Ltd.) and cDNA synthesis was carried out from 1 μl of RNA, using 5X PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd.; 4 μl, in total volume of 20 μl reaction mixture). PCR reactions were prepared using the SuperScript III Platinum One-Step qRT-PCR Kit (Thermo Fisher Scientific, Inc.). The PCR conditions were as follows: 95˚C for 56 sec, followed by 40 cycles of 95˚C for 14 sec and 58.5˚C for 30 sec. The primers used for PCR had the following sequences: Human LINK-A forward, 5'-TTC CCCCATTTTTCCTTTTC-3' and reverse, 5'-CTCTGGTTG GGTGACTGGTT-3'; β-actin forward, 5'-GACCTCTATGCC AACACAGT-3' and reverse, 5'-AGTACTTGCGCTCAGGAG GA-3'. Data were normalized using the $2^{-\Delta\Delta Cq}$ method (14).

ELISA. Plasma levels of ROCK1 were measured by ELISA using a ROCK1 ELISA Kit (human; cat. no. OKEH06554; AVIVA Systems Biology). All operations were performed following the manufacturer's protocol.

Cell lines, cell culture and transfection. The HPAF-II [American Type Culture Collection (ATCC)® CRL-1997™] and BxPC-3 (ATCC® CRL-1687™) human pancreatic adenocarcinoma cell lines were purchased from ATCC. Cells were cultured under conditions described in the manufacturer's protocol. LINK-A small interfering (si)RNA (5'-UGUCUA AGGUGGAGAUUAC-3') and negative control siRNA (5'-GGAATGCAGCTGAAAGATTCC-3') were purchased from GenePharma. LINK-A and ROCK1 expression pIRSE2 vectors and empty vectors were purchased from GeneCopoeia. Lipofectamine™ 2000 (11668-019; Thermo Fisher Scientific, Inc.) was used to transfect 15 nM vectors or 50 nM siRNA into cancer cells, and was further incubated for 15 min following the manufacturer's protocol. Empty vector or negative control siRNA transfection was performed in the negative control (NC) group. Cells without any transfections were used as control cells (C). An overexpression rate of >180% (180-245%) and a rate of silencing to <50% (30-50%) were confirmed by RT-qPCR prior to subsequent experiments.

Cell proliferation assay. Cell suspensions ($3x10^4$/ml) were prepared. A total of 100 μl cell suspension was added to each well of a 96-well plate. Cells were cultured and CCK-8 solution (10 μl) was added 24, 48, 72 and 96 h later. Cells were cultured for an additional 4 h, and optical density values at 450 nm were measured using a Fisherbrand™ accuSkan™ GO ultraviolet/visible light Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.).

Transwell® migration and invasion assay. Serum-free cell suspensions ($3x10^4$/ml) were prepared. Migration and invasion assays were performed using the same protocol except that Matrigel® (10 μg/ml, cat. no. 356234; EMD Millipore) was used to coat the upper chamber at 37˚C overnight prior to the invasion assay. The upper chamber was of the Transwell® plate (pore size, 0.45 μm; Corning, Inc.) was filled with 100 μl cell suspension, while the lower chamber was filled with medium containing 20% fetal calf serum (Sigma-Aldrich; Merck KGaA). Subsequently, the plates were incubated for 12 h, followed by membrane staining using 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min. Cells that had migrated or invaded to the lower side of the membrane were counted under an optical microscope (Olympus BX-51; Olympus Corporation).

Western blot analysis. Total protein was extracted using RIPA buffer and protein concentration was estimated by the BCA method. A total of 40 μg of protein per lane was loaded and separated using 10% SDS-PAGE. Following transfer to polyvinylidene difluoride membranes (EMD Millipore), blocking was performed in 5% skimmed milk at room temperature for 2 h. Subsequently, the membranes were incubated with rabbit anti-human primary antibodies to ROCK1 (1:1,200 dilution; cat. no. ab97582; Abcam) and GAPDH (1:1,200 dilution; cat. no. ab8245; Abcam) overnight at 4˚C. The next day, IgG-horseradish peroxidase secondary antibody (goat anti-rabbit; 1:1,000 dilution; cat. no. MBS435036; MyBioSource) was used to further incubate the membranes at room temperature for 1 h. Signals were developed using enhanced chemiluminescence (Sigma-Aldrich; Merck KGaA) and scanned with the MYECL™ Imager (Thermo Fisher Scientific, Inc.). Data normalization was performed with ImageJ v1.6 software (National Institutes of Health).

Statistical analysis. GraphPad Prism 6 (GraphPad Software, Inc.) was used for statistical analysis. Values are expressed as the mean ± standard deviation. Comparisons between two groups were performed by using Student's t-test. Comparisons among multiple groups were performed using one-way analysis of variance and Tukey's test. Correlation analysis was performed by determining Pearson's correlation coefficient. Receiver operating characteristics (ROC) curve analysis was performed to evaluate the diagnostic value. $P<0.05$ was considered to indicate statistical significance.

Results

Altered plasma levels of LINK-A lncRNA and ROCK1 in patients with pancreatic adenocarcinoma. Differential expression of genes in patients vs. healthy controls indicates the

Table I. Clinicopathological characteristics of the 42 patients.

Pathology	Number of cases, n (%)	Stage[a]
Pancreatic ductal adenocarcinoma	37 (88.1)	I A (3), I B (6), II A (14), II B (10), III (4)
Adenosquamous carcinoma	3 (7.1)	I A (1), I B (2)
Mucinous cystadenoma	1 (2.4)	II A (1)
Serous cystadenoma	1 (2.4)	II B (1)

[a]According to the AJCC Cancer Staging Manual (8th version).

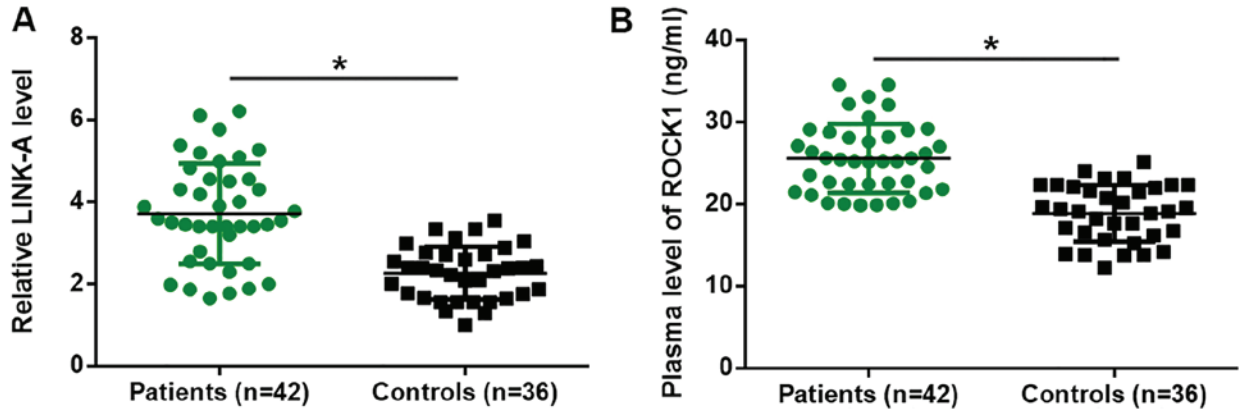

Figure 1. Altered plasma levels of LINK-A lncRNA and ROCK1 were observed in pancreatic adenocarcinoma patients. Compared with healthy controls, plasma levels of (A) LINK-A lncRNA and (B) ROCK1 were significantly increased in pancreatic adenocarcinoma patients. *P<0.05. ROCK1, Rho-associated protein kinase 1; lncRNA, long non-coding RNA.

involvement of the respective genes in diseases. In the present study, RT-qPCR analysis revealed that, compared with those in healthy controls, the plasma levels of LINK-A lncRNA were significantly increased in patients with pancreatic adenocarcinoma (P<0.05; Fig. 1A). In addition, ELISA demonstrated that the plasma levels of ROCK1 were also significantly higher in patients with pancreatic adenocarcinoma compared with those in healthy controls (P<0.05; Fig. 1B).

Plasma levels of LINK-A lncRNA and ROCK1 are positively correlated in patients with pancreatic adenocarcinoma. Pearson's correlation coefficient analysis revealed a significant positive correlation between the plasma levels of LINK-A lncRNA and ROCK1 in patients with pancreatic adenocarcinoma (Fig. 2A). However, the correlation between LINK-A lncRNA and ROCK1 was not significant in healthy controls (Fig. 2B).

Altered plasma levels of LINK-A lncRNA and ROCK1 distinguish early-stage pancreatic adenocarcinoma patients from healthy controls. ROC curve analysis was performed with pancreatic adenocarcinoma patients used as true-positive subjects and healthy controls as true-negative samples. As displayed in Fig. 3, the average area under the curve (AUC) for plasma LINK-A lncRNA was 0.8488 (sensitivity, 0.83; specificity, 0.85; standard error, 0.04449; 95% confidence interval, 0.7616-0.9360; P<0.0001), the cut-off value was determined from the point closest to the upper left-hand corner of the graph, the sensitivity was 94.7%. For plasma ROCK1, the average AUC was 0.8948 (sensitivity, 0.84; specificity, 0.90; standard error, 0.03421; 95% confidence interval, 0.8277-0.9618; P<0.0001), the cut-off value was determined from the point closest to the upper left-hand corner of the graph, the sensitivity was 90.1%.

LINK-A and ROCK1 participate in the proliferation, migration and invasion of pancreatic adenocarcinoma cells. Compared to the C and NC groups, LINK-A silencing led to significantly reduced proliferation (Fig. 4A), migration (Fig. 4B) and invasion (Fig. 4C) of HPAF-II and BxPC-3 human pancreatic adenocarcinoma cells (P<0.05). However, simultaneous overexpression ROCK1 significantly attenuated those inhibitory effects of LINK-A siRNA silencing on cancer cell proliferation (Fig. 4A), migration (Fig. 4B) and invasion (Fig. 4C; P<0.05).

LINK-A overexpression leads to upregulation of ROCK1 in pancreatic adenocarcinoma cells. Compared with that in the C and NC groups, LINK-A overexpression led to significantly increased expression of ROCK1, while siRNA-mediated silencing of LINK-A significantly inhibited the expression of ROCK1 in the HPAF-II and BxPC-3 cell lines (P<0.05; Fig. 5A). By contrast, ROCK1 vector transfection did not significantly affect the expression of LINK-A in those cells (Fig 5B).

Discussion

The present study was the first, to the best of our knowledge, to report on the involvement of LINK-A lncRNA in pancreatic adenocarcinoma, which is the major type of pancreatic

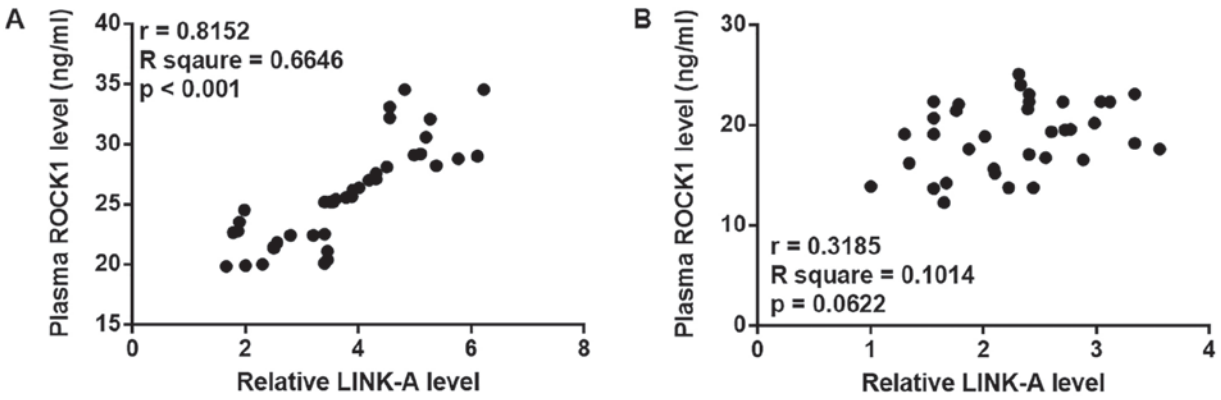

Figure 2. Plasma levels of LINK-A lncRNA and ROCK1 were positively correlated in pancreatic adenocarcinoma patients. Pearson's correlation coefficient analysis revealed a significantly positive correlation between plasma levels of LINK-A lncRNA and ROCK1 (A) in pancreatic adenocarcinoma patients but not in (B) healthy controls. ROCK1, Rho-associated protein kinase 1; lncRNA, long non-coding RNA.

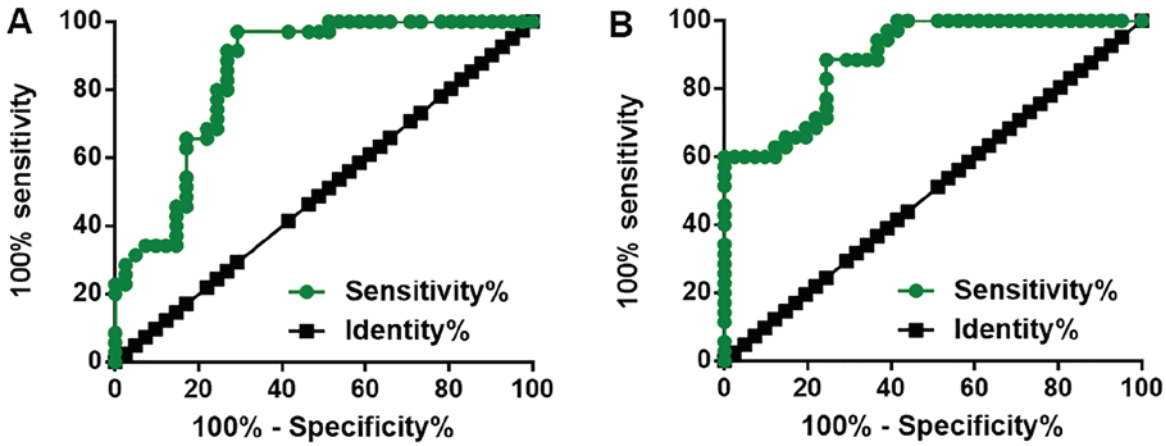

Figure 3. Altered plasma levels of LINK-A lncRNA and ROCK1 distinguished early-stage pancreatic adenocarcinoma patients from healthy controls. Receiver operating characteristics curve analysis revealed that altered plasma levels of (A) LINK-A lncRNA and (B) ROCK1 are of diagnostic value for pancreatic adenocarcinoma. ROCK1, Rho-associated protein kinase 1; lncRNA, long non-coding RNA.

cancer (15). The results revealed that LINK-A lncRNA influences the proliferation, migration and invasion of pancreatic adenocarcinoma cells, at least partially through affecting the expression of ROCK1, which is a well-characterized oncogenic serine/threonine kinase protein in various types of cancer.

To simplify the experimental design, only patients with pancreatic adenocarcinoma at stages IA-IIA, which represents the early stage of this disease in the absence of cancer metastasis, were enrolled in the present study. This design is based on the fact that tumor metastasis globally affects gene expression (16), which may bring uncertainties to the conclusions. Another purpose of only including patients at early stages was to investigate the early diagnostic value of LINK-A lncRNA for pancreatic adenocarcinoma. To date, altered expression of LINK-A has only been observed in triple-negative breast cancer (12) and ovarian carcinoma (13). The present study revealed that the plasma levels of LINK-A were significantly increased in pancreatic adenocarcinoma patients vs. healthy controls. It was demonstrated that elevated plasma levels of LINK-A were able to effectively distinguish patients with pancreatic adenocarcinoma from healthy controls. Therefore, plasma LINK-A may have a potential application for the early diagnosis of pancreatic adenocarcinoma. However, the expression of LINK-A may also be changed in other human diseases, such as ovarian carcinoma (13), diabetic nephropathy (17) and osteosarcoma (18). The diagnostic specificity may be improved by combined use of multiple biomarkers.

ROCK1 exerts oncogenic functions and activated ROCK1 signaling has been observed in various types of human malignancy (19,20). Consistent with previous studies, the present study also observed significantly increased plasma levels of ROCK1 in pancreatic adenocarcinoma patients compared with those in healthy controls. The present study focused on ROCK1 due to the observations from preliminary microarray data (unpublished; 24 patient samples, 24 healthy controls), which suggested that ROCK1 expression levels are likely positively correlated with the expression levels of LINK-A in pancreatic adenocarcinoma patients. In the present study, the existence of a significant positive correlation between ROCK1 and LINK-A in pancreatic adenocarcinoma was further proved. The regulation of ROCK1 by lncRNAs has been reported by a previous study (21). The present study proved that LINK-A is likely an upstream activator of ROCK1 to regulate the

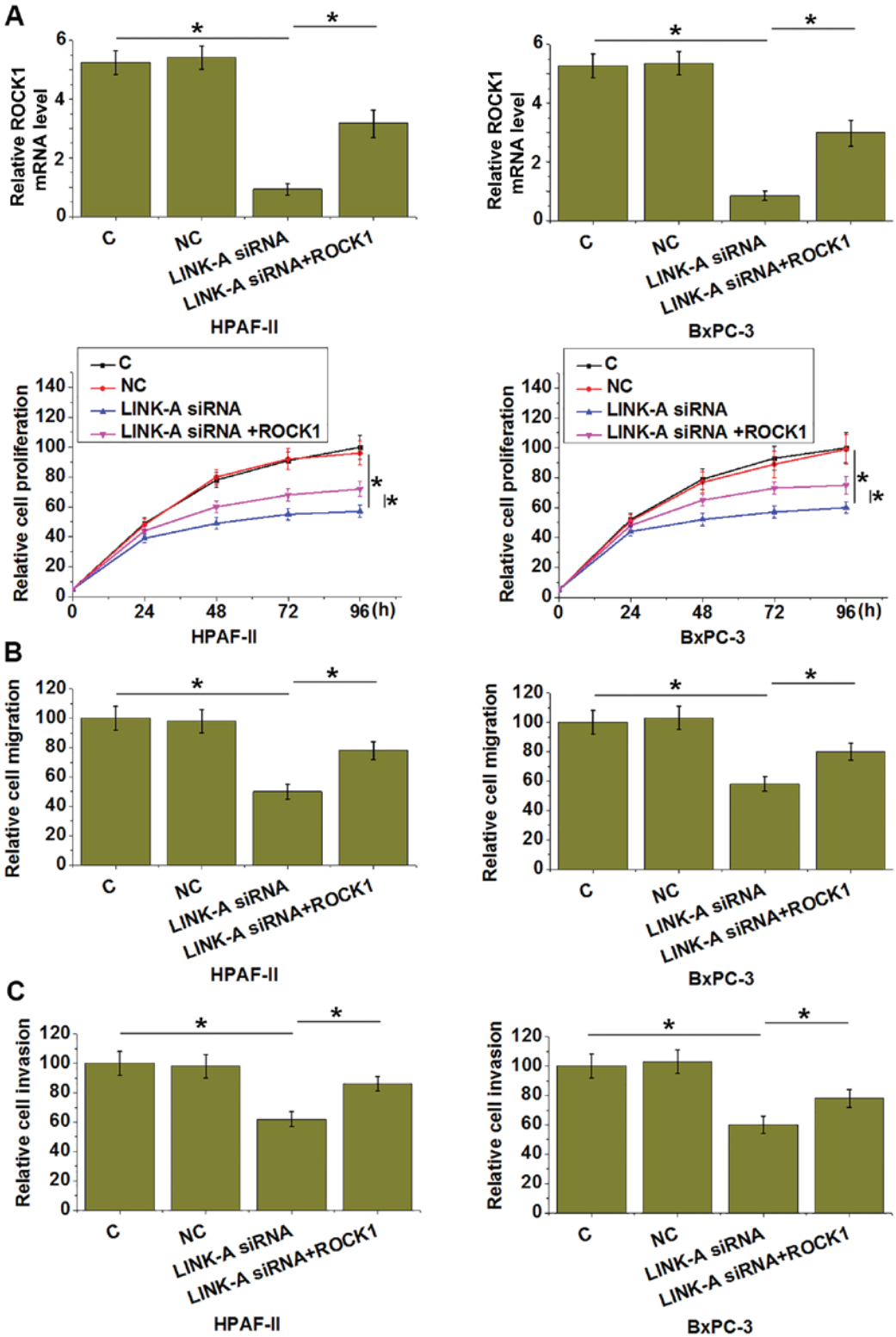

Figure 4. LINK-A and ROCK1 participate in the regulation of the proliferation, migration and invasion of pancreatic adenocarcinoma cells. LINK-A silencing led to significantly inhibited (A) proliferation, (B) migration and (C) invasion of cells of the HPAF-II and BxPC-3 cell lines, while those inhibitory effects were attenuated by ROCK1 overexpression. *P<0.05. ROCK1, Rho-associated protein kinase 1; siRNA, small interfering RNA; C, untreated control; NC, scrambled control.

proliferation, migration and invasion of pancreatic adenocarcinoma cells. This conclusion is based on following observations: i) LINK-A overexpression led to significantly upregulated ROCK1 expression; ii) ROCK1 overexpression did not significantly affect LINK-A expression; iii) ROCK1 overexpression attenuated the inhibitory effects of LINK-A overexpression on the proliferation, migration and invasion of pancreatic adenocarcinoma cells.

It can be hypothesized that the interaction between LINK-A and ROCK1 is indirect. This is based on the observation that plasma levels of LINK-A and ROCK1 are not significantly correlated in healthy controls, indicating

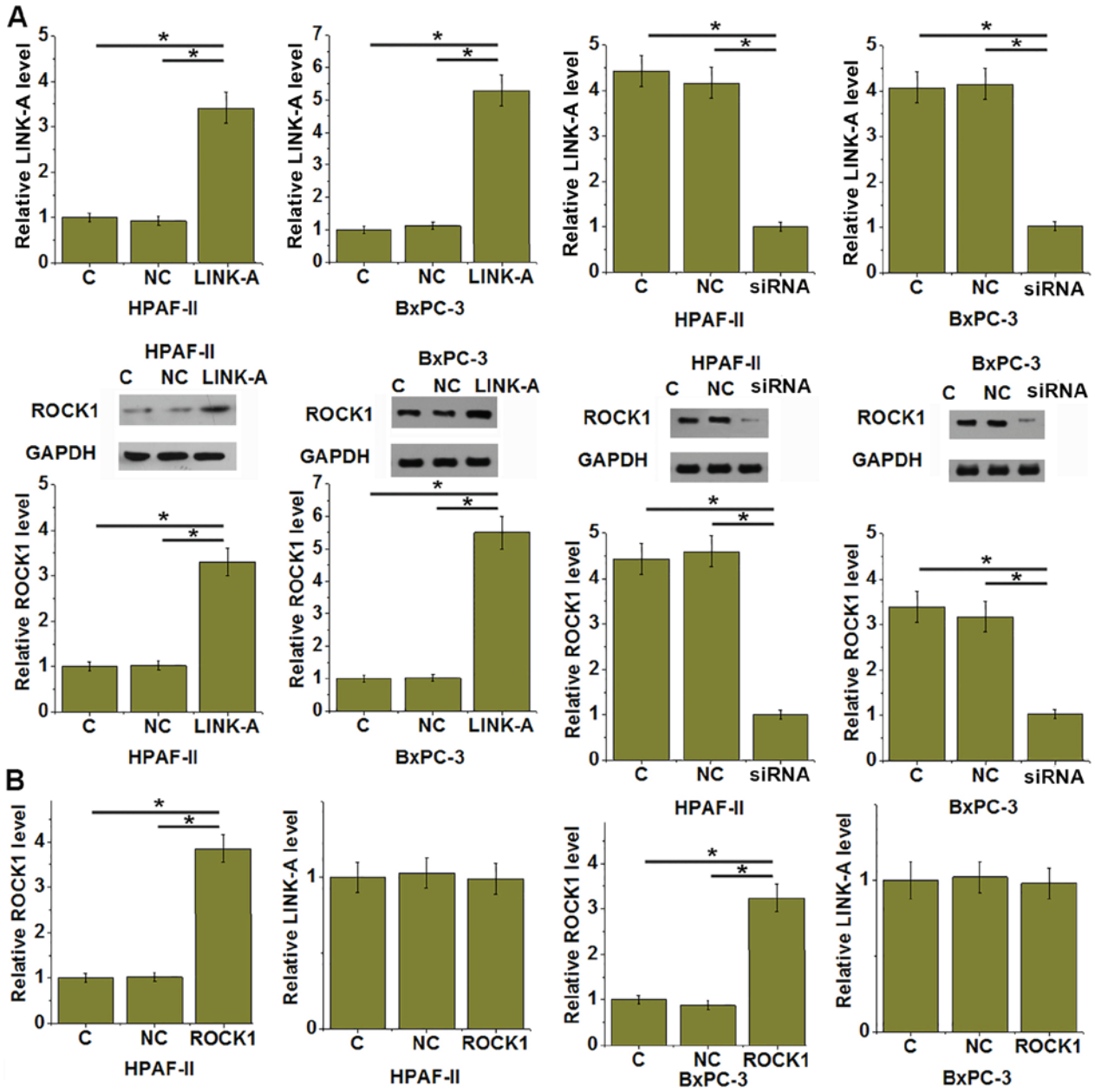

Figure 5. LINK-A overexpression leads to upregulation of ROCK1 expression in pancreatic adenocarcinoma cells. (A) Overexpression of LINK-A led to upregulation of ROCK1 expression, while (B) overexpression of ROCK1 failed to significantly affect LINK-A expression. *P<0.05. ROCK1, Rho-associated protein kinase 1; siRNA, small interfering RNA; C, untreated control; NC, scrambled control.

the existence of disease-associated mediators that mediate interaction between LINK-A and ROCK1 in pancreatic adenocarcinoma cells. LINK-A may also interact with other pathways to participate in the proliferation, migration and invasion of pancreatic adenocarcinoma cells due to the fact that ROCK1 overexpression only attenuated, but not totally reversed the inhibitory effects of LINK-A overexpression on the proliferation, migration and invasion of pancreatic adenocarcinoma cells. Further studies are required to elucidate the signaling pathways that mediate the interaction between LINK-A and ROCK1.

In conclusion, LINK-A and ROCK1 were upregulated in pancreatic adenocarcinoma. LINK-A may upregulate ROCK1 to participate in the proliferation, migration and invasion of pancreatic adenocarcinoma cells.

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Availability of data and materials

The datasets generated and analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

TW designed the experiments. MZ performed the experiments. MZ, RW, XZ and LL analyzed the data. XZ performed the statistical analysis. TW drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). All patients and healthy volunteers provided written informed consent prior to their inclusion in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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