

CREB1, a direct target of miR-122, promotes cell proliferation and invasion in bladder cancer

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Abstract. Bladder cancer (BC) is a prevalent cancer, which arises from the epithelial lining of the urinary bladder. CAMP-response element binding protein (CREB1) acts as a transcription factor, which regulates cell transcription through phosphorylation and dephosphorylation. The purpose of this study was to explore how miR-122 worked in BC on cell proliferation and invasion. RT-qPCR was applied to evaluate the mRNA levels of CREB1 and miR-122 in BC. CCK-8 and Transwell assays were employed to determine the migratory and invasive abilities. Dual luciferase reporter assay was applied to verify miR-122 targeting CREB1 in BC. CREB1 was upregulated in bladder tissues and T24, UM-UC-3 and J82 cells, while miR-122 upregulated and had negative correlation with CREB1. Moreover, knockdown of CREB1 inhibited cell proliferative and invasive capacities. In addition, CREB1 was directly targeted by miR-122 in BC and regulated its expression. We discovered that CREB1 could reverse partially the function of miR-122 on cell proliferation and invasion. CREB1 was mediated by miR-122, and regulated cell proliferation and invasiveness. The newly identified miR-122/CREB1 axis provides novel insight into the pathogenesis of BC.

Introduction

Bladder cancer (BC), one of the most prevalent cancers, arises from the epithelial lining of the urinary bladder, and is mainly caused by smoking and mutation (1). In current years, the standard treatment of invasive BC is radical cystectomy, although the therapeutic strategies have improved (2), the 5-year survival was 62% due to high rate of metastasis and invasion (3,4). Therefore, looking for the tumor markers for early diagnosis is particularly important for treatment of bladder tumors.

CAMP-response element binding protein (CREB1), a transcription factor, regulates gene transcription through phosphorylation and dephosphorylation. CREB1, as a proto-oncogenic transcription factor promotes expression of its target genes taking part in metabolism and DNA repair (5). Aberrant expression of CREB1 was reported to be connected with variety of cancers, including gastric, colorectal, ovarian and breast cancers (6-9). In colorectal cancer, Ye *et al* discovered that CREB1 was significantly upregulated and promoted cell migration and invasion (10). Similar findings were reported by Wang *et al* (11) knockdown of CREB1 inhibited cell proliferation and motility in prostate cells. Therefore, we strongly believe that CREB1 could play important roles in BC.

microRNAs (miRNAs) are a class of non-coding RNA sequences with 22-28 nucleotides, which could inhibit gene expression at post-transcriptional level by binding to the 3'-untranslated region (3'-UTR) of target mRNA (12,13). miRNAs are reported to be involved in several biological processes in BC, including miR-1, miR-126, miR-202 and miR-149 (14-17). There are several miRNAs binding to CREB1 and regulating the expression of CREB1 in cancers, including miR-590, miR-1224, miR-205 and miR-122 (5,18-20). miR-122, a novel microRNA, was predicted to be downregulated in many cancers including BC (21). In hepatocellular carcinoma, miR-122 inhibited epithelial-mesenchymal transition through snail1 and snail2 (22). Maierthaler *et al* discovered that miR-122 was a prognostic marker in colorectal cancer (23). Similar finding were reported by Wang *et al* that miR-122 inhibited tumor growth and angiogenesis by targeting VEGFC in BC (24). In addition, Rao *et al* discovered that miR-122 inhibited proliferation and invasion by targeting CREB1 in gastric cancer (20). Considering these functions, to the best of our knowledge, we first propose that miR-122 regulated CREB1 expression and mediated cell proliferation and invasion in BC.

Patients and methods

Patients and clinical samples. A collection of 47 BC tissues as well as corresponding healthy tissue samples (5 cm away from the tissues) were obtained from patients who underwent surgery at China-Japan Union Hospital of Jilin University from 2015 to 2017. All samples were snap-frozen in liquid nitrogen and saved in -80°C after resection. None of the patients had medication or radiation therapy. This study was

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approved by the Ethics Committee of China-Japan Union Hospital of Jilin University (Changchun, China) and obtained written informed consent from all the patients.

Cell lines and cell culture. Human BC cell lines T24, UM-UC-3 and J82 and normal bladder cells SV-HUC-1 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were maintained in RPMI-1640 with 10% FBS (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) then cultured in an incubator at 37°C with 5% CO₂.

RNA isolation and RT-qPCR. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was employed to isolate RNAs, which including miRNA from the cultured cells and tissues. OneStep PrimeScript® cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) was applied to perform reverse-transcription and synthesize first cDNA chain. Fast SYBR-Green Master Mix and TaqMan microRNA assay kits were employed to perform the RT-qPCR for CREB1 and miR-122, respectively, using ABI PRISM7900 Sequence Detection System (all from Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH and U6 were utilized as internal reference for CREB1 and miR-122, respectively. The specific stem-loop RT primers were used for reverse transcription reaction as follows: miR-122: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAAACACC-3'; U6: 5'-AACGCTTCACGAATTTGCGT-3'; CREB1: F: CTTT TCTCCGGAACACAGATTTC; R: GATTTGCCAAGTGGGAGGGA; GAPDH: F: 50-CACTCCTCCACCTTTGA-30, R: 50-CCACCACCCTGTTGCTG-3'. The thermocycling parameters were 95°C for 3 min and 40 cycles of 95°C for 15 sec followed by 60°C for 30 sec. The quantification of CREB1 or miR-122 mRNA levels was through measuring Cq values and normalized using the 2^{-ΔΔCq} method (25).

Protein extraction and western blotting. The cells were washed with cold PBS buffer and extracted using RIPA lysis buffer with protease inhibitor (Beyotime Institute of Biotechnology, Shanghai, China), followed by centrifugation for 20 min at 4°C with 12,000 x g. The supernatants were collected to determine the protein concentration using BCA reagent kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The protein lysates (50 μg/lane) were fractionated using 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA); The membrane was blocked using 5% non-fat dried milk for 1 h at room temperature. The blots were incubated with anti-CREB1 rabbit polyclonal antibody (dilution, 1:1,000; cat. no. SAB4300519; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and anti-GAPDH (dilution, 1:3,000; cat. no. G5262; Sigma-Aldrich; Merck KGaA) at 4°C overnight and anti-rabbit antibody (1:3,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature, which was normalized by GAPDH. Signal detection employed ECL detection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell proliferation assay. The cell proliferative ability was measured by Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). A total

of 100 μl T24 or J82 cells (2x10³ cells/well) were seeded in 96-well plates and cultured for 24, 48, 72 or 96 h, respectively. Then 10 μl of CCK-8 solution was added in each well and incubated at 37°C for 1 h. Microplate Reader (Epoch; BioTek Instruments, Inc., Winooski, VT, USA) was applied to determine the absorbance at 490 nm.

Transwell assay. Transwell chambers (Corning Costar, Beijing, China) with 8 μm pore size membranes were employed to assess invasive ability. Transwell chamber in 24-plate well with Matrigel (Clontech Laboratories, Inc., Mountainview, CA, USA) was coated. T24 or J82 cells with a density of 5x10⁴ were added in the upper chamber in 200 μl medium without FBS. Whereas, 500 μl normal medium with 15% FBS was added in the lower chamber for use as a chemoattractant. The cells were incubated for 24 h, and the unattached cells were removed using cotton swab. The invaded cells were fixed with methanol and then stained using 1% crystal violet; and cell counting was carried out under the microscope BX51 Olympus (Shenzhen, China).

Transfection. All vectors, including miR-122 mimic, miR-122 inhibitor, pcDNA3.1-CREB1 and luciferase reporter plasmids were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). miR-122 mimic or inhibitor were applied to overexpress or knockdown miR-122, while siRNA-CREB1 and pcDNA3.1-CREB1 were employed to knock down or overexpress CREB1. The sequences of miR-122 mimic/inhibitor and negative control (NC) were: 5'-UGGAGUGUGACAAUGGUGUUUG-3'; 5'-CAAACACCAUUGUCACACUCCA-3'; and 5'-UUCUCCGAACGUGUCACGUTT-3'. Scrambled nucleotide sequences were the NC of miR-184 inhibitor and miR-184 mimic.

Before transfection, T24 or J82 cells were seeded in 6-well plate and cultured overnight. A total of 4 μg vectors and 8 μl Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was mixed in 1.5 ml microcentrifuge tube and then let stand for 20 min; then mixed and added into cells and cultured at 37°C for 48 h.

Plasmid construction and luciferase reporter assay. 3'-UTR fragment of CREB1 mRNA, containing the putative miR-122 binding sequence was amplified by PCR and cloned into pmirGlo luciferase reporter vector (named pmirGlo-CREB1-WT; WT). QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for the site-directed mutagenesis of CREB1 3'-UTR (pmirGlo-CREB1-MUT; MUT) with WT as template.

For luciferase reporter assays, T24 cells were seeded into 6-well plate, miRNA-122 and WT or MUT reporter plasmid were transiently co-transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 48 h, luciferase activity was measured using a dual-luciferase assay system (Promega Corporation, Madison, WI, USA) according to the manufacturer's instruction, which was normalized by renilla-luciferase activity.

Statistical analysis. The data are presented as mean ± standard deviation. All statistical analyses were performed with SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA).

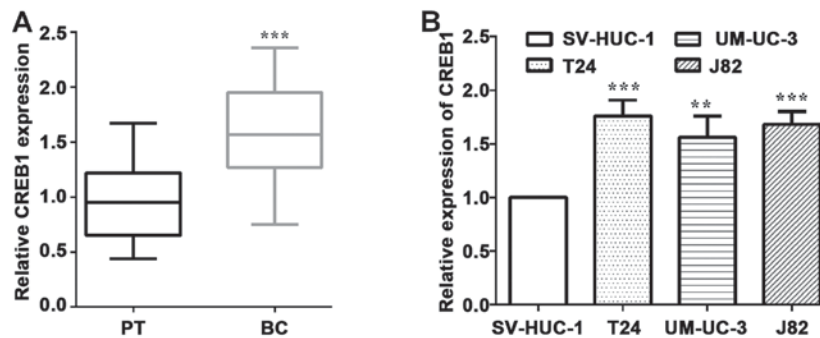


Figure 1. CREB1 is upregulated in BC tissues and cells. (A) The expression of CREB1 in BC tissues was higher than that in corresponding adjacent healthy tissues detected by RT-qPCR. (B) The levels of CREB1 were higher in BC cells T24, UM-UC-3 and J82 vs. normal cell SV-HUC-1. ** $P<0.01$; *** $P<0.001$. CREB1, CAMP-response element binding protein; BC, bladder cancer.

Two-tailed Student's t-test and one-way ANOVA followed by Tukey's post hoc test were employed to analyze two groups and three or more groups, respectively. Pearson's test analysis was applied to analyze the correlations between miR-122 and CREB1 mRNA expression. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CREB1 is upregulated in BC tissues and cells. To validate the expression of CREB1 in BC, we employed RT-qPCR to examine CREB1 expression in clinical specimens and cell lines. As shown in Fig. 1A, 49 paired of BC and corresponding healthy tissues were collected and measured the mRNA level of CREB1. As expected, CREB1 expression in BC tissues was significantly higher than that in the corresponding healthy tissues ($P<0.0001$) (Fig. 1A). Furthermore, CREB1 was expressed at prominently higher levels in bladder cancer patients with tumor ($P=0.030$), TNM stage ($P=0.014$), lymph node metastasis ($P=0.028$) and the expression of miR-122 ($P=0.014$), while it had tendency to have association with invasion ($P=0.053$) (Table I). We measured the mRNA levels of CREB1 in three human BC cells and a normal bladder cell, and found that CREB1 was upregulated in BC T24 ($P=0.0009$), UM-UC-3 ($P=0.0083$) and J82 cells ($P=0.0006$), compared to the normal SV-HUC-1 cells (Fig. 1B).

Knockdown of CREB1 inhibits cell proliferation and invasion. To explore the impact of CREB1, the cell proliferative and invasive abilities were measured after knockdown CREB1 in T24 cells. siRNA-CREB1 was applied to knockdown CREB1, and the CREB1 mRNA level was reduced in T24 ($P=0.0010$) and J82 ($P=0.0026$) cells as shown in Fig. 2A and C. When CREB1 was knocked down, the absorbance was reduced in T24 and J82 cells at 72 h ($P=0.0053$ and 0.0080) and 96 h ($P=0.0012$ and 0.0015) (Fig. 2B and D). On the other hand, the number of invasion was reduced ($P=0.0081$ and 0.0039) either in T24 or J82 cells (Fig. 2E), which suggested that knockdown CREB1 inhibited cell proliferation and invasion in BC T24 and J82 cells.

miR-122 is downregulated and targeted to CREB1 in BC. As we detected the effect of CREB1 on cell proliferation

Table I. CREB1 expression and clinicopathological features in 47 BC.

Clinicopathological features	Cases (n=47)	CREB1 expression		P-value ^a
		Low (%) (n=22)	High (%) (n=25)	
Sex				
Male	27	13 (48.1)	14 (51.9)	0.831
Female	20	9 (45.0)	11 (55.0)	
Age (years)				
<50	22	10 (45.6)	12 (54.4)	0.861
≥50	25	12 (48.0)	13 (52.0)	
Tumor size (mm)				
≤5.0	22	14 (63.6)	8 (36.4)	0.030 ^a
>5.0	25	8 (32.0)	17 (68.0)	
TNM stage				
I-II	21	14 (66.7)	7 (33.3)	0.014 ^a
III-IV	26	8 (30.8)	18 (67.2)	
Lymph-node metastasis				
0-2	24	15 (62.5)	9 (37.5)	0.028 ^a
>2	23	7 (30.4)	16 (69.6)	
Invasion				
No	25	15 (60.0)	10 (40.0)	0.053
Yes	22	7 (31.8)	15 (68.2)	
miR-122				
Low expression	26	8 (30.8)	18 (69.2)	0.014 ^a
High expression	21	14 (66.7)	7 (33.3)	

^aP-values are calculated with Chi-square test. CREB1, CAMP-response element binding protein; BC, bladder cancer.

an invasion, we considered what impacted CREB1 and then influenced cell proliferative and invasive abilities. Therefore, we predicted microRNAs by TargetScan, and we found miR-122 was upstream of CREB1. Then, we determined miR-122 level employing RT-qPCR and found that miR-122 was downregulated ($P<0.0001$) in BC tissues vs. healthy tissues (Fig. 3A). Furthermore, we analyzed the expression of CREB1 and miR-122 in BC tissue samples, and discovered

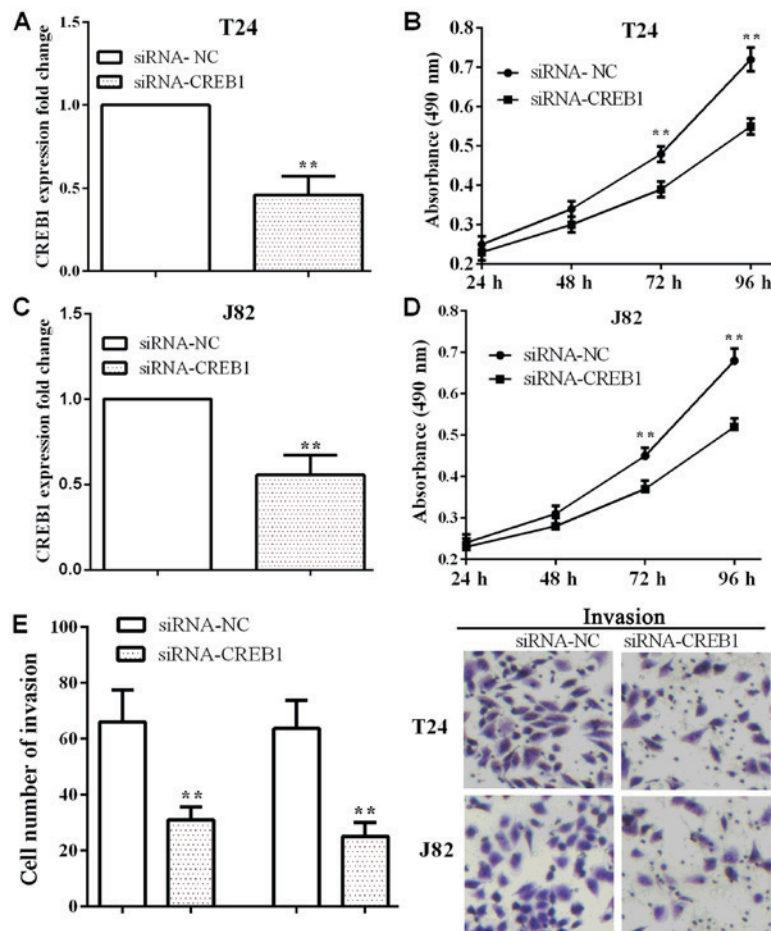


Figure 2. Knockdown CREB1 inhibits cell proliferation and invasion. (A and C) CREB1 expression was reduced in T24 and J82 cells transfected with siRNA-CREB1 compared to cells transfected with siRNA-NC detected by RT-qPCR. (B and D) Knockdown CREB1 significantly decreased the proliferative ability in T24 cells. (E) Knockdown CREB1 inhibited the invasive abilities of T24 and J82 cells (x200). ** $P < 0.01$. CREB1, CAMP-response element binding protein.

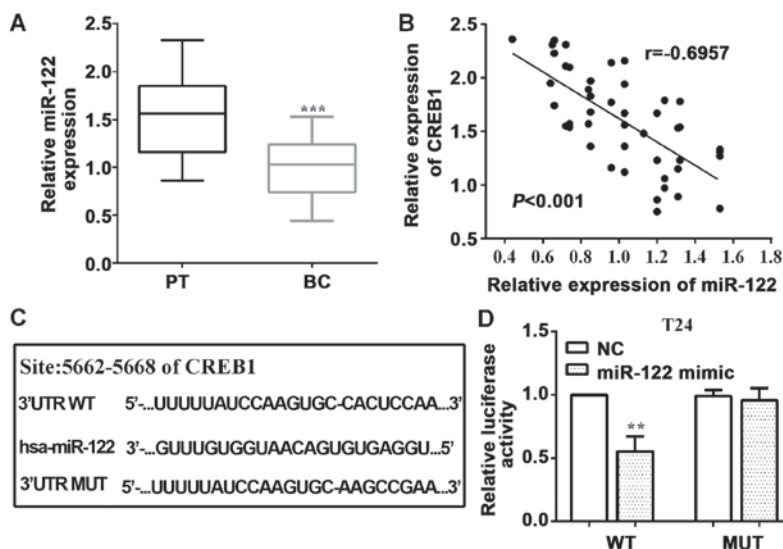


Figure 3. miR-122 is downregulated and targeted to CREB1 in BC. (A) The expression of miR-122 was determined to be downregulated in BC tissues versus healthy tissues. (B) The correlation between CREB1 and miR-122 had negative association in BC tissues explored by Pearson correlation analysis. (C) Putative seed-matching sites or mutant sites between miR-122 and 3'-UTR of CREB1. (D) Luciferase activity of A498 cells transfected with Dicer 3'-UTR luciferase reporter vector was reduced by wild-type, while the mutant 3'-UTR was not. WT, wild-type of CREB1 3'-UTR; MUT, mutant of CREB1 3'-UTR; CREB1, CAMP-response element binding protein; BC, bladder cancer. ** $P < 0.01$; *** $P < 0.001$.

that they had a negative association between them ($P < 0.0001$, $r = -0.6957$) (Fig. 3B).

In addition, we mutated the binding sequences of miR-122 on CREB1 3'-UTR from 5'-CACUCCA-3' (WT)

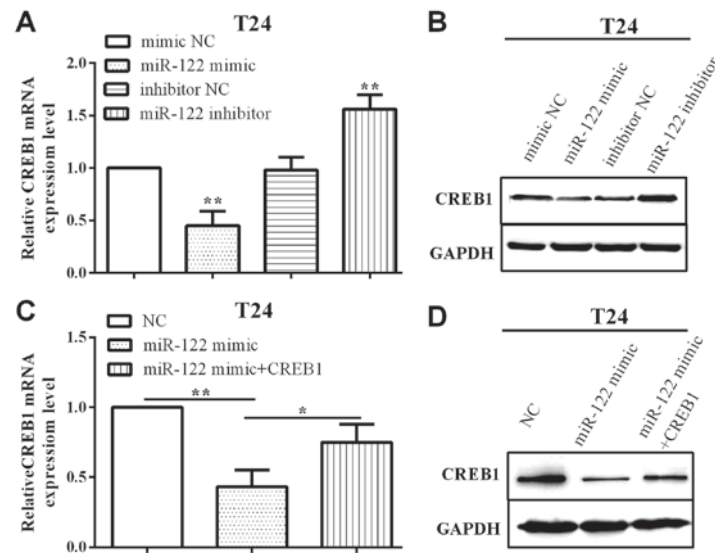


Figure 4. The changes of CREB1 mRNA and protein in altered miR-122 in T24 cells. (A and B) RT-qPCR and western blotting determined the transfection of miR-122 mimic and inhibitor on CREB1 expression. (C and D) Re-expressed CREB1 revealed the reduction by miR-122. * $P<0.05$; ** $P<0.01$. CREB1, CAMP-response element binding protein.

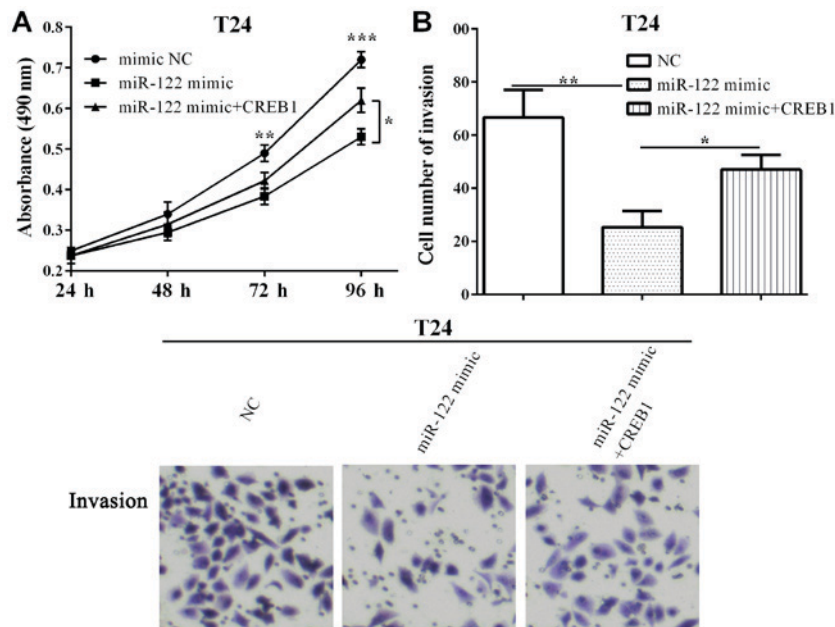


Figure 5. CREB1 mediated by miR-122 and regulation of cell proliferation and invasion in BC. (A) The proliferative ability was decreased when transfected with miR-122, which was reversed by re-expressed CREB1. (B) CREB1 could counteract the inhibitory effect of miR-122 on cell invasion in T24 cells. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. CREB1, CAMP-response element binding protein; BC, bladder cancer.

to 5'-AAGCCGA-3' (MUT), which were inserted into pmirGlo vector, as shown in Fig. 3C. To confirm miR-122 direct targeting to CREB1, luciferase reporter assay was performed. As expected, the luciferase activity was reduced ($P=0.0027$) by wild-type, while the mutant was not in T24 cells ($P=0.6194$) (Fig. 3D).

CREB1 is mediated by miR-122 and regulates cell proliferation and invasion in BC. To evaluate the impact of miR-122 on CREB1, we employed miR-122 mimic and inhibitor to over-express or knock down miR-122 and measured the CREB1 expression in T24 cells. As shown in Fig. 4A and B, when

transfected with miR-122 mimic, both CREB1 mRNA and protein levels were decreased ($P=0.0024$), whereas increased ($P=0.0055$) when transfected with miR-122 inhibitor.

In addition, when overexpressed CREB1 was used pcDNA 3.1-CREB1, CREB1 expression was increased ($P=0.0351$), based on the reduction by miR-122 evaluated in T24 cells by RT-qPCR and western blotting (Fig. 4C and D).

Furthermore, the proliferative ability was decreased ($P=0.0029$ and 0.0003) when transfected with miR-122 after 72 h and 96 h, and was reversed ($P=0.0124$) by re-expressing CREB1 at 96 h (Fig. 5A). Additionally, we determined the invasive ability that CREB1 could counteract ($P=0.0105$) and

the inhibitory ($P=0.0039$) effect of miR-122 on cell invasion in T24 cells (Fig. 5B).

Discussion

BC is one of the most prevalent cancers, which arises from the epithelial lining of the urinary bladder (1). Moreover, although the therapeutic strategies have improved, the 5-year survival is 62% due to high rate of metastasis and invasion (3,4). Thus, identifying new targets for the development of effective therapeutics for bladder tumors is urgent. In our study, we found that CREB1 was upregulated in bladder tissues and T24, UM-UC-3 and J82 cells, while miR-122 was upregulated and had a negative correlation with CREB1. Knockdown of CREB1 inhibited T24 and J82 cell proliferative and invasive capacities. In addition, CREB1 was directly targeted by miR-122 in BC and regulated its expression. We discovered that CREB1 could reverse the function of miR-122 partially on cell proliferation and invasion in T24 cells.

CREB1 acted as a proto-oncogenic transcription factor, promoted gene transcription through phosphorylation and dephosphorylation (5). CREB1 acted as predictor of prostate cancer recurrence and a critical driver of pro-survival, cell cycle and metabolic transcription programs (26). CREB1 was upregulated and promoted cell proliferation, invasion and acted as an independent prognostic factor in various cancers (10,11). Even in BC, CREB1 was involved in EMT (27), but the underlying molecular mechanisms are still elusive. Our findings are consistent with the above findings, CREB1 was upregulated in BC tissues and cell lines. Further investigation in our present research found similar results that CREB1 promoted BC T24 and J82 cell proliferation and invasion using MTT and Transwell assays, indicating that CREB1 can be an oncogene in BC. The data uncovered that the proliferation and invasion activity were significantly suppressed in T24 cells transfected with siRNA-CREB1 as compared with the NC group. However, the biological mechanism is unclear. The results were consistent with the findings of Shabestari *et al* in human pre-B acute lymphoblastic leukemia cells (28). However, due to the limitation of conditions we did not do IHC to evaluate the expression of CREB1.

miRNAs are non-coding RNAs, which inhibited gene expression at post-transcriptional level through targeting the 3'-untranslated region (3'-UTR) of target mRNA (12,13). miR-122 was predicted to downregulate and inhibit cell proliferation, invasion and EMT in many cancers (22-24). In gastric cancer, CREB1 was a direct target gene of miR-122 and miR-122 regulated the expression of CREB1 (20). Consistent with previous findings of Wang *et al* (24), we discovered that miR-122 was downregulated in BC. Moreover, consistent with Rao *et al* (20), miR-122 had a negative correlation with CREB1 in BC tissues, which, to the best of our knowledge, was the first time to propose the connection between CREB1 and miR-122 in BC. We hypothesized that CREB1 may ablate the inhibitory effects of miR-122 on cell proliferation and invasion in BC based on these results. In order to prove this hypothesis, we examined the rescue experiments in BC T24 cells. We found that the overexpression of miR-122 inhibited cell proliferation and invasion in T24 cells, and CREB1 partially reversed the role of miR-122 on cell proliferation

and invasion in BC. In the present study, we first proposed the relationship between CREB1 and miR-122; and we used rescue experiments to verify that miR-122 regulated cell proliferation and invasion through targeting CREB1, which was the novelty of the study.

In conclusion, we demonstrated that CREB1 acts as an oncogene in BC by reducing cancer growth and invasion. Moreover, we indicated that CREB1 has an inverse correlation with miR-122 and the expression of CREB1 by miR-122 in T24 cells. The newly identified CREB1 may provide further insight into the progression of BC and offers a promising therapeutic target for the treatment of BC. The newly identified miR-122/CREB1 axis provides a novel insight into the pathogenesis of BC.

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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

LG performed the data analyses and wrote the manuscript. MY contributed significantly to analysis and manuscript preparation. YW contributed to the conception and design of the study. All authors have read and approved the final study.

Ethics approval and consent to participate

The Ethics Committee of China-Japan Union Hospital of Jilin University (Changchun, China) approved the study, and informed consent was obtained by all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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