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ORIGINAL ARTICLE

miR-19a promotes invasion and epithelial to mesenchymal transition of bladder cancer cells by targeting RhoB

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Summary

Purpose: *Bladder cancer is one of the most frequent carcinomas worldwide, the incidence of which ranks fourth in males. MiR-19a is identified as one of the key miRs associated with tumorigenesis and has been documented to act as an oncogene in various types of tumors. The aim of this study was to explore the effects of miR-19a and RhoB in bladder cancer.*

Methods: *The expression of miR-19a was analyzed from 54 paired of bladder cancer and paracancer tissues by real-time quantitative polymerase chain reaction (RT-qPCR). Transwell assay was employed to confirm the invasion capacity in bladder cancer. Dual luciferase reporter assay was utilized to analyze the association between the expression of miR-19a and RhoB. Western blot analyses were performed to detect the relative protein expression.*

Results: *miR-19a was clearly overexpressed in bladder cancer tissues and cells, while the expression of RhoB was lower in bladder cancer tissues and cells as compared with corresponding paracancer tissues and normal bladder cells. Also, miR-19a could promote the invasion and epithelial-mesenchymal transition (EMT) of bladder cancer cells through inhibiting the expression of RhoB. Moreover RhoB was identified as a direct target of miR-19a and the inverse relationship between them was also observed.*

Conclusions: *This data demonstrated that miR-19a promoted the invasion and EMT through targeting RhoB. It is suggested that miR-19a/RhoB axis may show a new way for the treatment of bladder cancer.*

Key words: *bladder cancer, EMT, invasion, miR-19a, RhoB*

Introduction

Bladder cancer, the incidence of which ranks fourth in males, is one of the most frequent carcinomas worldwide. It has been reported that there are lots of factors leading to bladder cancer, among which smoking is affirmed to be one of them as 30-50% of bladder cancer is caused by smoking. According to pathology characteristic, bladder cancer can be divided into three main: bladder urothelial carcinoma (UC), bladder squamous cell carcinoma and bladder adenocarcinoma [1-3]. As the pathophysiological mechanisms are unclear, the treatment is limited. Therefore, the identification of novel molecular mechanisms involving tumorigenesis and progression of bladder cancer and the possible therapeutic targets for its treatment are urgently needed.

MicroRNAs (miRNAs) are noncoding RNAs with 22-28 nucleotides in length [4-6]. miRNAs regulate the expression of target genes by inhibiting their translation or cutting of mRNA transcripts at post-transcriptional level [7,8]. At present, miR-19a, identified as one of the key members related to tumorigenesis, has been documented to act as an oncogene in various types of tumors including lung cancer, glioma, colorectal cancer and even bladder cancer [9-13]. MiR-19a can promote the proliferation and migration in colorectal cancer cells and accelerate tumor growth in xenografted

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mice [12]. What's more, miR-19a modulates cell growth, metastasis and cell cycle of lung cancer [13]. In addition, miR-19a acts as oncogene, promoting the proliferation of bladder cancer cells [14]. In glioma cells, miR-19a promoted cell proliferation and invasion through targeting RhoB. However, the effect of miR-19a and RhoB on the proliferation and invasion of bladder cancer cell is still unclear, demanding urgent detection of the functions of miR-19a and RhoB in bladder cancer.

RhoB, a kind of GTP-binding protein, is a member of Rho family which belongs to the Ras superfamily. Unlike RhoA and RhoC, RhoB is usually lowly expressed in carcinomas with high metastatic potential and poor differentiation [15]. RhoB has been reported to be downregulated in gastric cancer and suppresses cell proliferation, invasion, promotes cell apoptosis and increases chemosensitivity [16]. Furthermore, RhoB prevents early metastasis by inhibiting the EMT-derived invasiveness in non-small cell lung cancer [17].

In this study, 59 bladder cancer patients were included from whom pairs of bladder cancer and paracancer tissues were obtained to measure the expression of miR-19a and RhoB and explore the relevant mechanisms.

Methods

Patients and tumor samples

Pairs of bladder cancer and paracancer specimens were obtained from Jinan Central Hospital Affiliated to Shandong University from 59 patients who were operated. All the tissues were stored in liquid nitrogen at -80℃ immediately after surgery. Two urinary pathologists performed the histological diagnoses of bladder cancer. Neither radiotherapy nor chemotherapy therapy was used in any patients before surgery. Of these patients, 26 were at advance disease stage (III/V), while the rest were at early stage (I/II) according to TNM stage. All patients signed informed consent and the study was approved by the Ethics Committee of Jinan Central Hospital Affiliated to Shandong University.

Cell lines and culture conditions

Normal bladder cell line SV-HUC-1 and two bladder cancer cell lines (J82 and T24) were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were stored in liquid nitrogen until use. All the cells were cultured in Roswell Park Memorial Institute 1640 (RMIP-1640) medium (Gibco, Rockville, MD, USA) supplemented with 10% foetal bovine serum (FBS; HyClone, South Logan, UT, USA) at 37℃ in a humidified atmosphere of 5% CO^2.

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

The TRIeasy™ Total RNA Extraction Reagent TRIeasy™ (Yesen, Shanghai, China) and the MIRcute Extraction and Separation of miRNAs Kit (Tiangen, Beijing, China) were utilized to extract total RNA and miRNA. Next, we reverse-transcribed the purified RNA into complementary DNA (cDNA) using a PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Otsu, Japan) according to the manufacturer's instructions. The primers for RT-PCR were as follows: miR-19a, 5'-TGCGGTTCACAGTGGCTAAG-3'; U6 forward, 5'-TGCGGGTGCTCGCTTCGGCAGC-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'; RhoB forward: 5'-GGACACCGACGTCATTCTCA-3' and reverse: 5'-ACCAGGATGATGGGCACATT-3'; GAPDH forward: CGAGCCACATCGCTCAGACA and reverse: GTGGTGAAGACGCCAGTGGA. Then, the SYBR Premix kit or the SYBR Prime Script miRNA RT-PCR Kit (both purchased from TaKaRa, Otsu, Japan) were applied to perform RT-PCR. The glyceraldheyde 3-phosphate dehydrogenase (GAPDH) and U6 acted as the internal control of mRNA and miRNA respectively. The relative level of mRNA and miRNA was calculated according to the $2^{-\Delta\Delta Ct}$ method.

Protein extraction and Western blotting

For western blot analysis, after washing with phosphate buffered saline (PBS; Sigma-Aldrich, Missouri, USA), the cells were lysed on ice used lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) (both from Beyotime, Shanghai, China). Next, the concentration of the protein was calculated by Bicinchoninic Acid Reagent Kit (BCA; Solarbio, Beijing, China) after centrifugation. In addition, the proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Later, the blots were incubated by specific primary antibodies against RhoB, N-cadherin, E-cadherin and Vimentin (both antibodies diluted at 1:1000; Abcam, London, UK) at 4℃ overnight after blocking with 5% bovine serum albumin (BSA; Roche, Shanghai, China). Then, the blots were washed with tris buffered saline-tween (TBST) and incubated with mouse anti-human IgG (1:4000; Applygen, Beijing, China) at indoor temperature for about 2 hrs. The protein bands were detected by chemiluminescence through Bio-Rad Gel Doc XR system (Bio-Rad, Hercules, CA, USA).

Transwell assay

The capacity of cell invasion was assessed by Transwell assay. First, the transwell chamber was covered with Matrigel (Clontech, Mountain View, CA, USA). Two hundred ml of cell suspension were seeded into the upper chamber; meanwhile 500 μL medium was added in the lower chamber with 15% serum acting as chemoattractant. After 24-h culture, the cells in the upper chamber were removed with cotton swabs, while the cells that migrated in the lower chamber were fixed with methanol for 20 min and subsequently stained with crystal violet stain. The cell number was counted in five random fields and the average of per field of view was calculated.

Plasmid construction and Luciferase reporter assay

TargetScan (www.targetscan.org) was employed to predict the target genes and we found RhoB was predicted to be a candidate target gene of miR-19a. The

fragment of 3'UTR of RhoB mRNA (pmirGLO-RhoB-WT) and miR-19a mimic were inserted into pmirGLO plasmid. Meanwhile, the binding sequences were designed from 5'-UUUGCACA-3' to 5'-UUUCGUCA-3' and cloned into pmirGLO vector (pmirGLO-RhoB-MUT). The pmirGLO-RhoB-WT and miR-19a mimic or control were co-transfected into bladder cancer cells J82 and T24. Also, the pmirGLO-RhoB-MUT and miR-19a mimic or control were co-transfected into the bladder cancer cells. Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) was employed to detect luciferase activity.

Transfection

miR-19a inhibitor was used to knockdown the expression of miR-19a while pcDNA3.1-RhoB was employed to overexpress the RhoB. After the bladder cancer cells were transfected with miR-19a inhibitor or pcDNA3.1-RhoB, we detected the change of invasive ability and the expression of EMT associated proteins that were N-Ca, E-Ca and Vimentin.

The specific operation of transfection was as follows: at first, 2 mL 1×10^5/mL cell suspensions were inserted into 6-well plate. After culturing the cells for 24 hrs, the RMIP-1640 medium was replaced by fresh medium without serum. After that, 4μg specific plasmid and 10μg Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) were diluted into 150 μL serum-free medium respectively, the two solutions were then mixed for 20 min. In addition, the mixture was added into the bladder cells and 6 hrs later the RMIP-1640 medium was replaced with fresh medium with serum.

Statistics

Each experiment was repeated at least three times, and all quantitative data were expressed as the mean ± standard deviation (SD). The data were analysed by one way analysis of variance (ANOVA) using GraphPad Prism6 (GraphPad Software, Inc., San Diego, CA, USA). The statistical analyses were evaluated by independent samples t-test and ANOVA. $p\leq0.05$ was considered as statistically significant.

Results

MiR-19a was overexpressed in bladder cancer and had negative correlation with RhoB

To analyze the important roles of miR-19a, the expression of miR-19a of 59 pairs of bladder cancer tissues and paracancer tissues was detected by

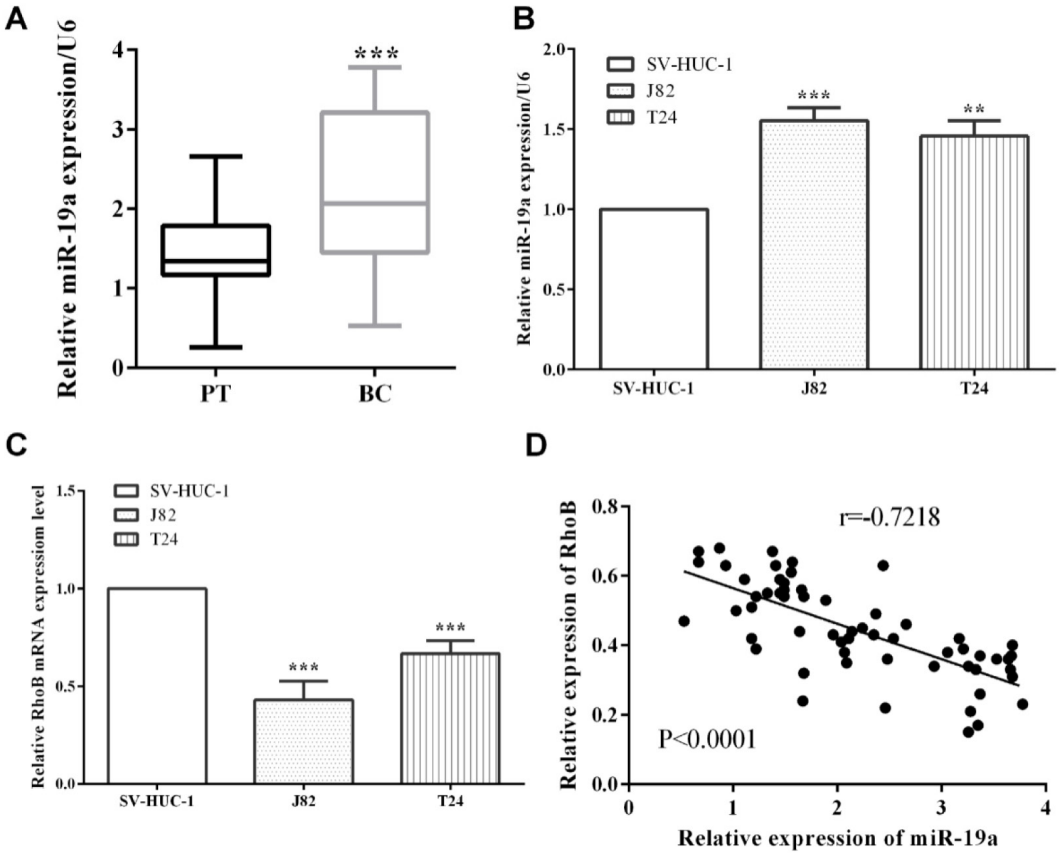

Figure 1. The expression of miR-19a was high in bladder cancer and had negative association with RhoB. **(A):** The miR-19a was highly expressed in bladder cancer and paracancer tissues. **(B):** The expression of miR-19a in bladder cancer cell lines J82 and T24 and normal bladder cell SV-HUC-1. **(C):** The mRNA level of RhoB in bladder cancer cells J82 and T24 and normal bladder cell SV-HUC-1. **(D):** The relationship between the expression of miR-19a and RhoB. **p<0.01; ***p<0.001. PT: paracancer tissues; BC: bladder cancer.

RT-qPCR. As expected, we found that it was highly expressed in bladder cancer tissues compared with the levels in paracancer tissues (p<0.001) (Figure 1A). We also noted that the expression of miR-19a was significantly higher in bladder cancer cell lines J82 (p=0.0003) and T24 (p=0.0012) than that in normal bladder cell line SV-HUC-1 (Figure 1B). On the contrary, the mRNA level of RhoB was lowly expressed in bladder cancer cell lines J82 (p=0.0005) and T24 (p=0.0010) versus normal bladder cell line SV-HUC-1 (Figure 1C). In addition, a connection between the expression of miR-19a and RhoB was detected, and we discovered that the expression of miR-19a had negative relation with the expression of RhoB in bladder cancer tissues (p<0.001, r=-0.7218) (Figure 1D).

MiR-19a promoted the invasion and epithelial to mesenchymal transition (EMT) of bladder cancer cells

Since we detected the miR-19a was overexpressed in bladder cancer cells, we knocked-down the miR-19a by miR-19a inhibitor and detected miR-19a expression level by RT-qPCR in J82 (p=0.0002) and T24 (p=0.0008) cells (Figure 2A). The Transwell and Western blot assays were used to evaluate the biological function of miR-19a on cell invasiveness and EMT. The results suggested that downregulation of miR-19a inhibited the invasion both in J82 (p=0.0048) and T24 (p=0.0104) cells (Figure 2B). The proteins associated with EMT including N-cadherin (N-Ca), Vimentin and E-cadherin (E-Ca) were detected by Western blot and it was found that knockdown of miR-19a could reduce the proteins expression of N-cadherin and Vimentin, while it increased the expression of E-cadherin, suggesting that knockdown of miR-19a inhibited the EMT of bladder cancer cells (Figure 2C).

RhoB was a direct target of miR-19a and mediated by miR-19a

RhoB was supposed to be a direct target of miR-19a predicted by TargetScan with binding sites located at 857–864 of 3'UTR. Thus, the binding region was designed from 5'-UUUGCACA-3' to 5'-UUUCGUCA-3', both of which were inserted into pmirGlo luciferase vectors, which were named pmirGLO-RhoB-wild type (WT) and

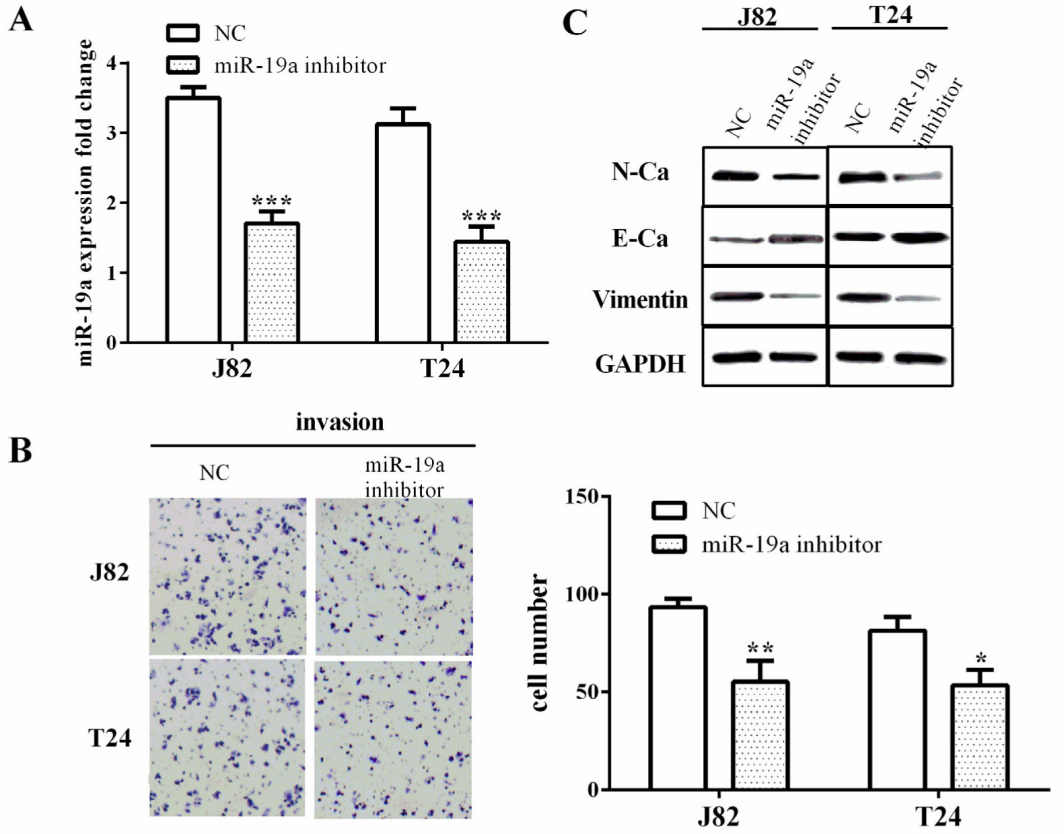

Figure 2. miR-19a promoted the invasion and EMT of bladder cancer cells. **(A):** RT-qPCR detected the miR-19a level after transfection with the miR-19a inhibitor in J82 and T24 cells. **(B):** The invasive capacity was measured by Transwell assay after knockdown of miR-19a in J82 and T24 cells. This figure shows that downregulation of mir-19a inhibits the invasion both in J82 and T24 cells. **(C):** Western blotting showed that the EMT ability was reversed by miR-19a in J82 and T24 cells. *p<0.05; **p<0.01; ***p<0.001. NC: normal control.

pmirGLO-RhoB-mutant type (MUT), respectively (Figure 3A). The bladder cancer cells J82 and T24 were cotransfected with the WT or MUT and the miR-19a mimic or negative control, and then the changes of relative luciferase activity were measured. The relative luciferase activity was reduced when co-transfected with the WT and the miR-19a mimic compared with the cotransfection with the WT and negative control in J82 (p=0.0029) and T24 (p=0.0003) respectively, while it almost has no suppressive effect when cotransfected with the MUT and the miR-19a mimic versus the MUT and the negative control both in J82 (p=0.7066) and T24 (p=0.5147) (Figure 3B).

In order to further confirm the regulation of miR-19a on RhoB, the miR-19a inhibitor was transfected into the bladder cancer cells J82 and T24. Following this, the expressions of RhoB were detected after transfection with miR-19a inhibitor or negative control in J82 and T24 cells, and we found it was reduced both in mRNA (p=0.0005 and 0.0018 respectively) and protein levels in J82 and T24 cells, respectively (Figure 3C). Taken together, all these data suggest that RhoB is a direct target of miR-19a in bladder cancer.

Overexpressed RhoB could inhibit the invasion and EMT of bladder cancer

To further verify that miR-19a effects the invasion and EMT through RhoB, the RhoB was overexpressed in J82 and T24 cells, and the transfection efficiency was measured by RT-qPCR (p=0.0011 and 0.0006 of J82 and T24, respectively) and western blot (Figure 4A).

Then, the ability of cell invasion was measured by Transwell assay and it was found that overexpression of RhoB decreased the invaded J82 (p=0.0032) and T24 (p=0.0018) cell number, sug-

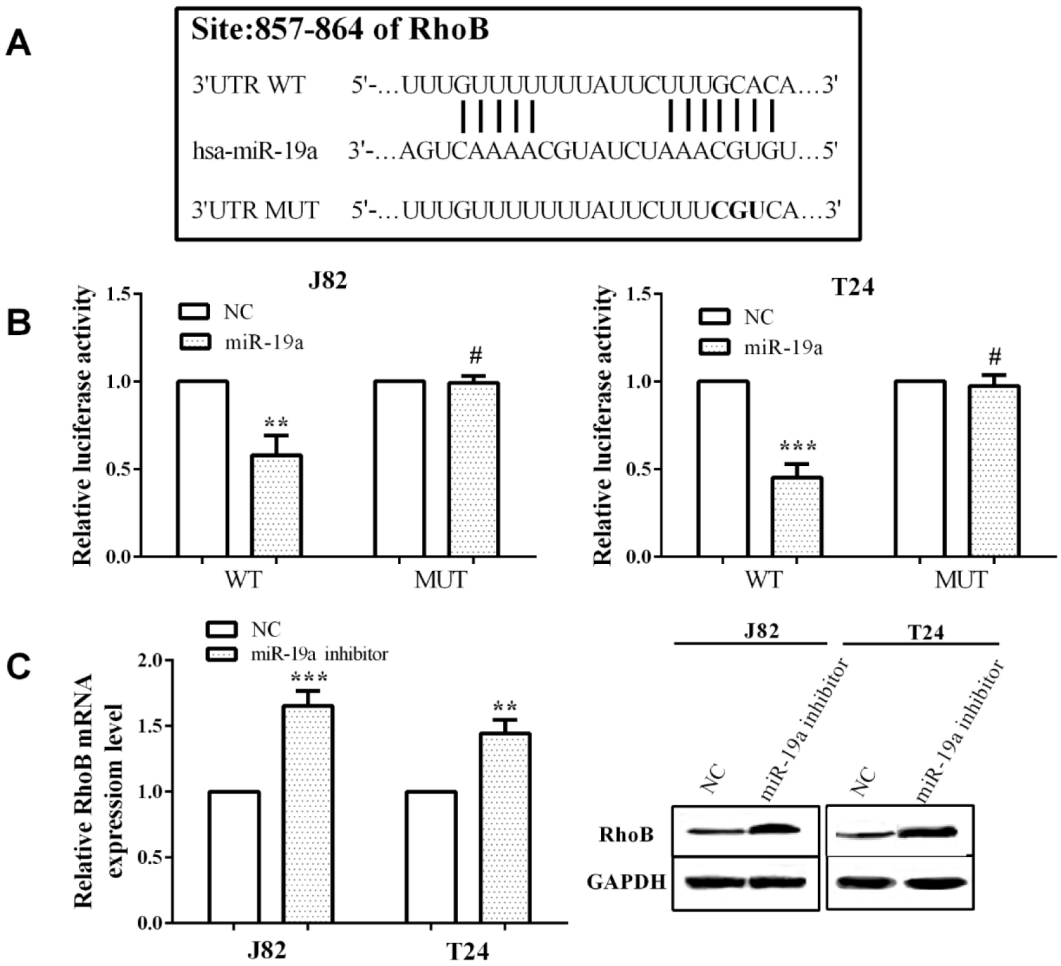

Figure 3. RhoB was a direct target of miR-19a and regulated its expression by miR-19a. **(A):** According to the TargetScan, the miR-19a target site in the sequence of RhoB was predicted. **(B):** The fluorescence activity of the RhoB 3'UTR was measured by the luciferase reporter gene assay in J82 and T24 cells that were cotransfected with wild-type RhoB 3'UTR and miR-19a or mutational type RhoB 3'UTR and miR-19a, respectively. The figure shows that RhoB is a direct target of miR-19a. **(C)**: RT-qPCR and Western blotting were applied to detect the mRNA and protein levels of RhoB in J82 and T24 cells after transfection with miR-19a inhibitor or negative control. The figure further shows that RhoB is a direct target of MiR-19a. **p<0.01; ***p<0.001; #p<0.05.

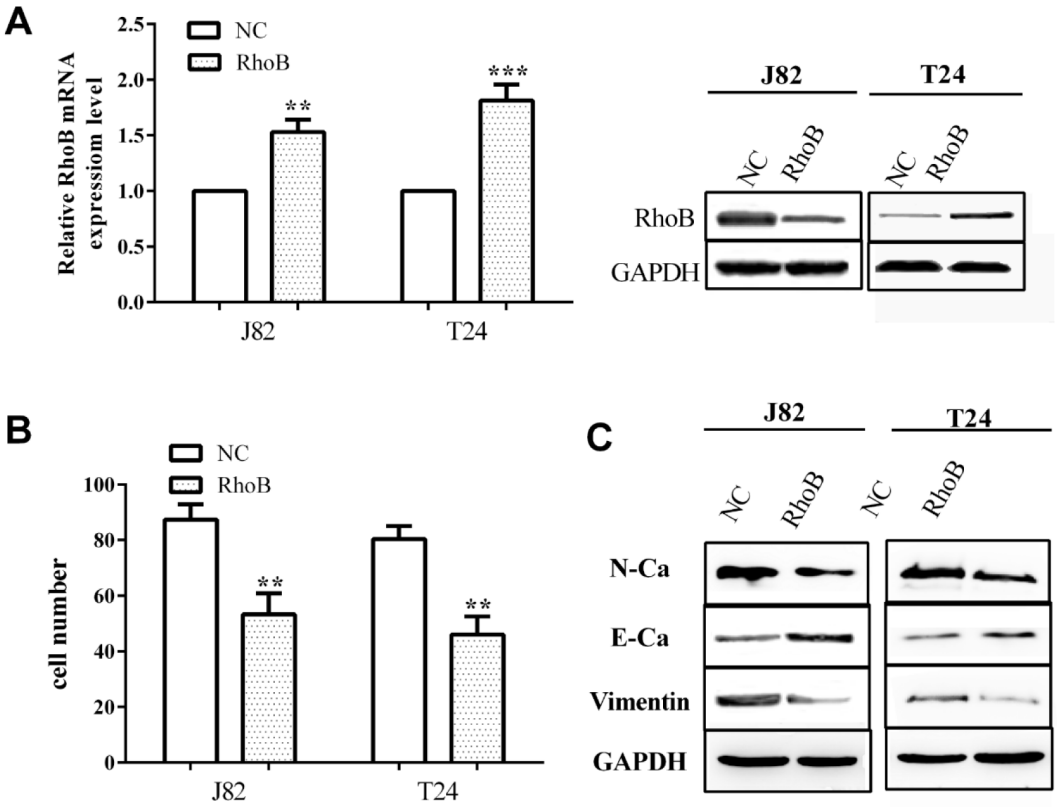

Figure 4. Overexpression of RhoB partially reversed the function of miR-19a on the invasive and EMT capabilities. **(A):** The transfection efficiency was detected by RT-qPCR and Western blot with transfection of RhoB in J82 and T24 cells. **(B):** The ability of cell invasion was measured by Transwell assay when transfected with RhoB in J82 and T24 cells. **(C):** The expression of N-cadherin, E-cadherin and Vimentin was detected in J82 and T24 cells after transfection with RhoB. This figure shows that overexpressed RhoB inhibits the EMT of bladder cancer cells. **p<0.01; ***p<0.001.

gesting that the ability of invasion was reduced by overexpressing RhoB (Figure 4B). What's more, the EMT associated proteins N-Ca, E-Ca and Vimentin were detected, and we noted that when transfected with RhoB, the levels of N-Ca and Vimentin decreased, while E-Ca increased, indicating that overexpressed RhoB inhibited the EMT of bladder cancer cells (Figure 4C).

Discussion

In this study, we demonstrated that miR-19a was conspicuously highly expressed in bladder cancer cells (J82 and T24) and tissues. What's more, we discovered that miR-19a acted as an oncogene and could promote the invasion and EMT of bladder cancer cells. In addition, RhoB was predicted to be a direct target of miR-19a as verified by luciferase reporter assay.

Bladder cancer is one of the most prevalent carcinomas, the incidence of which is ranked fourth in males. However, the molecular mechanism by which miR-19a regulates the progression and development of bladder cancer has not been thoroughly investigated. MiRs regulate gene expression by inhibiting translation or cutting mRNA transcripts at post-transcriptional level. A large number of miRs have been reported to play important roles in bladder cancer, including miR-9, miR-21 and miR-19a [14,18-20]. MiR-19a has been identified as one of the key members related to tumorigenesis, which acts as an oncogene in various types of tumours. Hu et al. proved that miR-19a could facilitate the proliferation and metastasis in human colorectal and lung cancer cells [13]. What's more, miR-19a was significantly highly expressed and promoted proliferation in bladder cancer cells [14]. However, the functions of miR-19a have not been thoroughly investigated and there are only few studies exploring the mechanism of miR-19a on invasion and the EMT in bladder cancer cells. In our study, we showed the expression of miR-19a was upregulated in bladder cancer which was consistent with a previous study [14]. In addition, knockdown of miR-19a reduced the invasion and EMT capacity versus the control.

miRs usually regulate cell proliferation, apoptosis and metabolism by direct combination with the target genes [21]. Several genes involved in the progression of tumours, such as PTEN, SOCS1

and RhoB have been reported as the target genes of miR-19a [11,22,23]. Chen et al. had discovered that RhoB was a direct target of miR-19a and promoted the proliferation and invasion of glioma cells [11]. Accordingly, we also measured the mRNA level of RhoB and found it was lowly expressed in bladder cancer cells. In addition, we also discovered that the expression of RhoB was negatively associated with miR-19a in bladder cancer tissues. To further confirm that miR-19a regulates the expression of RhoB, TargetScan was employed to verify the binding sites of miR-19a on the 3'UTR of RhoB mRNA. Besides, we found that the luciferase reporter activity was repressed in bladder cells after co-transfection with miR-19a mimic and pmirGLO-RhoB-WT, while there were no significant differences when co-transfected with pmirGLO-RhoB-MUT and miR-19a mimic. Furthermore, RhoB was upregulated both in mRNA and protein levels after repression of miR-19a in bladder cancer cell lines J82 and T24. The results illustrated that miR-19a could mediate the expression of RhoB by direct targeting the 3'UTR of RhoB mRNA.

RhoB, a member of Rho family, is a GTP-binding protein that is usually lowly expressed in carcinomas with high metastatic potential and poor differentiation [15]. Zhou et al. have reported that RhoB inhibited gastric cancer cell growth and migration [16]. Also, Calvayrac et al. [24] and Bousquet et al. [17] have reported that RhoB could modulate the mesenchymal phenotype and invasion in non-small cell lung cancer. Our results were consistent with previous findings, that overexpression of RhoB suppressed the invasion capacity and EMT in J82 and T24.

Conclusions

miR-19a was found to be highly expressed in bladder cancer tissues and cells compared with normal tissues and cells. The function of miR-19a was negatively connected with RhoB in bladder cancer tissues. Knockdown of miR-19a or upregulation of RhoB suppressed the EMT and the invasion of bladder cancer cells J82 and T24. All these results implied that miR-19a mediated the EMT and invasion through direct targeting the 3'UTR of RhoB mRNA in bladder cancer cell lines.

Conflict of interests

The authors declare no conflict of interests.

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