Long non-coding RNA EGFR-AS1 sponges micorRNA-381 to upregulate ROCK2 in bladder cancer

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Abstract. The present study aimed to investigate the role of the long non-coding RNA EGFR-AS1 in bladder cancer (BC). In this study gene expression of both BC and non-tumor tissues from BC patients were measured by quantitative PCR. Cell transfections were performed to analyze gene interactions in HT-1197 cells. Transwell assays were performed to analyze cell invasion and migration of HT-1197 cells. It was revealed that epidermal growth factor receptor-antisense RNA 1 (EGFR-AS1) was upregulated in BC and positively associated with rho associated coiled-coil containing protein kinase 2 (ROCK2). Analysis of data collected in follow-ups indicated that EGFR-AS1 expression was significantly associated with poorer overall survival of patients with BC. Moreover, in bladder cancer cells, EGFR-AS1 overexpression mediated the upregulation of ROCK2, while microRNA (miR)-381 mediated the downregulation of ROCK2. However, EGFR-AS1 and ROCK2 failed to affect each other. Bioinformatics analysis indicated that miR-381 binds EGFR-AS1. In addition, EGFR-AS1 and ROCK2 overexpression resulted in the promotion of cell invasiveness and migration of HT-1197 BC cells. Conversely, miR-381 was revealed to partially reverse the effect of EGFR-AS1 overexpression. Therefore, EGFR-AS1 may sponge miR-381 to upregulate ROCK2 in BC, thereby promoting cell invasion and migration.

Introduction

Bladder cancer (BC) is a common malignancy with an incidence rate four times higher in males than in females (1,2). In 2018, GLOBOCAN statistics revealed a total of 549,393 new cases of bladder cancer, accounting for ~3.0% of all cancer cases, and resulting in ~199,922 mortalities (2.1% of all cancer-associated mortalities) worldwide (3). Tobacco smoking is a major risk factor for BC (4). Besides that, other factors, such as infections of *Schistosoma haematobium*, are also closely associated with the tumorigenesis of BC (1,4). However, the molecular pathogenesis of BC is yet to be elucidated (5), and this represents a major challenge in the development of novel preventative and therapeutic approaches.

Genetic alterations are frequently observed in patients with BC (6,7). Rho associated coiled-coil containing protein kinase 2 (ROCK2) is a serine/threonine kinase that serves a critical role in smooth muscle contraction and cytokinesis (8). ROCK2 is also implicated in cancer biology and influences the metastasis of BC via the promotion of cancer cell migration and invasiveness (9,10). In effect, inactivation of ROCK2 signaling represents a promising target for cancer treatment (9). It is well established that certain miRNAs, such as miR-381, serve tumor-suppressive roles via the inhibition of ROCK2 (11). Epidermal growth factor receptor-antisense RNA 1 (EGFR-AS1) is a recently identified oncogenic lncRNA in several types of cancer, such as liver cancer (12). In the present study, bioinformatics analysis indicated that EGFR-AS1 may interact with miR-381 by forming base pairing between the complementary sequences. Therefore, it was hypothesized that EGFR-AS1 may interact with miR-381 and indirectly regulate ROCK2, thereby influencing the progression BC. Therefore, the current study was performed to investigate the possible interactions between EGFR-AS1 and miR-381 in BC and explore the consequent effects on the expression level of ROCK2.

Materials and methods

Patients. A total of 145 patients with BC were admitted to The Second People's Hospital of Liaocheng (Linqing, China) between January 2011 and April 2014. Of these patients, 70 cases [52 males and 18 females; 53.1±6.0 (SEM) years; range, 41-67 years]. The present study was approved by the Ethics Committee of The Second People's Hospital of Liaocheng (Linqing, China). Patients were selected according to the following inclusion criteria: i) Newly diagnosed BC cases; and ii) treatment was completed and 5 year follow-up was conducted. The exclusion criteria were: i) Recurrent cases of BC; ii) patients had been transferred from other hospitals; iii) clinical disorders

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other than BC were diagnosed; and iv) therapies had been initiated prior to patient admission. All patients were informed of the design of experiments and the potential publication of this paper, and written form informed consent was provided by all participants. According to clinical findings, all patients were staged in line with the guidelines established by the American Joint Committee on Cancer (13). There were 8, 17, 21 and 24 cases at stage I-IV, respectively.

BC tissue specimens and cell line. Under the guidance of MRI, bladder biopsy was performed on all BC patients (prior to the initiation of any therapies) to retrieve BC tumor tissues and adjacent paracancerous tissues (collected ≤3 cm from the tumor border) from each BC patient. All tissue samples were verified via histopathological biopsy. Fresh tissues were stored in liquid nitrogen before use.

The human BC cell line HT-1197 (American Type Culture Collection) was used. Cells were cultured in a mixture of 10% FBS (Sigma-Aldrich; Merck KGaA) and 90% Eagle's Minimum Essential Medium (Sigma-Aldrich; Merck KGaA). The culture conditions were 95% humidity, 5% CO_2 and 37˚C.

Therapies and follow-up. Patients were subjected to different therapies according to their conditions. Therapies included surgical resection, chemotherapy, radiotherapy or targeted therapy. Initiating at the day of admission, all patients were followed-up for 5 years. The following patients were excluded from the survival analysis: i) Patients who experienced mortality due to other causes; ii) patients that were unwilling to complete the follow-up.

RNA-RNA interaction prediction. The interactions between miR-381 and EGFR-AS1 by base pairing were predicted by IntaRNA 2.0 (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp). The sequence of EGFR-AS1 was used as the long sequence and the sequence of miR-381 was used as the short sequence. All other parameters were default.

Transient transfections. HT-1197 cells were harvested at ~80% confluency and Lipofectamine 2000 (Sigma-Aldrich; Merck KGaA) was used to transfect the following vectors or miRNAs into $1x10^6$ cells: i) 10 nM EGFR-AS1 or ROCK2 expression vector (empty vector as the negative control, NC); and ii) 50 nM miRNA (5'-AGCGAGGUUGCCCUUUGUAUAU-3') with NC miRNA (5'-GUAGCUCGUGAUGCAUACGUGU-3', targest no genes in the human genome) as NC group. EGFR-AS1 and ROCK2 expression vectors were constructed using pcDNA3.1 vector (Sangon Biotech Co., Ltd.). NC miRNA and miR-381 mimic were purchased from Guangzhou Ribobio Co., Ltd. In all groups, untransfected cells were used as control cells. Cells were harvested at 24 h post-transfection to be used in subsequent analyses.

Total RNA extractions and treatments. All tissue samples (0.05 g, both BC and non-tumor) were ground in liquid nitrogen. Total RNAs in tissue samples and $1x10^5$ HT-1197 cells (collected at 24 h post-transfection) were extracted using RNAzol (Sigma-Aldrich; Merck KGaA). In the precipitation step, 85% of ethanol was used to harvest miRNAs. All RNA samples were digested with DNase I for 2 h at 37˚C to remove genomic DNAs.

Quantitative PCR (qPCR). Total RNA was reverse transcribed using Tetro Reverse Transcriptase (Bioline; Meridian Bioscience Inc.) following manufacturer's protocol. To measure the expression levels of EGFR-AS1 and ROCK2 mRNA, all qPCR assays were performed using TB Green Advantage qPCR Premix from Clontech Laboratories, Inc. following the manufacturer's protocol. GAPDH was used as an endogenous control. The measure the expression level of mature miR-381, the addition of poly (A) was performed, followed by miRNA reverse transcription and qPCR assays. All these steps were completed using All-in-One™ miRNA qRT-PCR Detection kit (GeneCopoeia, Inc.), following manufacturer's protocol. The following primer sequences were used: EGFR-AS1 forward, 5'-GGCCATCACGTAGGCTTCC-3' and reverse, 5'-GCGTCTTCACCTGGAAGGG-3'; ROCK2 forward, 5'-TGATTGGTGGTCTGTAG-3' and reverse, 5'-CTGCCGTTTCTCTTATG-3'; and GADPH forward, 5'-ACAACTTTGGTATCGTGGAAG-3' and reverse, 5'-GCCATCACGCCACAGTTT-3'. Universal miRNA reverse primer and U6 primers were from the All-in-One™ miRNA qRT-PCR Detection kit. All Cq values were normalized using the $2^{-\Delta\Delta Cq}$ method (14), and all PCR reactions were repeated 3 times.

Western blot analysis. To measure the expression levels of ROCK2 in HT-1197 cells, HT-1197 cells were harvested at 24 h post-transfection and cells were counted. Total protein was extracted from $1x10^5$ cells using RIPA solution, and quantified using a bicinchoninic acid assay (both Sangon Biotech Co., Ltd.). Protein denaturation was performed in boiling water for 10 min, followed by SDS-PAGE electrophoresis, which was performed on a 12% gel with 30 μg of protein sample per lane. Subsequently, proteins were transferred to PVDF membranes, and blocking was performed in 5% non-fat milk with PBS (Sigma-Aldrich; Merck KGaA) for 2 h at room temperature. The membranes were first incubated with rabbit anti-ROCK2 (1:1,600; cat. no. ab71598) and anti-endogenous control GAPDH (1:1,300; cat. no. ab37168; both Abcam) for 18 h at 4˚C, followed by incubation with goat HRP (IgG; 1:1,300; cat. no. ab6721; Abcam) secondary antibody for 2 h at 22˚C. After that, membranes were incubated with an enhanced chemiluminescence kit (Sigma-Aldrich; Merck KGaA) for 15 min at 22˚C to develop the signals. The Image J v1.46 software (National Institutes of Health) was used to normalize signals.

Transwell assay. Transwell assays were performed to analyze the effects of transfections on the invasion and migration of HT-1197 cells. To mimic *in vivo* invasion, Transwell membranes were coated with Matrigel (Corning, Inc.) for 6 h at 37˚C, prior to the invasion assay. To prepare single-cell suspensions, 1 ml serum-free Eagle's Minimum Essential Medium was used to resuspend $3x10^3$ transfected cells. The suspension was then plated in the upper Transwell chamber (96-well, 0.1 ml per well), and the lower chamber was filled with a mixture of 80% Eagle's Minimum Essential Medium and 20% FBS. Cells were cultivated under the aforementioned conditions for 16 h. Subsequently, cells were stained using 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) at 22˚C for 20 min. Cells were observed under a light microscope (magnification x40) and quantified using Image J v1.46 software.

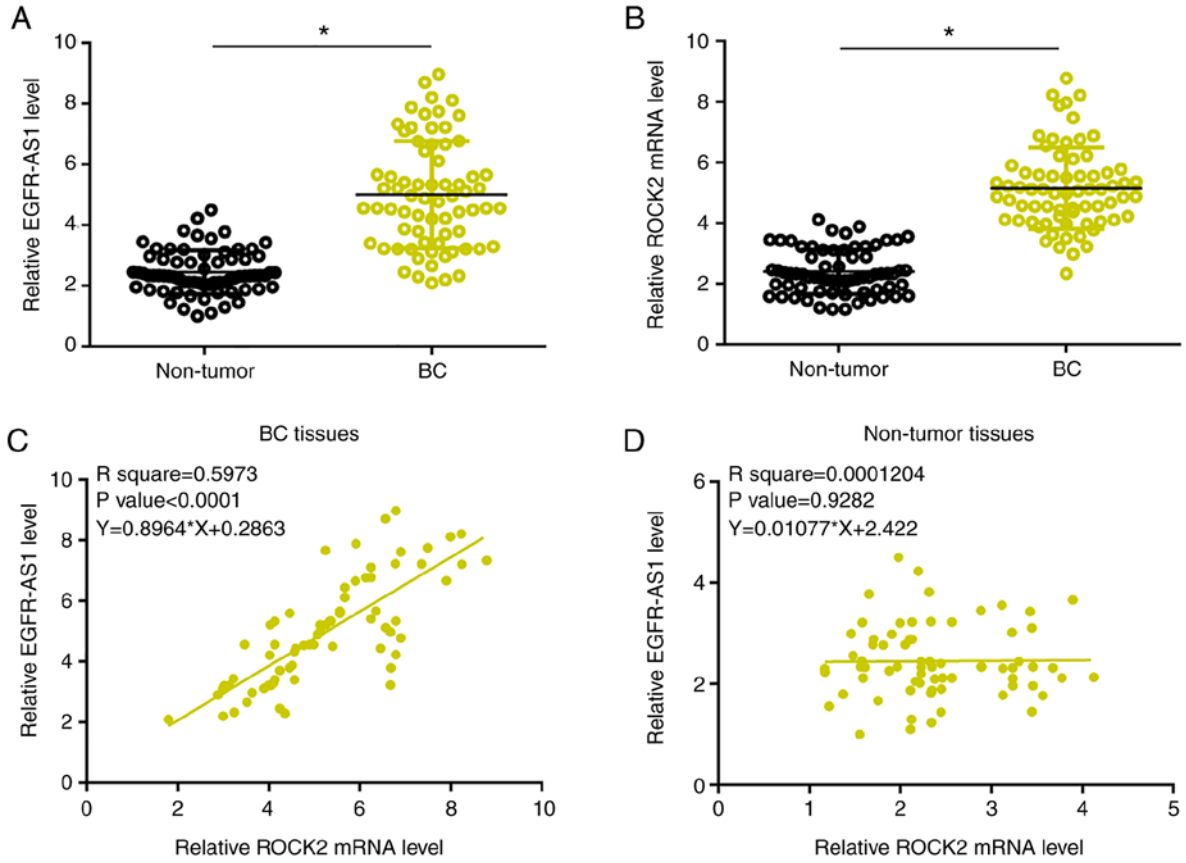

Figure 1. EGFR-AS1 and ROCK2 mRNA are upregulated and positively associated in BC. Levels of (A) EGFR-AS1 and (B) ROCK2 mRNA expression were measured and compared between non-tumor and BC tissues by performing qPCR and paired t-test. Associations between EGFR-AS1 and ROCK2 mRNA in (C) BC and (D) non-tumor tissues were analyzed by Linear regression. Three replicates were included and mean values (±SEM) are presented, *P<0.05. EGFR-AS1, epidermal growth factor receptor-antisense RNA 1; ROCK2, rho associated coiled-coil containing protein kinase 2; BC, bladder cancer.

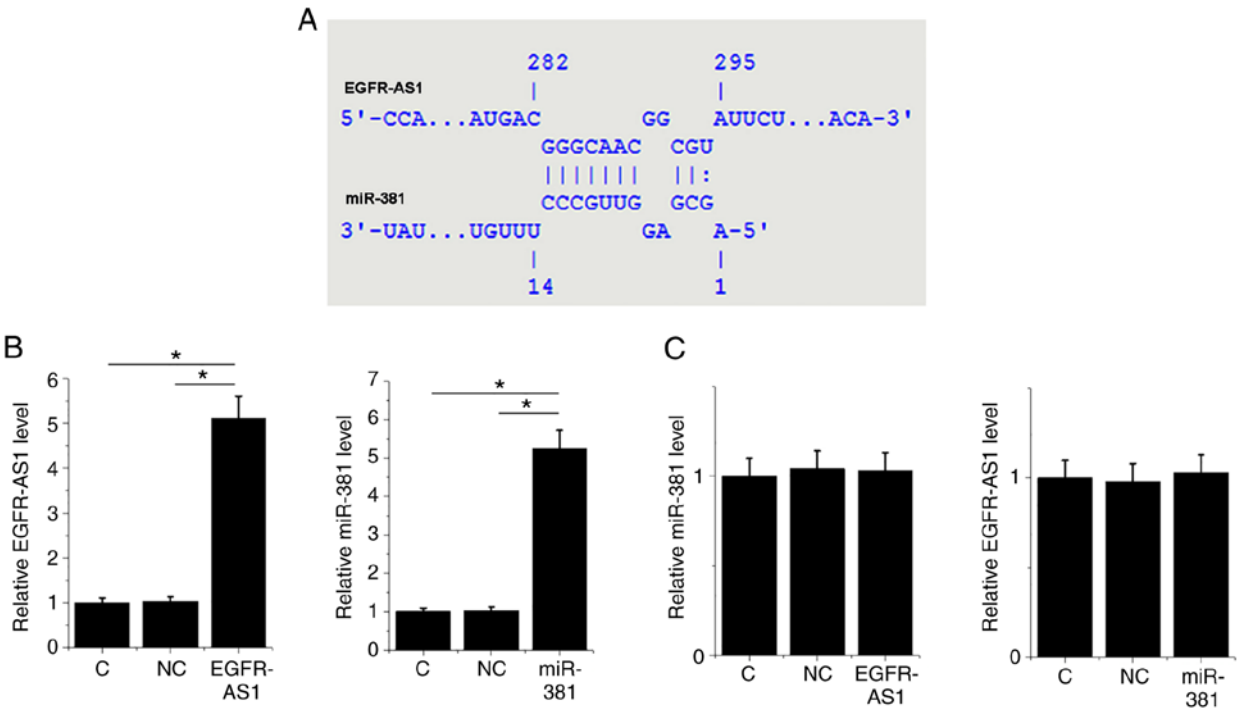

Figure 2. EGFR-AS1 may interact with miR-381 but failed to regulate its expression. (A) Bioinformatics analysis performed using IntaRNA revealed that miR-381 can form base pairing with EGFR-AS1. (B) To further analyze the interactions between them, HT-1197 cells were transfected with miR-381 mimic and EGFR-AS1 expression vector. Overexpression of miR-381 and EGFR-AS1 was confirmed by qPCR at 24 h post-transfection. (C) Interaction between miR-381 and EGFR-AS1 was also analyzed by qPCR. Three replicates were included and mean values are presented. *P<0.05. EGFR-AS1, epidermal growth factor receptor-antisense RNA 1; ROCK2, rho associated coiled-coil containing protein kinase 2; miR, microRNA; pPCR, quantitative PCR.

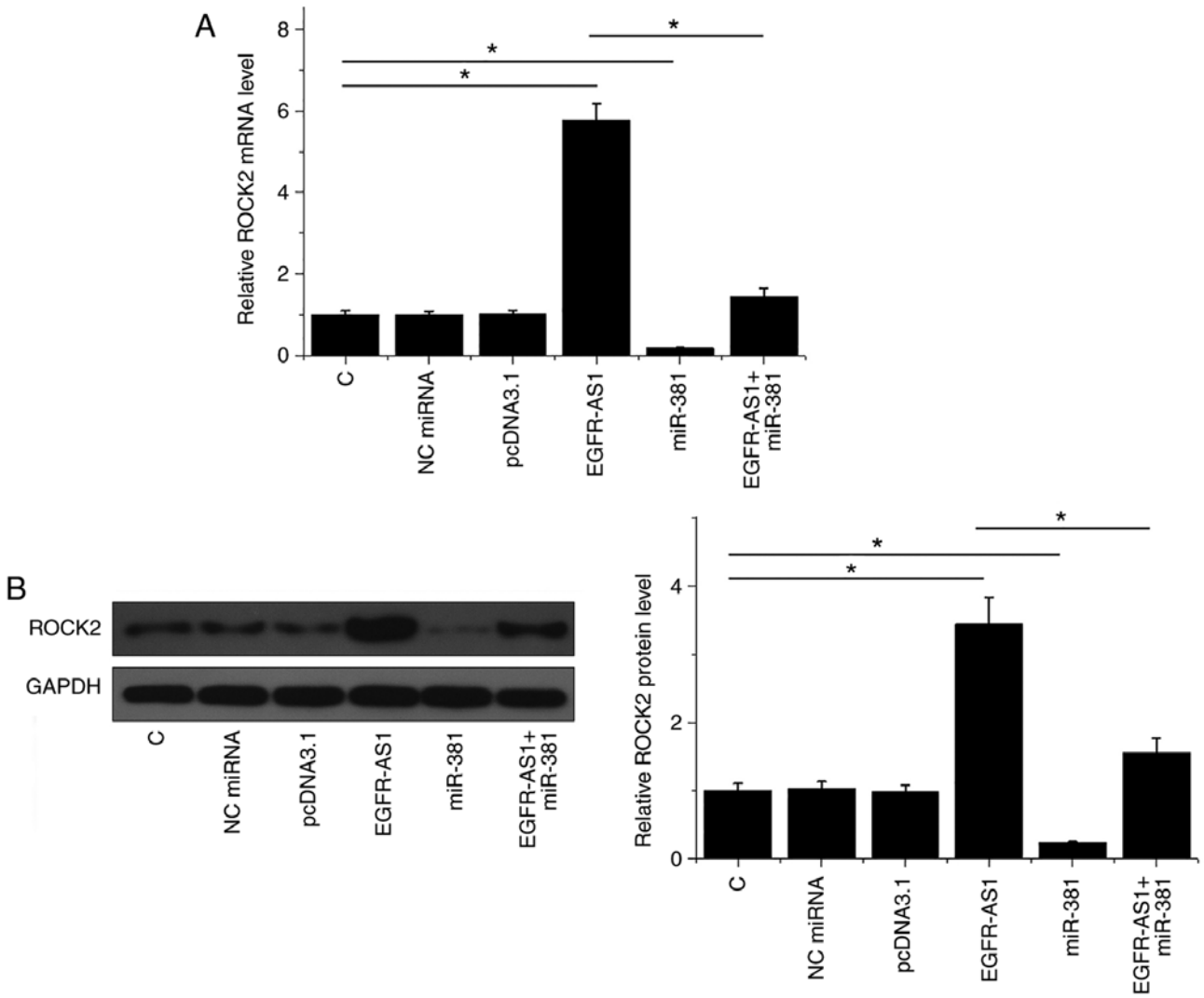

Figure 3. EGFR-AS1 may sponge miR-381 to upregulate ROCK2. Western blot and quantitative PCR were performed to analyze the effects of miR-381 and ROCK2 overexpression on ROCK expression at (A) mRNA and (B) protein levels, respectively. Three replicates were included and mean values are presented. *P<0.05. EGFR-AS1, epidermal growth factor receptor-antisense RNA 1; ROCK2, rho associated coiled-coil containing protein kinase 2; miR, microRNA.

Statistical analysis. Three biological replicates were included in each experiment and mean values were calculated and used in all data analyses. The GraphPad Prism 6 software (GraphPad Software, Inc.) was used for statistical analysis. Associations were analyzed using linear regression. To perform survival analysis, the 70 BC patients were divided into high and low (both n=35) EGFR-AS1 level groups with the median level of EGFR-AS1 in patients with BC used as the cut-off value (4.23). The Kaplan-Meier plotter and the log-rank test were used to plot and compare survival curves. Differences were explored between two tissue types or among cell transfection groups by paired Student's t-test and one-way ANOVA (followed by Tukey's post-hoc test), respectively. The χ^2 test was used to compare clinical stages between 2 groups. P<0.05 was considered to indicate a statistically significant difference.

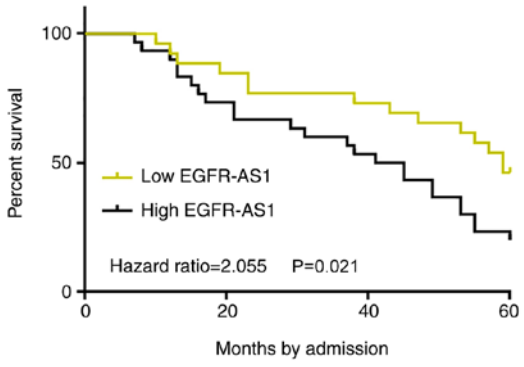

Figure 4. High levels of EGFR-AS1 in BC predicted poor survival. To perform survival analysis, the 70 BC patients were divided into high and low (n=35) EGFR-AS1 level groups with the median level of EGFR-AS1 in BC as the cutoff value. Kaplan-Meier plotter and log-rank test were used to plot and compare survival curves. EGFR-AS1, epidermal growth factor receptor-antisense RNA 1; miR, microRNA; BC, bladder cancer.

Results

EGFR-AS1 and ROCK2 mRNA were upregulated and positively associated in BC. Expression levels of EGFR-AS1 and ROCK2 mRNA were measured and compared between BC and adjacent paracancerous tissues by performing qPCR and a paired t-test. Compared with paracancerous tissues,

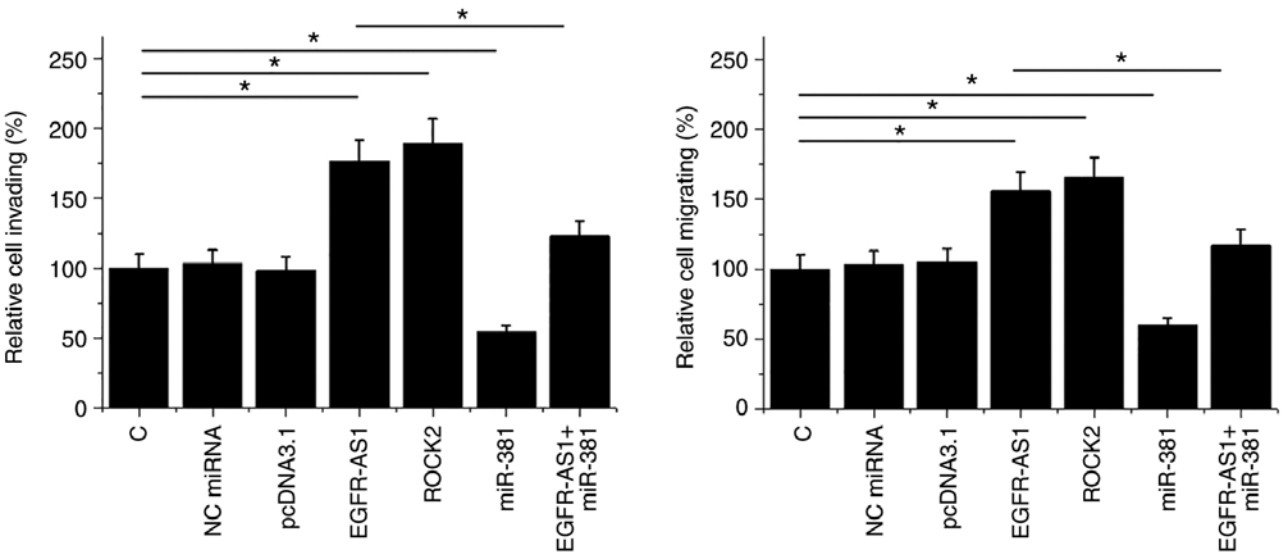

Figure 5. EGFR-AS1 promoted HT-1197 cell invasion and migration through miR-381 and ROCK2. Effects of transfections on the invasion and migration of HT-1197 cells were analyzed by Transwell assays. Three replicates were included and mean values are presented. *P<0.05. EGFR-AS1, epidermal growth factor receptor-antisense RNA 1; ROCK2, rho associated coiled-coil containing protein kinase 2; miR, microRNA; C, control; NC, negative control.

significantly higher EGFR-AS1 (Fig. 1A) and ROCK2 mRNA levels were observed in BC tissues (Fig. 1B; P<0.05). Associations between EGFR-AS1 and ROCK2 mRNA expression were analyzed using linear regression. Expression levels of EGFR-AS1 were significantly and positively associated with expression levels of ROCK2 mRNA in BC tissues (P<0.0001; Fig. 1C). Moreover, the interaction between them was not significant in the paracancerous tissues (Fig. 1D).

EGFR-AS1 may interact with miR-381 but failed to regulate its expression. It is known that miR-381 is able to target ROCK2 (11). A bioinformatics analysis performed using IntaRNA 2.0 (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp) revealed that miR-381 can form a base pairing with EGFR-AS1 (Fig. 2A). To further analyze the interactions between them, HT-1197 cells were transfected with either a miR-381 mimic or EGFR-AS1 expression vector. Overexpression of miR-381 and EGFR-AS1 was confirmed by qPCR at 24 h post-transfection. Compared with the control and NC groups, expression levels of miR-381 and EGFR-AS1 mRNA were significantly elevated post-transfection (Fig. 2B; P<0.05). However, overexpression of miR-381 and EGFR-AS1 did not influence the expression of each other (Fig. 2C).

EGFR-AS1 may sponge miR-381 to upregulate ROCK2. Western blotting and qPCR were performed to analyze the effects of miR-381 and ROCK2 overexpression on ROCK expression at the mRNA and protein level, respectively. Compared with the control and NC (NC miRNA or empty pcDNA3.1) groups, EGFR-AS1 overexpression mediated the upregulation of ROCK2 at both mRNA (P<0.05; Fig. 3A) and protein (P<0.05; Fig. 3B) levels, while miR-381 mediated the downregulation of ROCK2 and attenuated the effects of EGFR-AS1 overexpression (P<0.05).

High expression levels of EGFR-AS1 in BC predicted poor survival. Survival curves were plotted and compared through the aforementioned methods. Low EGFR-AS1 level group included 3, 10, 12 and 10 cases at stage I-IV, respectively. High EGFR-AS1 level group included 5, 7, 9 and 14 cases at stage I-IV, respectively. No significant differences in clinical stages were found between two groups (χ^2 test, P=0.55). Treatments are mainly determined by clinical stages. Therefore, it is likely that no significant differences in therapeutic approaches would exist between high and low EGFR-AS1 level groups. Notably, compared with patients in the low-EGFR-AS1 expression level group, the overall survival rate of patients in the high-EGFR-AS1 expression level group was significantly lower (P=0.021; Fig. 4). The current data indicated that the upregulation of EGFR-AS1 in patients with BC predicted poor survival.

EGFR-AS1 promotes HT-1197 cell invasion and migration via miR-381 and ROCK2. The effect of transfections on the invasion and migration of HT-1197 cells were analyzed by Transwell assays. ROCK2 overexpression was achieved after transfection (Fig. S1). Compared with the control and NC (NC miRNA or empty pcDNA3.1) groups, EGFR-AS1, and ROCK2 overexpression promoted cancer cell invasion (Fig. 5A) and migration (Fig. 5B). In addition, miR-381 overexpression played the opposite role and attenuated the effects of EGFR-AS1 overexpression (P<0.05).

Discussion

The present study predominantly investigated the role of EGFR-AS1 in BC. It was revealed that EGFR-AS1 was upregulated in BC and predicted poor survival. In addition, it was also determined that EGFR-AS1 may act as a sponge for miR-381 and consequently upregulate ROCK2 expression, promoting cancer cell invasion and migration abilities.

The roles of EGFR-AS1 have been investigated in several types of malignancies. In both hepatocellular carcinoma and gastric cancer, EGFR-AS1 is upregulated, and can in turn

upregulate the expression of epidermal growth factor receptor (EGFR) by increasing the stability of EGFR mRNA, thereby promoting cancer cell proliferation (12,15). Moreover, in squamous cell carcinoma it was observed that EGFR-AS1 can mediate EGFR addiction and regulate therapeutic responses (16). The present study is the first to report the overexpression of EGFR-AS1 in BC. In addition, accelerated cancer cell invasion and migration following EGFR-AS1 overexpression. Furthermore, high levels of EGFR-AS1 were found to be significantly associated with the poor survival of BC patients. The current data suggest that EGFR-AS1 serves as an oncogenic lncRNA in BC. Notably, a preliminary CCK-8 assay revealed that EGFR-AS1 exerted no significant effects on the proliferation of BC cells (data not shown). Therefore, EGFR-AS1 may have different functions dependent on the cancer type.

All previous studies on EGFR-AS1 focused on its interactions with EGFR (12,15,16). In the present study, it was revealed that EGFR-AS1 may form base pairing with miR-381, which is a well-characterized tumor-suppressive miRNA in many types of cancer, such as epithelial ovarian cancer (17) and breast cancer (18). In a recent study, Xie *et al* (11) reported that miR-381 can directly target ROCK2 to suppress the progression of gastric cancer. In the current study an association was observed between the downregulation of ROCK2 in BC cells and miR-381 overexpression, indicating that miR-381 may also target ROCK2 in BC. It was also predicted that miR-381 may form base pairing with EGFR-AS1. However, overexpression experiments revealed that miR-381 did not serve a regulatory role in the expression of EGFR-AS1. Therefore, miR-381 may not target EGFR-AS1. Increasing evidence has shown that lncRNAs may serve as the sponge of miRNAs to reduce their role in targeting downstream genes (19). In the present study, EGFR-AS1 was revealed to act as a sponge to miR-381, resulting in the upregulation of ROCK2; however, a limitation in the study design was the failure to analyze the direct interaction between EGFR-AS1 and miR-381 by dual-luciferase assay. Future studies should include this assay to further strengthen the conclusions reported in the current study. In addition, animal model experiments, such as tumor xenograft experiments are needed to further analyze the effects of EGFR-AS1 on tumor metastasis. In conclusion, EGFR-AS1 was upregulated in BC and may upregulate ROCK2 by sponging miR-381 to promote cancer cell invasion and migration.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SY designed the experiments. SY, XL and HC performed experiments. XS and XZ collected and analyzed data. SY drafted the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Second People's Hospital of Liaocheng (Linqing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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