

LncRNA UCA1 promotes the invasion and EMT of bladder cancer cells by regulating the miR-143/HMGB1 pathway

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Abstract. The long non-coding RNA (lncRNA) urothelial carcinoma associated 1 (UCA1) is an oncogenic lncRNA in bladder cancer, and its upregulation is associated with enhanced cell invasion. However, the underlying mechanism remains to be elucidated. The present study demonstrated that UCA1 was positively associated with cell invasion ability and promoted epithelial-mesenchymal transition (EMT) of bladder cancer cells by inducing high mobility group box 1 (HMGB1). Furthermore, bioinformatics and luciferase reporter assays demonstrated binding sites of the tumor suppressive miR-143 within UCA1 and the 3'untranslated region of HMGB1. UCA1 negatively regulated miR-143 expression in a dose-dependent manner in bladder cancer cells. In addition, UCA1 and HMGB1 were upregulated and miR-143 was downregulated in bladder cancer specimens. Overall, the data suggested that UCA1 may promote the invasion and EMT of bladder cancer cells by regulating the miR-143/HMGB1 pathway, which exhibits an important regulatory role in the pathology of bladder cancer.

Introduction

Bladder cancer is one of the most frequent malignancies in the world. Despite the significant advances in the development of diagnostic and therapeutic methods in recent years, patients with invasive bladder cancer still have a poor 5-year survival rate of less than 60% (1,2). Epithelial-mesenchymal transition (EMT), which is characterized by loss of cell adhesion and gain of migratory and invasive capability, is an important mechanism for metastatic potential and the initial stage of cancer metastasis, including bladder cancer (3,4).

Long non-coding RNAs (lncRNAs) are a class of noncoding regulatory RNA molecules that are longer than 200 nucleotides. Previous studies have focused on the regulatory roles of lncRNAs in several biological processes, such as chromosome inactivation, genomic imprinting and development (5-7). Recently, increasing reports have revealed that lncRNAs are frequently dysregulated in various human cancers, including bladder cancer (8,9). Of note, many dysregulated lncRNAs are involved in EMT and metastasis in human cancer. For example, high expression of the lncRNA PVT1 promotes invasion by inducing EMT in esophageal cancer (10), and increased lncRNA PANDAR expression indicates a poor prognosis for colorectal cancer and promotes metastasis by the EMT pathway (11). LncRNAs can also function as a competing endogenous RNA to sponge microRNAs (12-14). For example, the lncRNA H19 promotes EMT by functioning as a miRNA sponge to miR-138 and miR-200a in colorectal cancer (15). Therefore, by interacting with specific miRNAs, lncRNAs can play an important role in cancer development and progression.

UCA1 was first identified as an oncogenic lncRNA in bladder cancer (16). UCA1 is highly expressed in diverse cancer types, such as breast cancer (17), pancreatic cancer (18), gastric cancer (19), and colorectal cancer (20,21), suggesting that high expression of UCA1 might serve as a molecular marker for predicting metastasis and prognosis in these cancers (22). Several studies also demonstrated that upregulation of UCA1 is associated with EMT in breast cancer (23) and bladder cancer (24), but the underlying mechanism remains largely unknown.

HMGB1, a member of the high mobility group box (HMG-box) subfamily, is involved in many cancers (25,26). HMGB1 also functions as an inducer of EMT in human cancer cells (27-31). HMGB1 expression was much higher in bladder cancer cells than normal urethra epithelial cells and was associated with cell invasion (32,33). However, the mechanism of HMGB1 in bladder cancer is largely unknown.

MicroRNAs (miRNAs) play important roles during tumorigenesis, and dysregulated miRNAs are also involved in EMT in human cancer. In the past decades, the involvement of miRNAs in human bladder cancer has been widely studied. We previously reported that miR-143 acts as a tumor suppressor in human bladder cancer (34).

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In the present study, UCA1 and HMGB1 were upregulated and miR-143 was downregulated in bladder cancer specimens. Bioinformatics found that binding sites of the tumor suppressive miR-143 within UCA1 and the 3'UTR of HMGB1. And taken the functional examinations together, we hypothesis that UCA1 may promote the invasion and EMT of bladder cancer cells by regulating the miR-143/HMGB1 pathway.

Materials and methods

Tissue specimens. A total of 81 bladder tissue specimens, including 52 tumor tissues and 29 adjacent noncancerous tissues (at least 2.5 cm away from the tumor), were obtained from our hospital. All tissues were collected from bladder cancer at the time of radical cystectomy, snap-frozen in liquid nitrogen and stored at -70°C until use. All patients gave informed consent prior to collection of specimens according to institutional guidelines. The study was approved by the Institute Research Ethics Committee at Peking University Shenzhen Hospital.

Cell culture. The human bladder cancer cell lines (T24, 5637, J82, RT4 and HT1376) were purchased from China Academia Sinica Cell Repository (Shanghai, China) and cultured in DMEM (Invitrogen, Carlsbad, CA, USA) or RPMI 1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ incubator.

RNA isolation and qRT-PCR. Total RNA was extracted by TRIzol reagent (Invitrogen) and reverse transcribed using the Primer Script RT Reagent kit (Takara Bio, Tokyo, Japan). UCA1 and miR-381 expression levels were determined using the SYBR-Green qRT-PCR assay (BioRad, Berkeley, CA, USA) and mirVana qRT-PCR miRNA Detection kit (Ambion, Austin, TX, USA), respectively. GAPDH and U6 snRNA were used as endogenous controls for UCA1 and miR-143 relative expression, respectively. Primers used are as follows: UCA1-F, 5'-CTCTCCATTGGGTTACCATTC-3', UCA1-R, 5'-GCG GCAGGTCTTAAGAGATGAG-3'; HMGB1-F, 5'-ATCCCA ATGCACCCAAGAGGCCT-3', HMGB1-R, 5'-TTCGCAACA TCACCAATGGACAGG-3'; β -actin-F, 5'-AGGGGCCGGACT CGTCATACT-3', β -actin-R, 5'-GGCGGCACCACCATGTAC CCT-3'; miR-143-F, 5'-CCTGGCCTGAGATGAAGCAC-3', miR-143-R, 5'-CAGTGCTGGGTCCGAGTGA-3'; and U6-F, 5'-CTCGCTTCGGCAGCACA-3', U6-R, 5'-AACGCTTCA CGAATTTGCGT-3'. The relative levels were measured in triplicate using the 2^{- $\Delta\Delta C_q$} method (35).

Luciferase reporter assay. Fragments of the 3'UTR of HMGB1 and UCA1 containing miR-143 binding sites were amplified and cloned into the psiCHECK-2 reporter vector (Promega, Shanghai, China). The mutated versions of the 3'UTR of HMGB1 and UCA1 (with substitutions of four bases in the miR-143 seed binding sites) were generated using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). T24 cells were co-transfected with the reporter vector (0.5 μ g) and miR-143 mimics or scrambled mimics (50 nM) using Lipofectamine 2000 (Invitrogen). After 48 h, the Dual Luciferase Reporter assay system (Promega,

Fitchburg, WI, USA) was used to examine luciferase activity. MiR-143, scramble mimics and miR-143 inhibitor mimics were purchased from RiboBio (Guangzhou, China).

Vector construction and siRNA. To construct the UCA1 expression vector, we amplified UCA1 cDNA with flanking sequences (forward primer, 5'-ATGCACCTTGTGACT CCCTCCTCT-3'; reverse primer, 5'-CACCTCATCAGA CTGCCTTTGG-3') and cloned it into the pcDNA3.1 vector in BamHI/XhoI sites (Invitrogen). We used the following siRNAs for knockdown experiments: si-UCA1, 5'-GAGCCG AUCAGACAAACAAUU-3' (S), 5'-UUGUUUGUCUGAUCG GCUCUU-3' (AS); si-HMGB1, 5'-CCCGUUAUGAAAGAG AAAUUU-3' (S), 5'-AUUUCUCUUUCAUAAUGGGUU-3' (AS); si-NC (control), 5'-UUCGUCUGUACUCCACAUATT-3' (S), 5'-GAUGUCUUCUACAGUCCGATT-3' (AS). We used Lipofectamine 2000 Reagent (Invitrogen) for cell transfection.

Cell invasion assays. Cell invasion was assessed by Transwell assays with matrigel (BD Bioscience, San Diego, CA, USA). Briefly, 5x10⁴ cells in 200 μ l of serum-free media were added into the upper chamber and 500 μ l media with 10% fetal bovine serum were added to the bottom chamber. After 24 h, the cells that invaded through the membrane were fixed with 3% formaldehyde, stained with 0.5% crystal violet, and imaged under an inverted microscope (Olympus, Tokyo, Japan).

Western blot analysis. Total cellular extracts were prepared using lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). Approximately 50 μ g of total protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated with anti-E-cadherin (1:500), anti-N-cadherin (1:1,000) and anti-Vimentin (1:500) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by the HRP-conjugated secondary antibody (Santa Cruz Biotechnology). Signals were visualized using the ECL detection system (SuperSignal West Femto, Pierce, USA).

Statistical analysis. Statistical analyses were carried out using the SPSS 19.0 software (IBM SPSS, Armonk, NY, USA). Student's t-test was used to analyze experimental significance. Spearman's correlation analysis was used to analyze the correlation between miR-143 and UCA1 and HMGB1 expression.

Results

UCA1 modulated the invasion and EMT of bladder cancer cells. Previous studies revealed that UCA1 expression is associated with enhanced invasion ability of bladder cancer cells (24,36,37). We first investigated the relationship between the expression of UCA1 and the invasion ability of five bladder cancer cell lines. Comparison of Transwell assay results and qRT-PCR analysis of UCA1 expression showed that UCA1 expression was positively correlated with cell invasion ability (Fig. 1A-C). T24 cells showed stronger invasion capability and higher UCA1 expression levels, while RT4 cells showed relatively weaker invasion capability and lower UCA1 expression levels among the five tested bladder cancer cell lines. Knockdown of endogenous UCA1 in T24 cells by siRNA significantly (P<0.05) reduced the number of invading cells,

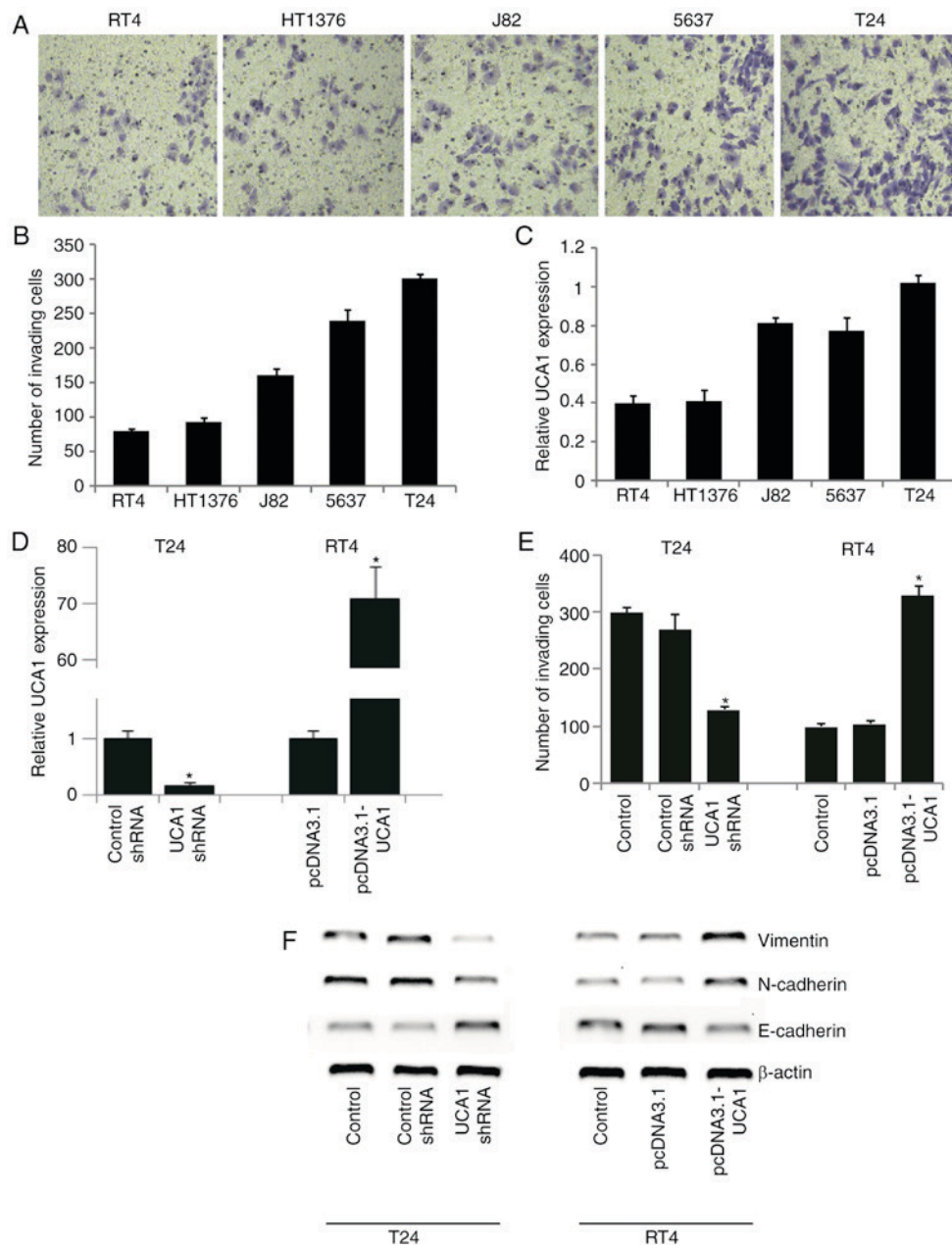


Figure 1. UCA1 modulated the invasion and EMT of bladder cancer cell lines. (A and B) Invasion capability of five bladder cancer cell lines was evaluated by Transwell assays. Crystal violet staining is shown in (A) and quantification of cells is shown in (B). (C) UCA1 mRNA expression levels in bladder cancer cell lines were evaluated by qRT-PCR. (D) T24 cells were transfected with UCA1 siRNA or control siRNA, and RT4 cells were transfected with UCA1-expressing pcDNA3.1 (pcDNA3.1-UCA1) or empty pcDNA3.1 vector (pcDNA3.1). Knockdown of UCA1 in T24 cells and overexpression of UCA1 in RT4 cells were validated by qRT-PCR. (E) Transwell assays in T24 and RT4 cells with UCA1 knockdown or overexpression. * $P < 0.05$ vs. the control. (F) Western blot analysis of EMT markers E-cadherin, N-cadherin and Vimentin in response to UCA1 knockdown or overexpression. UCA1, urothelial carcinoma associated 1; EMT, epithelial-mesenchymal transition.

while UCA1 overexpression in RT4 cells increased the number of invading cells significantly ($P < 0.05$) (Fig. 1D and E), suggesting that UCA1 is closely involved in the invasion ability of bladder cancer cells.

EMT is an important mechanism of enhanced invasion of bladder cancer cells. Therefore, we further investigated whether UCA1 modulated EMT in bladder cancer cells. The epithelial marker E-cadherin and mesenchymal markers N-cadherin and vimentin were examined by western blot analysis (Fig. 1F). UCA1 knockdown in T24 cells led to increased E-cadherin and decreased N-cadherin and vimentin, while UCA1 overexpression in RT4 cells inhibited E-cadherin and enhanced

N-cadherin and Vimentin protein expression. Together these results suggest that UCA1 may modulate invasion and EMT of bladder cancer cells.

UCA1 promoted the invasion and EMT of bladder cancer cells by regulating HMGB1. To investigate the potential mechanism by which UCA1 regulates EMT in bladder cancer cells, we examined the effect of UCA1 on the expression of HMGB1, which is reported to be associated with the invasion ability of many types of human cancers including bladder cancer (24). qRT-PCR showed that HMGB1 mRNA expression was significantly ($P < 0.05$) reduced by knockdown of

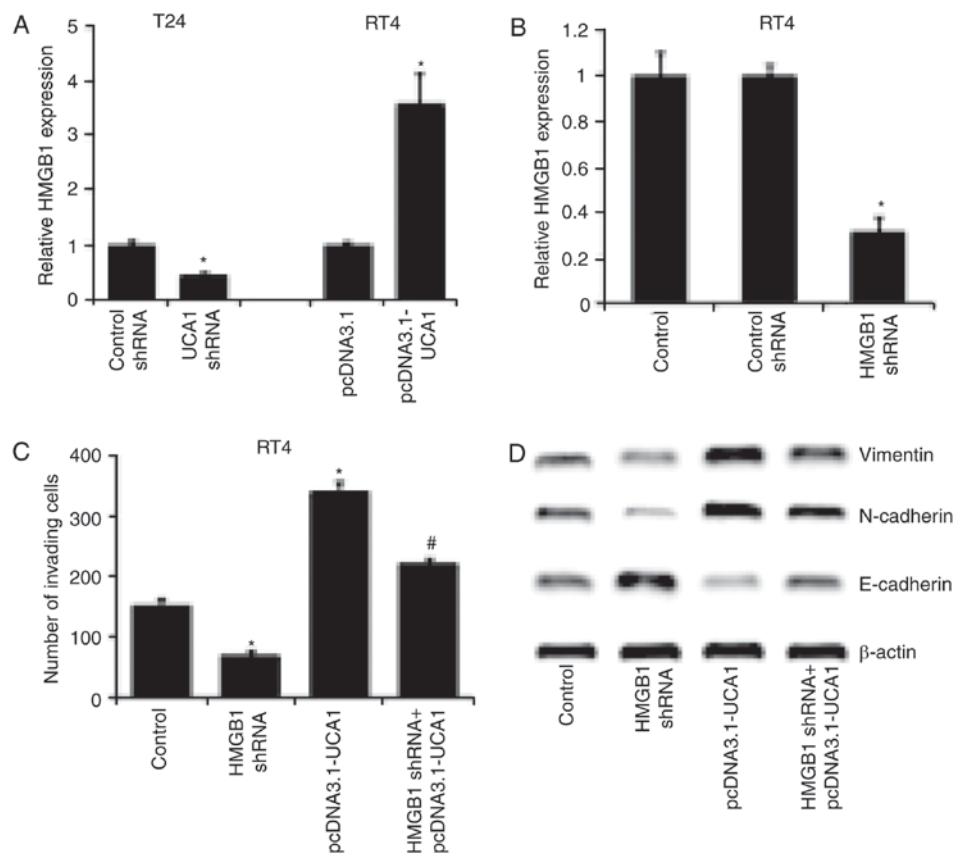


Figure 2. UCA1 promoted the invasion and EMT of bladder cancer cells by regulating HMGB1. (A) qRT-PCR analysis of HMGB1 mRNA expression in UCA1 knockdown and overexpressing T24 and RT4 cells, respectively. (B) Confirmation of HMGB1 knockdown using siRNA in RT4 cells by qRT-PCR. (C) Transwell assays in RT4 cells with HMGB1 knockdown (HMGB1 siRNA), UCA1 overexpression (pcDNA3.1-UCA1) or HMGB1 knockdown combined with UCA1 overexpression (HMGB1 shRNA+pcDNA3.1-UCA1). (D) Western blot analysis of EMT related markers E-cadherin, N-cadherin and Vimentin expression in RT4 cells with HMGB1 knockdown (HMGB1 siRNA), UCA1 overexpression (pcDNA3.1-UCA1) or HMGB1 knockdown combined with UCA1 overexpression (HMGB1 shRNA+pcDNA3.1-UCA1). * $P < 0.05$ vs. the control, # $P < 0.05$ vs. pcDNA3.1-UCA1. UCA1, urothelial carcinoma associated 1; EMT, epithelial-mesenchymal transition; HMGB1, high mobility group box 1.

UCA1 in T24 cells and increased by overexpression of UCA1 in RT4 cells (Fig. 2A). In addition, Transwell assays revealed that knockdown of HMGB1 by specific siRNA significantly inhibited the invasion of RT4 cells (Fig. 2B and C), similar to the effects of UCA1 knockdown. Of note, we found that knockdown of HMGB1 and overexpression of UCA1 significantly attenuated the effect of UCA1 on invasion and EMT of T24 cells (Fig. 2C and D). These results illustrated that UCA1 promoted the invasion and EMT of bladder cancer cells at least in part by regulating HMGB1.

UCA1 induced HMGB1 expression through directly interacting with miR-143. Bioinformatics revealed putative binding sites for the tumor suppressive miR-143 within UCA1 and the 3'UTR of HMGB1 (Fig. 3A). Luciferase reporter assay, which was a standard way to prove the direct interaction between microRNAs and target genes (38), was used to determine whether miR-143 targets UCA1 and the 3'UTR of HMGB1. We cloned the full-length UCA1 and a fragment of the HMGB1 3'UTR containing miR-143 binding sites into luciferase reporter plasmids (psiCheck-2) and co-transfected the reporter plasmids with miR-143 mimics or scrambled mimics (miR-NC) into T24 cells. Luciferase assays showed that miR-143 mimics significantly reduced the activity of psiCheck-2-UCA1 and psiCheck-2-HMGB1 (Fig. 3B). Of note,

mutations of the miR-143 binding sites in psiCheck-2-UCA1 and psiCheck-2-HMGB1 significantly abolished the effects on luciferase activity mediated by miR-143 mimics (Fig. 3B). In addition, miR-143 mimics significantly decreased UCA1 and HMGB1 expression, whereas miR-143 inhibitor mimics significantly increased UCA1 and HMGB1 expression in T24 cells (Fig. 3C). These data revealed that miR-143 could directly target and inhibit UCA1 and HMGB1 in T24 cells. We also observed that UCA1 overexpression in RT4 cells significantly repressed miR-143 expression in a dose-dependent manner (Fig. 3D). Taken together, our data suggest that UCA1 may act as a competing endogenous RNA of miR-143 and inhibit miR-143 to upregulate HMGB1 in bladder cancer cells.

UCA1 and HMGB1 were upregulated and miR-143 was downregulated in bladder tumors. To examine the clinical significance of UCA1, HMGB1 and miR-143 in human bladder cancer development, qRT-PCR was performed to evaluate the expression levels of UCA1, miR-143 and HMGB1 mRNAs in a cohort of 81 bladder tissue specimens including 52 tumor tissues and 29 adjacent noncancerous tissues. As shown in Fig. 4A, UCA1 and HMGB1 were significantly upregulated while miR-143 was significantly downregulated in tumors compared with adjacent noncancerous tissues. In addition, correlation analysis revealed that UCA1 and HMGB1

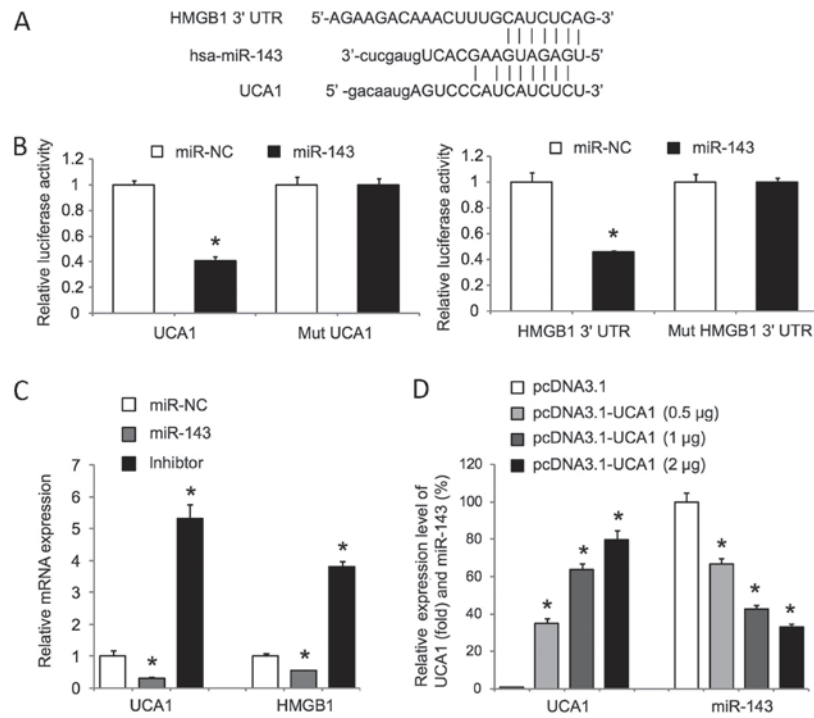


Figure 3. UCA1 induced HMGB1 expression through directly interacting with miR-143. (A) The putative binding sites of miR-143 in UCA1 and the 3'UTR of HMGB1. (B) Luciferase assays in T24 cells co-transfected with wild-type UCA1 (left) or HMGB1 3'UTR (right) luciferase vectors or mutant reporter vectors together with either miR-143 mimics (miR-143) or scrambled mimics (miR-NC). (C) qRT-PCR was used to detect the expression levels of UCA1 and HMGB1 mRNA in T24 cells transfected with scrambled mimics, miR-143 mimics or miR-143 inhibitor mimics. (D) qRT-PCR was performed to examine UCA1 and miR-143 mRNA expression levels in T24 cells transfected with pcDNA3.1 or pcDNA3.1-UCA1 expression plasmid at the indicated concentrations. *P<0.05. UCA1, urothelial carcinoma associated 1; EMT, epithelial-mesenchymal transition; HMGB1, high mobility group box 1.

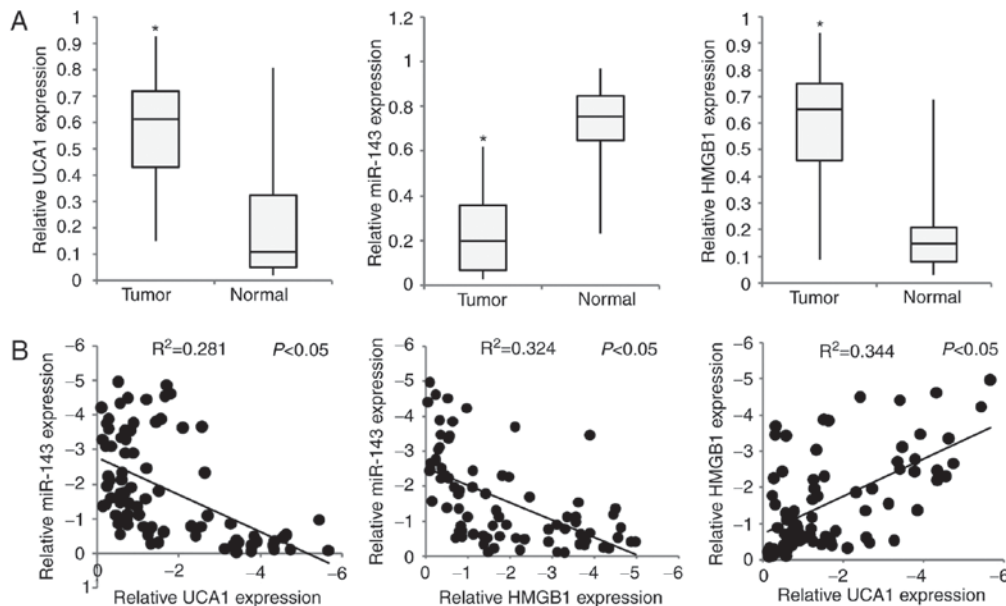


Figure 4. Expression levels of miR-143, UCA1 and HMGB1 in bladder cancer tissues. (A) Relative expression of miR-143, UCA1 and HMGB1 in 52 bladder tumor tissues (tumor) and 29 adjacent noncancerous tissues (normal). (B) Spearman's correlation analysis was carried out between miR-143, UCA1 and HMGB1 expression in 81 samples (52 bladder tumor tissues and 29 adjacent noncancerous tissues). *P<0.05. UCA1, urothelial carcinoma associated 1; EMT, epithelial-mesenchymal transition; HMGB1, high mobility group box 1.

expression levels had a positive correlation, but were both inversely correlated with miR-143 expression (Fig. 4B). Taken together, our observations suggest that dysregulation of the UCA1/miR-143/HMGB1 axis may be associated with the development and progression of bladder cancer.

Discussion

Increasing reports have revealed that dysregulated expression of lncRNAs is closely involved in the pathological development of a wide variety of human cancers including bladder cancer (8,9).

UCA1 is a sensitive and specific oncogenic lncRNA in bladder cancer (16). Consistent with previous observations that high UCA1 expression was involved in cell invasion in many cancers as well as bladder cancer (24,36,37), our results clearly showed that cells with stronger invasion capability had higher UCA1 expression levels in bladder cancer cell lines *in vitro*. EMT is a well-characterized process that facilitates invasion and metastatic dissemination of human cancers (3,4). Therefore, we further investigated whether UCA1 could modulate EMT of bladder cancer cells. Gain and loss of function analysis showed that UCA1 knockdown increased E-cadherin and decreased N-cadherin and vimentin, while UCA1 overexpression led to the opposite results. These data suggest that UCA1 may modulate cell invasion by promoting EMT in bladder cancer cells.

The HMGB family could be divided into 3 subgroups, which are HMGB1, HMGB2 and HMGB3 (39). HMGB1 is widely distributed in the lymphoid tissue, brain, liver, lung, heart, spleen, kidney tissues, and exists in most organizations in the nucleus (40); The HMGB2/3 distribution is limited. HMGB2 is mainly distributed in the testicular and lymphoid tissues (41,42), while HMGB3 is found mainly in embryos (43). This is why we chose HMGB1 as our candidate.

To investigate the potential mechanism by which UCA1 regulates EMT in bladder cancer cells, we examined the effect of UCA1 on the expression of HMGB1. We found that HMGB1 mRNA expression was significantly reduced by knockdown of UCA1 in T24 cells and increased by overexpression of UCA1 in RT4 cells, suggesting that UCA1 can positively regulate HMGB1 mRNA expression. Cellular function assays further revealed that knockdown of HMGB1 significantly inhibited the invasion of RT4 cells and attenuated the effect of UCA1 on invasion and EMT of T24 cells. These results revealed that UCA1 promoted the invasion and EMT of bladder cancer cells, at least in part by inducing HMGB1. And in order to look into the biological function of HMGB1 in bladder cancer, we might pursue another research on how the protein level of HMGB1 affected by UCA1.

lncRNAs interact with miRNAs to function as miRNA sponges or inhibitors and also modulate the repression of miRNA targets. Increasing reports have revealed that UCA1 functions as an oncogenic lncRNA by interacting with and inhibiting tumor suppressive miRNAs such as miR-507 (44), miR-145 (24), miR-16 (45) and miR-216b (46). Based on bioinformatics and luciferase reporter assays, we confirmed binding sites of the tumor suppressive miR-143 within UCA1 and the 3'UTR of HMGB1. In addition, we observed that UCA1 overexpression significantly repressed miR-143 expression in a dose-dependent manner in RT4 cells. Our observation was closely consistent with a recently published report demonstrating that UCA1 promoted bladder cancer cell migration and invasion by the hsa-miR145-ZEB1/2-FSCN1 pathway (24). Similar to miR-143, miR-145 was also reported to be a tumor suppressive gene and a negative regulator of EMT (47-49). Thus, UCA1 might function as an oncogene by sponging EMT-related miRNAs.

miR-143 was already known to act as a tumor suppressor in multiple cancers, including bladder cancer (34). According to the literature found in Pubmed, miR-143 was found to suppress EMT and cell invasion in multiple cancers, including spinal glioblastoma (50), esophageal squamous cell carcinoma (51), and breast cancer (52). Our research showed that UCA1 may

act as a competing endogenous RNA of miR-143 and inhibit miR-143 to upregulate HMGB1, result in promoting the invasion and EMT of bladder cancer cells. Taken together, we believe that miR-143 might also affect EMT in bladder cancer. Further studies were needed to look into the function of miR-143 on EMT and invasion in bladder cancer.

Expression analysis on clinical bladder cancer specimens also revealed that UCA1 and HMGB1 expression levels were positively correlated but were both inversely correlated with miR-143 expression. Taken together, our data suggest that UCA1 may act as a miRNA sponge to miR-143 and inhibit miR-143 to upregulate HMGB1 in bladder cancer cells. The limitation in this research was that this study only included *in vitro* models. Our future studies will pursue these findings in an *in vivo* model.

In conclusion, our study provided evidence that UCA1 promotes the invasion and EMT of bladder cancer by regulating the miR-143/HMGB1 pathway, which may play an important regulatory role in the pathology of bladder cancer.

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