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LncRNA LINC00355 promotes EMT and metastasis of bladder cancer cells through the miR-424-5p/HMGA2 axis

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Bladder cancer is a common malignant tumor with a high recurrence rate and mortality, while the detailed mechanisms for bladder cancer progression and metastasis are unknown. Recently, long non-coding RNAs (lncRNAs) have been reported to be involved in the development of cancers. In this study, we aim to investigate the role of lncRNA LINC00355 in bladder cancer progression and metastasis. The association between LINC00355 and the prognosis of bladder cancer patients was determined by Kaplan-Meier survival analysis. Cell migration and invasion ability were detected using the Transwell migration and invasion assay. The relationships of LINC00355, miR-424-5p, and High Mobility Group AT-Hook 2 (HMGA2) were verified through the luciferase assay and RNA pull-down assay. Xenograft tumor was established to evaluate tumor lung metastasis in vivo. qRT-PCR and western blot were used to detect gene expression. LINC00355 was upregulated in bladder cancer patients, especially in patients with higher TNM stage. Elevated LINC00355 was correlated with the poor prognosis of bladder cancer patients. Besides, overexpressed LINC00355 promoted migration, invasion, and epithelial-mesenchymal transition (EMT) ability of bladder cancer cells. Contrarily, decreased LINC00355 suppressed migration, invasion, and EMT ability of bladder cancer cells, and lung metastasis of xenograft tumors. Furthermore, LINC00355 could regulate HMGA2 expression by acting as a sponge for miR-424-5p. Overexpression of HMGA2 induced EMT of bladder cancer cells. Additionally, LINC00355 regulated the migration, invasion, and EMT ability of bladder cancer cells through modulating HMGA2 expression via sponging miR-424-5p. LINC00355 promoted migration, invasion, and EMT ability of bladder cancer through elevating HMGA2 expression via acting as a sponge for miR-424-5p.

Key words: bladder cancer, LINC00355, miR-424-5p, HMGA2

Bladder cancer is a frequently diagnosed tumor in urological systems [1]. Bladder cancer has the ninth incidence in malignant tumors and the second incidence in men [2]. In 2018, approximately 550,000 new cases were diagnosed and 20,000 cases died of bladder cancer [3, 4]. Bladder cancer affects over two million cases worldwide now [5]. At present, the primary therapeutic strategies for bladder cancer are surgery, chemotherapy, and radiation [6]. Although the progression has been made in bladder cancer therapy, the 5-year survival of bladder cancer patients is only 50–60% and the recurrence rate remains high due to cancer metastasis [6]. Therefore, it is imperative to investigate the potential molecular mechanism of bladder cancer development and metastasis and to explore the valuable therapeutic strategies for bladder cancer patients.

Increasing evidence has demonstrated that lncRNAs are participating in multiple biological functions [7–9]. For instance, lncRNA MIR31HG regulated the senescence-associated secretory phenotype [7]. LncRNA P53RRA promoted cell ferroptosis and apoptosis in cancer [8]. LncRNA NORAD could induce Pumilio phase separation, which was necessary for genomic stability [9]. Besides, lncRNAs have aberrant expression patterns in cancers and are involved in cancer development and metastasis [10]. For example, lncRNA MIR22HG presented elevated expression in glioblastoma, and interfering with MIR22HG suppressed glioblastoma progression [11]. LncRNA H19 was highly expressed in abdominal aortic aneurysm and knockdown of H19 limited abdominal aortic aneurysm development [12]. Downregulated GIAT4RA acted as a tumor suppressor in non-small cell lung cancer [13]. In bladder cancers, the vital roles of lncRNAs also emerged in recent years [14]. For example, lncRNA KCNQ1 opposite strand/antisense transcript 1 (KCNQ1OT1) was notably increased in bladder cancer,

and highly expressed KCNQ1OT1 regulated the malignant phenotypes of bladder cancer cells [15]. LncRNA PTENP1 was downregulated in bladder cancer tissues and exosomes from bladder cancer patients' plasma and exosomal PTENP1 could regulate cancer cell apoptosis, migration, and invasion ability [16]. Furthermore, accumulating evidence proved that lncRNAs modulated disease development through the competing endogenous RNA (ceRNA) mechanism [17]. The ceRNA hypothesis proposed that lncRNAs might function as ceRNAs to sponge the microRNAs (miRNAs), thereby alleviating the suppression influences of miRNAs on target mRNAs post-transcriptional level [18]. For instance, lncRNA differentiation antagonizing non-protein coding RNA (DANCR) promoted bladder cancer progression via sponge miR-149 to regulate the expression of MSI2 [19].

As a member of the lncRNAs family, lncRNA LINC00355 showed abnormal expression, it was proven to modulate development in some cancers [20–22]. In head and neck squamous cell carcinoma (HNSCC), LINC00355 was robustly increased in HNSCC, knockdown of LINC00355 suppressed HNSCC invasion, migration, and EMT ability *in vitro* and tumor growth *in vivo* through modulating HOXA10 via acting as the miR-195 sponge [20, 21]. In gastric cancer, LINC00355 was highly expressed in tumor tissues and cancer cells, which promoted the proliferation and invasion of gastric cancer cells *in vivo* and *in vitro* [21]. Besides, LINC00355 was also increased in colon cancer cells and overexpressed LINC00355 elevated IGFBP2 and GTF2B expression, thereby promoting the proliferation, invasion, migration, and chemotaxis ability of colon cancer cells [22]. However, the role of LINC00355 in bladder cancer remains unclear now.

Thus, this study aimed to investigate the effect of LINC00355 on bladder cancer progression and metastasis and explore the underlying mechanism of action.

Patients and methods

Clinical specimens and cell culture. A total of 59 bladder cancer patients undergoing surgical resection in the Suzhou First People's Hospital were enrolled in this study. None of the patients underwent preoperative chemoradiotherapy. Tumor tissues and adjacent normal tissue samples were collected and stored in liquid nitrogen until use. All patients signed informed consent. The Ethics Committee of the Suzhou First People's Hospital permitted the study.

Human normal bladder epithelial cell line SV-HUC-1 and bladder cancer lines T24, HT-1197, SW780, HT-1376, UM-UC-3, TCCSUP, KU1919, and VMCUB1 were acquired from American Type Culture Collection (ATCC). All cells were maintained in RPMI-1640 medium plus 10% FBS at 37 °C with 5% CO_2.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA extraction was conducted by TRIzol reagent and cDNA was produced using the cDNA Synthesis Kit (Sigma, St. Louis, MO, USA). Subsequently, the qPCR assay was performed using SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on an ABI 7300 System with the following program: 2 min at 95 °C; 40 cycles of 10 s at 95 °C, 30 s at 60 °C and 10 s at 72 °C; and 10 min at 72 °C for the final extension. The primers used in this study were: LINC00355: Forward 5'-TGGGTCTCCTCTGAGCTGTT-3', Reverse 5'-TGTCCTGTGTCCAGGATGAA-3' [23]; miR-424-5p: Forward 5'-ACACTCCAGCTGGGCAGCAGCAATTCATGT-3', Reverse 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTCAAAAC-3' [24]; HMGA2: Forward 5'-AAAGCAGCTCAAAAGAAAGCA-3', Reverse 5'-TGTTGTGGCCATTTCCTAGGT-3' [25]. *GAPDH* and *U6* were chosen as control genes for data normalization by the $2^{-\Delta\Delta Ct}$ method.

Cell transfection. The shRNA against LINC00355 (sh-LINC00355), miR-424-5p mimics, and inhibitors were acquired from Ribobio (Ribobio, Guangzhou, China). The transfection of sh-LINC00355, miR-424-5p mimics, and inhibitors into bladder cancer cells was performed using Lipofectamine 3000 (Thermo Scientific, Waltham, MA, USA) in line with the manufacturer's instructions. Lentiviruses expressing LINC00355, HMGA2, and corresponding control vectors were obtained from GenePharma (GenePharma, Shanghai, China) and were used for infection of bladder cancer cells for 48 h. The transfection efficiency was determined using qRT-PCR.

Transwell migration assay. To determine cell migration ability, bladder cancer cells (1×10^5) were plated into the upper chamber of Transwell with the serum-free medium. The lower chamber of Transwell was filled the complete medium. Cells were then cultured for 24 h at 37 °C with 5% CO_2. Afterward, the migrated bladder cancer cells were fixed using 4% paraformaldehyde and dyed with 0.1% crystal violet for 10 min. The migration cell number was recorded in five random fields under the light microscope.

Transwell invasion assay. To determine cell invasion ability, bladder cancer cells (1×10^5) were inoculated into the upper chamber of Transwell, which was pre-covered with Matrigel (BD Biosciences, USA), and cultured in the serum-free medium. The lower chamber of Transwell was filled with the complete medium. Cells were then cultured for 24 h at 37 °C with 5% CO_2. Afterward, the invaded bladder cancer cells were stained with Giemsa dye. The invasion cell number was recorded in five random fields under the light microscope.

Western blot. Proteins extraction was conducted in the presence of RIPA buffer (Sigma, St. Louis, MO, USA). The concentration of proteins was determined using a BCA kit (Sigma, St. Louis, MO, USA). Subsequently, proteins were subjected to SDS-PAGE and transferred to the PVDF membranes. After blocking the nonspecific binding using 4% non-fat milk, the membranes were incubated with anti-ZEB1 (ab 245283, 1:3000), E-cadherin (ab76055, 1:1000), Vimentin (ab137321, 1:3000), HMGA2 (ab97276, 1:3000), and GAPDH

(ab9485, 1:2500) antibodies (Abcam, Cambridge, MA, UK) at 4 °C for 12 h. Afterward, the membranes were further incubated with IgG H&L (HRP) (Abcam, Cambridge, MA, UK) for 2 h at room temperature. GAPDH was used as the control protein. The bands were visualized using the ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and quantified by ImageJ.

Xenograft assays in nude mice. Fifteen BALB/c nude mice aged 4 weeks were purchased from Beijing Laboratory Animal Research Center (Beijing, China). All mice were divided into three groups, including the sh-LINC00355-1 group, sh-LINC00355-2 group, shRNA negative control (sh-ctrl) group. HT-1376 cells were stably transduced with sh-LINC00355-1, sh-LINC00355-2, or sh-ctrl lentivirus. To generate xenograft tumors, all mice in the three groups were injected with HT-1376 cells via the tail vein. After 42 days of injection, all mice were euthanized and the lung tissues were resected to observe tumor metastasis. Afterward, the tumors in lung tissues were separated to perform immunohistochemistry. Animal experiments complied with the national and international regulations and policies and the Experimental Animal Ethics of the Suzhou First People's Hospital permitted the study.

Immunohistochemistry. After mice were euthanized, the tumor samples were collected, fixed using paraformaldehyde, embedded by paraffin, and cut into 4 μm sections. After that, the tissue sections were probed with anti-ZEB1 (ab245283, 1:200) and E-cadherin (ab76055, 1:200) antibodies at 4 °C for 12 h, followed by incubating with IgG H&L (HRP) secondary antibody. The sections were then dyed with the DAB (R&D Systems, Minneapolis, MN, USA). The expression levels of ZEB1 and E-cadherin were observed under a microscope.

Luciferase assay. The wild and mutant sequences of LINC00355 binding to miR-424-5p or wild and mutant 3'-UTR of HMGA2 were fused into the psiCHECK-2 vector (Promega, Madison, WI, USA). The cloned psiCHECK-2 plasmids and miR-424-5p mimics were transfected into HEK293T cells. After 48 h, the cells were collected to determine the relative luciferase activity using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

RNA pull-down assay. Biotinylated wild miR-424-5p, mutant miR-424-5p, and negative control (NC) were acquired from RiboBio (RiboBio, Guangzhou, China), and were then transfected into HEK293T cells. After 48 h, cell lysates were obtained and then incubated with Dynabeads M-280 Streptavidin (Invitrogen, USA) at 4 °C for 12 h. After the washing step using wash/binding buffer, the absorbed RNAs were collected to perform qRT-PCR.

Statistical analysis. All data are shown as mean ± standard deviation (SD) and processed by SPSS Statistics 22.0 (SPSS, Chicago, IL, USA). One-way ANOVA with LSD's post hoc test or Student's t-test assessed the differences among groups. The difference between tumor tissues and adjacent normal tissues was determined using the paired t-test. The survival of bladder cancer patients was determined using Kaplan-Meier analysis with the log-rank test. The difference was judged as statistically significant when $p<0.05$.

Results

LINC00355 was upregulated in bladder cancer patients and correlated with a poor prognosis. To investigate the effect of LINC00355 in bladder cancer, the expression of LINC00355 in 59 bladder cancer tissues was determined using qRT-PCR. It was observed that LINC00355 was highly expressed in tumor tissues compared to adjacent normal tissues ($p<0.01$, Figure 1A). Besides, Kaplan-Meier analysis revealed that the bladder cancer patients with high LINC00355 expression presented poor overall survival and recurrence-free survival ($p<0.05$, Figures 1B, 1C). Furthermore, the expression pattern of LINC00355 in TNM stages of bladder cancer patients was explored. Results showed that LINC00355 was significantly upregulated in advanced TNM III and IV stages ($p<0.05$, Figure 1D). Moreover, the expression of LINC00355 was also upregulated in bladder cancer cell lines, including T24, HT-1197, SW780, HT-1376, UM-UC-3, TCCSUP, KU1919, and VMCUB1, compared to normal bladder epithelial cell line SV-HUC-1 ($p<0.05$, Figure 1E). Therefore, LINC00355 was upregulated in bladder cancer patients and correlated with a poor prognosis.

Enforced LINC00355 expression promoted migration, invasion, and EMT of bladder cancer cells. T24 and SW780 cells presented elevated LINC00355 expression compared to normal bladder epithelial cells but lowest LINC00355 expression among the eight bladder cell lines (Figure 1E), therefore, T24 and SW780 cells were used to explore the effect of elevated LINC00355 on bladder cancer progression after transfected with lentiviruses expressing LINC00355. The transfection efficacy of lentiviruses expressing LINC00355 in T24 and SW780 cells was confirmed using qRT-PCR ($p<0.01$, Figure 2A). Thereafter, the action of enforced LINC00355 expression on bladder cancer cell aggressive behaviors was studied. Transwell migration assay revealed that overexpressed LINC00355 significantly increased the migration ability of T24 ($p<0.01$) and SW780 ($p<0.05$) cells (Figures 2B, 2C). Transwell invasion assay found that elevated LINC00355 greatly enhanced the invasion ability of T24 ($p<0.01$) and SW780 ($p<0.01$) cells (Figures 2D, 2E). Besides, western blot and qRT-PCR results identified the EMT markers ZEB1 and Vimentin were significantly upregulated in T24 and SW780 cells with LINC00355 overexpression ($p<0.01$, Figures 2F–2H). Furthermore, overexpression of LINC00355 suppressed the protein and mRNA levels of E-cadherin in T24 and SW780 cells ($p<0.05$, Figures 2F–2H). Thus, enforced LINC00355 expression promoted migration, invasion, and EMT of bladder cancer cells.

Silencing LINC00355 suppressed migration, invasion, EMT, and lung metastasis of bladder cancer cells. To further interpret the role of LINC00355 in bladder cancer progression, two shRNAs against LINC00355 were trans-

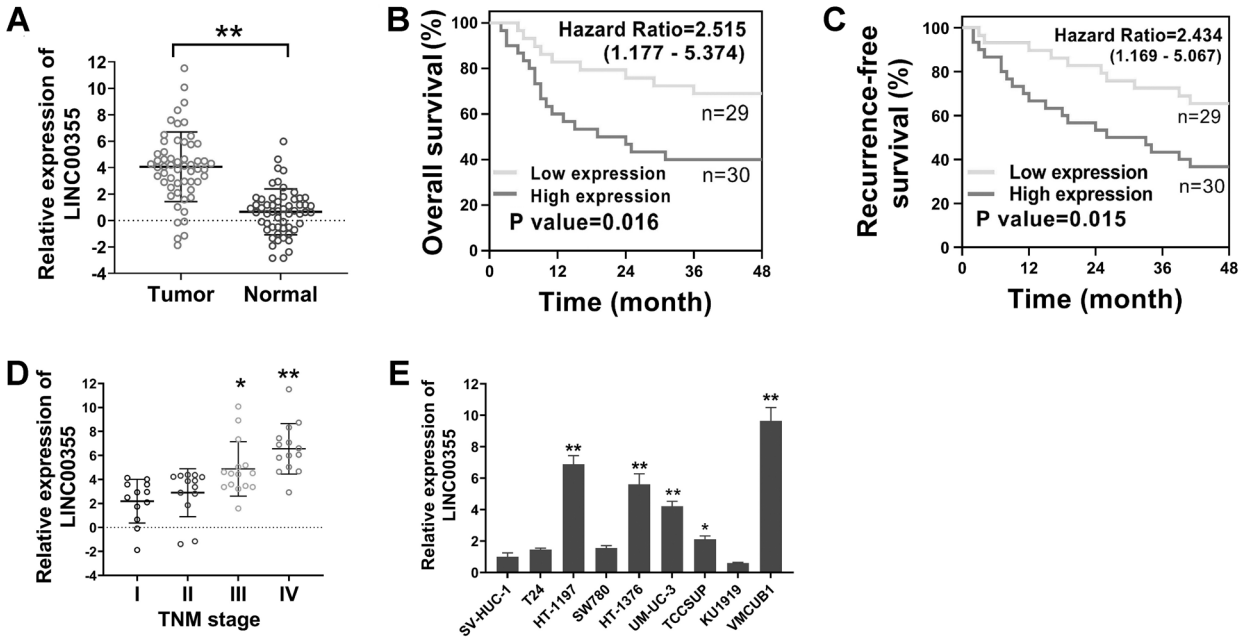

Figure 1. LINC00355 is upregulated in bladder cancer patients and correlated with a poor prognosis. A) Expression of LINC00355 in tissues and paired adjacent normal tissues of 59 bladder cancer patients was evaluated by qRT-PCR. B) The relationship of LINC00355 expression and overall survival of bladder cancer patients was analyzed using Kaplan-Meier survival analysis. C) The association of LINC00355 expression and recurrence-free survival of bladder cancer patients was analyzed using Kaplan-Meier survival analysis. D) Expression of LINC00355 in different TNM stages of bladder cancers was detected by qRT-PCR. E) Expression of LINC00355 in bladder cancer cell lines and normal bladder epithelial cell line was detected by qRT-PCR. *p<0.05; **p<0.01

fected into HT-1376 cells, which presented high LINC00355 expression among eight bladder cancer cell lines (Figure 1E). The transfection efficacies of two kinds of sh-LINC00355 were verified by qRT-PCR (p<0.01, Figure 3A). Thereafter, the influence of decreased LINC00355 on bladder cancer cell aggressive behaviors was explored. Results demonstrated that silencing LINC00355 significantly suppressed migration (p<0.05) and invasion (p<0.05) ability of HT-1376 cells (Figures 3B–3D). Besides, decreased LINC00355 inhibited the levels of ZEB1 (p<0.05) and Vimentin (p<0.01) but increased the level of E-cadherin (p<0.01) in HT-1376 cells (Figures 3E, 3F). Furthermore, HT-1376 cells transfected with lentivirus expressing sh-LINC00355 were injected into the mouse tail vein, then tumor lung metastasis was evaluated. Results showed that silencing LINC00355 suppressed the lung metastasis tumor numbers (p<0.05, Figures 3G, 3H). Moreover, the immunohistochemistry results showed that ZEB1 expression was downregulated and E-cadherin expression was increased in tumor tissues of mice transfected with sh-LINC00355 (Figure 3I). These results suggested that silencing LINC00355 suppressed migration, invasion, EMT, and lung metastasis of bladder cancer cells.

LINC00355 regulated HMGA2 expression by acting as a molecular sponge for miR-424-5p. LncRNAs usually regulate gene expression by acting as the miRNA sponges [18]. Bioinformatics analysis revealed that LINC00355 might target miR-424-5p, and the putative binding sites between LINC00355 and miR-424-5p are listed in Figure 4A. qRT-PCR results revealed that silencing LINC00355 significantly increased the expression of miR-424-5p, and overexpressed LINC00355 considerably decreased the expression of miR-424-5p (p<0.01, Figures 4B, 4C). Besides, the luciferase assay proved that overexpressed miR-424-5p significantly decreased wild-type LINC00355 reporter activity but did not affect the activity of mutant LINC00355 reporter (p<0.05, Figure 4D). In addition, RNA pull-down assay revealed a significantly higher enrichment level of LINC00355 using the wild biotinylated-miR-424-5p compared to mutant biotinylated-miR-424-5p (p<0.001, Figure 4E).

miRNAs usually exerted their roles via binding to target mRNAs. Bioinformatics analysis revealed that miR-424-5p might target HMGA2, and the putative binding sites were presented in Figure 4F. qRT-PCR results showed that overexpression of miR-424-5p inhibited the expression of HMGA2 (p<0.01, Figure 4G). Luciferase assay found that overexpressed miR-424-5p significantly decreased wild 3'-UTR of HMGA2 reporter activity but did not affect the activity of mutant 3'-UTR of HMGA2 reporter (p<0.05, Figure 4H). RNA pull-down assay revealed that wild biotinylated-miR-424-5p enriched a higher level of HMGA2 compared to mutant biotinylated-miR-424-5p (p<0.01, Figure 4I). Besides, we found that overexpression of LINC00355 significantly increased the mRNA and protein levels of HMGA2, which was reversed by overexpressed miR-424-5p (p<0.01,

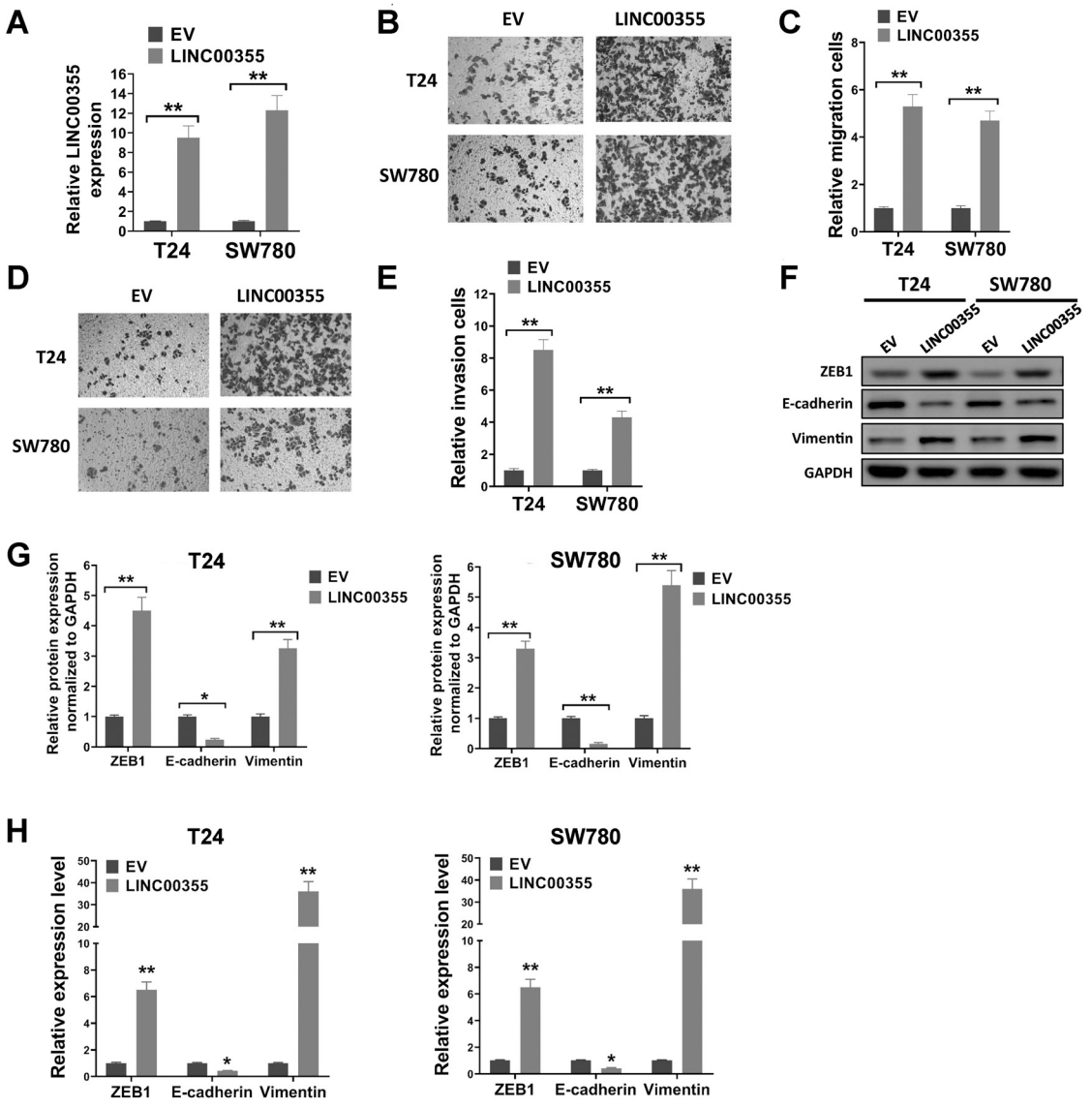

Figure 2. Enforced LINC00355 expression promotes migration, invasion, and EMT of bladder cancer cells. A) The transfection efficacy of expressing LINC00355 lentiviruses T24 and SW780 cells was confirmed using qRT-PCR. B, C) The migration ability of T24 and SW780 cells with overexpression of LINC00355 was determined using the Transwell migration assay. D, E) The invasion ability of T24 and SW780 cells with overexpression of LINC00355 was determined using the Transwell invasion assay. F, G) The protein levels of EMT markers ZEB1, E-cadherin, and Vimentin in T24 and SW780 cells with overexpression of LINC00355 were detected by western blot. H) The mRNA expression levels of EMT markers ZEB1, E-cadherin, and Vimentin in T24 and SW780 cells with overexpression of LINC00355 were detected by qRT-PCR. *p<0.05; **p<0.01

Figures 4J, 4K). Hence, LINC00355 regulated HMGA2 expression via acting as the miR-424-5p sponge.

Ectopic HMGA2 expression induced EMT of bladder cancer cells. To investigate the role of HMGA2 on bladder cancer cell aggressive behaviors, lentiviruses expressing HMGA2 were transfected into T24 and SW780 cells. The transfection efficacy was verified by western blot (Figure 5B). Overexpression of HMGA2 increased the mRNA and protein levels of ZEB1 and Vimentin but decreased the mRNA and protein levels of E-cadherin in T24 and SW780 cells (Figures 5A–5C). Taken together, ectopic HMGA2 expression induced EMT of bladder cancer cells.

HMGA2 overexpression or miR-424-5p knockdown abolished the effects of silencing LINC00355 on bladder cancer cells. In light of the relationship of LINC00355, miR-424-5p, and HMGA2 in the above findings, we inferred that miR-424-5p and HMGA2 might mediate the regulation of LINC00355 on bladder cancer progression. Therefore, miR-424-5p inhibitor or lentiviruses expressing HMGA2 were co-transfected with lentiviruses expressing

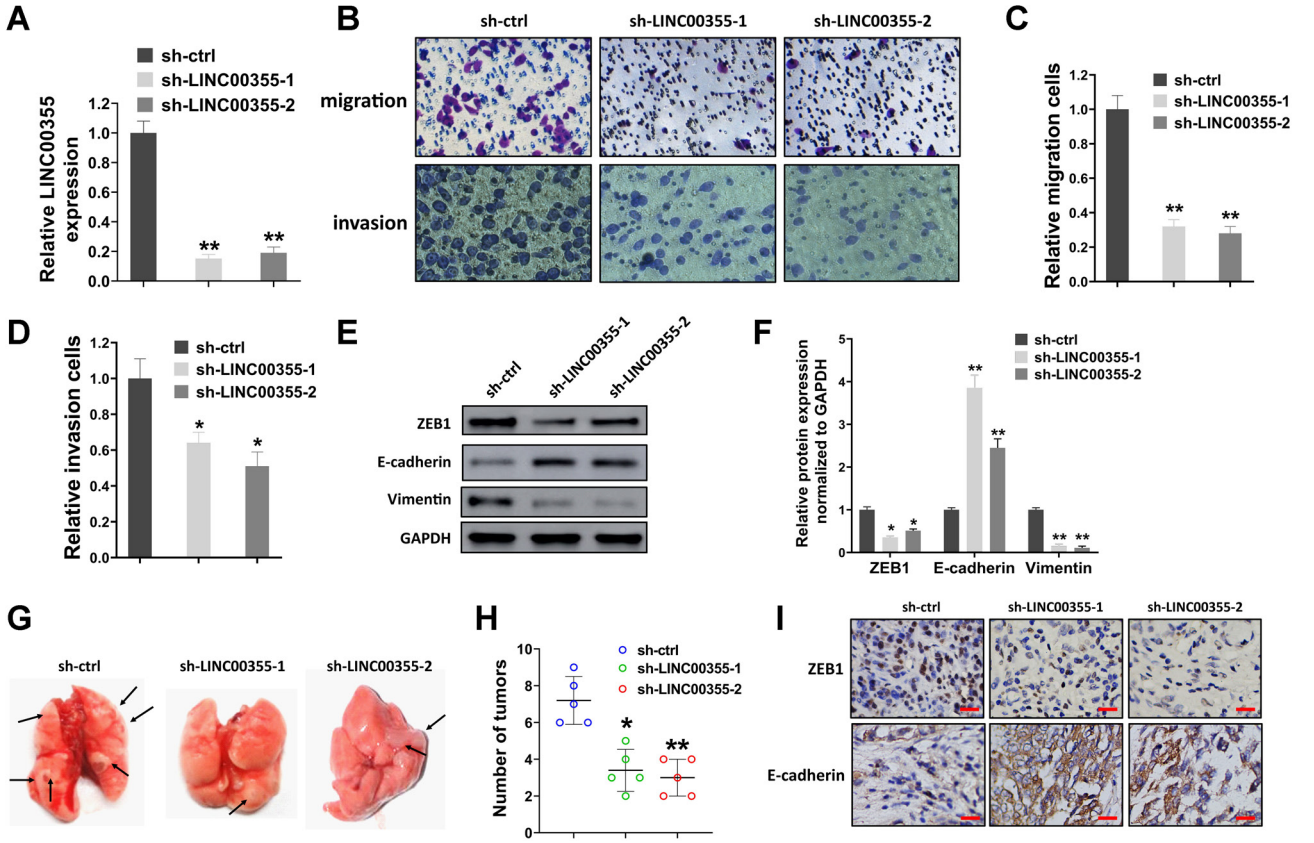

Figure 3. Silencing LINC00355 suppresses migration, invasion, EMT, and lung metastasis of bladder cancer cells. A) The transfection efficacy of expressing sh-LINC00355 lentiviruses in HT-1376 cells was confirmed using qRT-PCR. B–D) The migration and invasion ability of HT-1376 cells with silencing LINC00355 was determined using Transwell migration and invasion assay. E, F) The protein levels of EMT markers ZEB1, E-cadherin, and Vimentin in HT-1376 cells with silencing LINC00355 were detected by western blot. G, H) HT-1376 cells were transduced with sh-LINC00355-1, sh-LINC00355-2, or sh-ctrl lentivirus, cells were then injected into mouse tail vein, and tumor lung metastasis was evaluated. Represent images of metastasis tumors in the lung (G), and the number of tumors (H) are shown. I) The expression levels of ZEB1 and E-cadherin in lung metastasis tumor sections were detected by immunohistochemistry. *p<0.05; **p<0.01

sh-LINC00355 into HT-1376 cells. The transfection efficacy of the miR-424-5p inhibitor was detected using qRT-PCR ($p<0.01$, Figure 6A). Transwell assay found that silencing LINC00355 inhibited migration and invasion ability of HT-1376 cells, which was abolished by overexpressed HMGA2 or decreased miR-424-5p ($p<0.01$, Figures 6B, 6C). Besides, silencing LINC00355 inhibited ZEB1 and Vimentin levels but increased E-cadherin level in HT-1376 cells, which were reversed by overexpressed HMGA2 or decreased miR-424-5p ($p<0.05$, Figures 6B, 6C). Collectively, HMGA2 overexpression or miR-424-5p knockdown abolished the effects of silencing LINC00355 on bladder cancer cells.

Discussion

Bladder cancer was a common malignant tumor with high incidence and poor prognosis [1, 6]. It is essential to explore the mechanism of bladder cancer progression. Overwhelming evidence revealed that lncRNAs were involved in tumor development and metastasis in recent years [26, 27]. Previous studies reported that LINC00355 acted as an oncogene in some cancers such as HNSCC, gastric cancer, and colon cancer [20–22]. Therefore, we inferred that LINC00355 might modulate the development and metastasis of bladder cancer.

To investigate the role of LINC00355 in bladder cancer development, the expression of LINC00355 in bladder cancer was first explored. Our results revealed that LINC00355 was elevated in tumor tissues of bladder cancer and bladder cancer cells. These results were consistent with the findings reported in previous studies [20–22, 28]. The high expression of LINC00355 was reported in tumor tissues and cells of HNSCC, gastric cancer, colon cancer, lung adenocarcinoma [20–22, 28]. The study performed by Seitz et al. found that LINC00355 was increased in bladder cancer tissues using RNA sequencing [29]. Besides, the expression level of LINC00355 was increased with the advance of TNM stages. Contrary to these results, LINC00355 was not associated

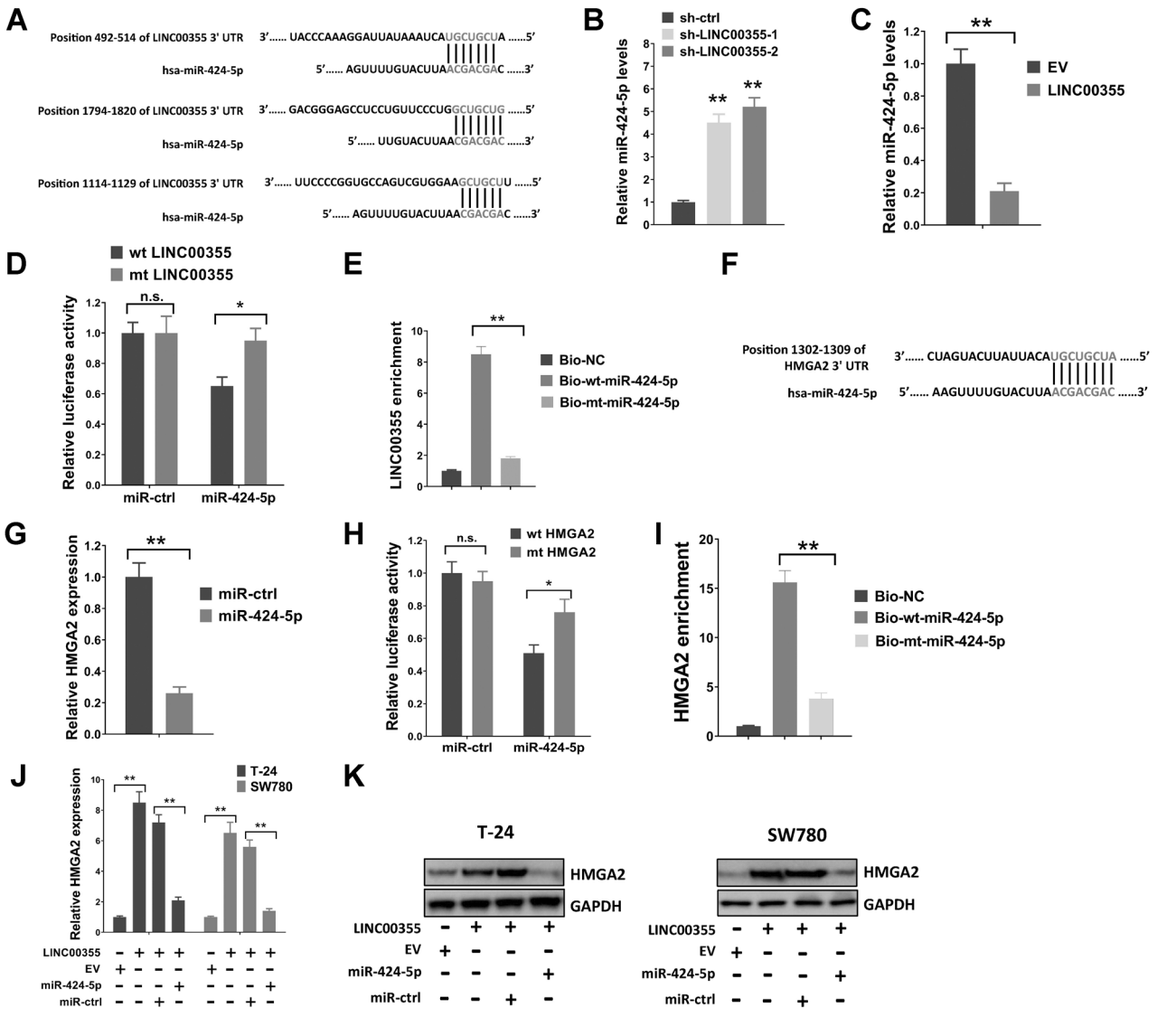

Figure 4. LINC00355 regulates HMGA2 expression by acting as a molecular sponge for miR-424-5p. A) The putative binding sites of LINC00355 and miR-424-5p were presented. B) Expression of miR-424-5p in HT-1376 cells with silencing LINC00355 was detected by qRT-PCR. C) Expression of miR-424-5p in T24 cells with overexpression of LINC00355 was detected by qRT-PCR. D) The relationship of LINC00355 and miR-424-5p was verified using luciferase assay. E) The relationship of LINC00355 and miR-424-5p was further determined by RNA pull-down assay. F) The putative binding sites of miR-424-5p and HMGA2 are shown. G) Expression of HMGA2 mRNA was detected by qRT-PCR with overexpression of miR-424-5p. H) The relationship of miR-424-5p and HMGA2 was verified using luciferase assay. I) The relationship of miR-424-5p and HMGA2 was further determined by RNA pull-down assay. J) The mRNA expression of HMGA2 in T24 and SW780 cells with overexpression of LINC00355 and miR-424-5p was detected by qRT-PCR. K) The protein levels of HMGA2 in T24 and SW780 cells with overexpression of LINC00355 and miR-424-5p were detected by western blot. *p<0.05; **p<0.01

with the TNM stage of colorectal cancer [30], which might be caused by differences in disease types. However, Zhao et al. revealed that the expression of LINC00355 was higher in TNM III and IV stages compared to I and II stages, which indicated that LINC00355 expression was positively associated with TNM stage [21]. Furthermore, we found that the bladder cancer patients with high LINC00355 expression presented poor overall survival and recurrence-free survival. Similarly, Zhao et al. found that gastric cancer patients with low expression of LINC00355 had more prolonged survival than patients with LINC00355 high expression [21]. Sun et al. reported that overexpressed LINC00355 was positively associated with poor overall survival in lung squamous cell carcinoma (LSCC) patients [31]. Taken together, LINC00355 is upregulated in bladder cancer patients and correlated with a poor prognosis.

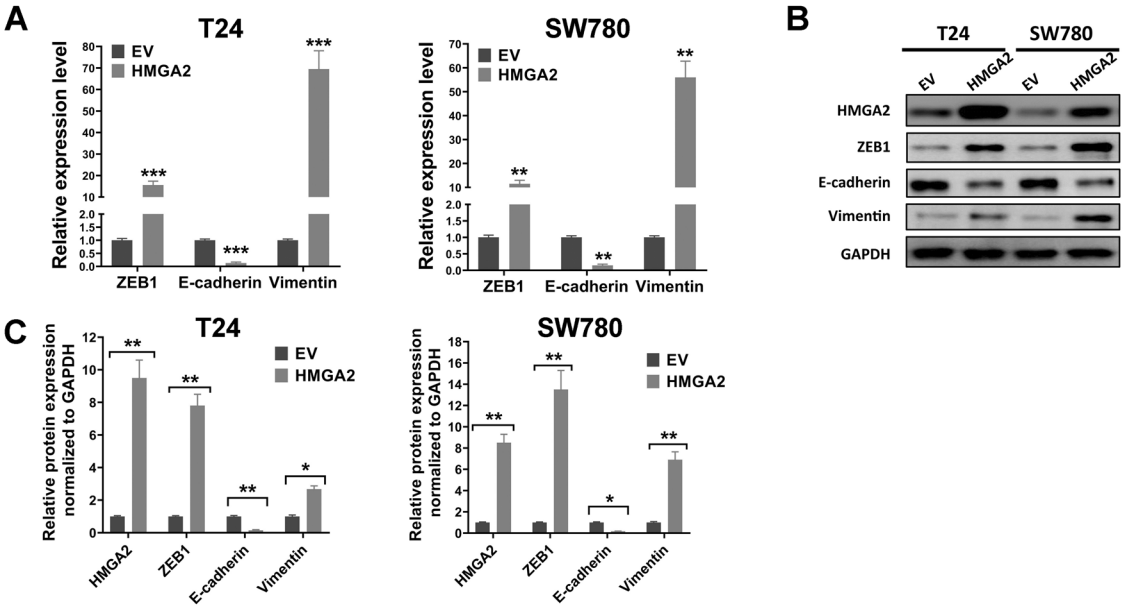

Figure 5. Ectopic HMGA2 expression induces EMT of bladder cancer cells. A) The expression levels of ZEB1, E-cadherin, and Vimentin in T24 and SW780 cells transfected with lentiviruses expressing HMGA2 were detected by qRT-PCR. B, C) The protein levels of ZEB1, E-cadherin, and Vimentin in T24 and SW780 cells transfected with lentiviruses expressing HMGA2 were detected by western blot. *p<0.05; **p<0.01

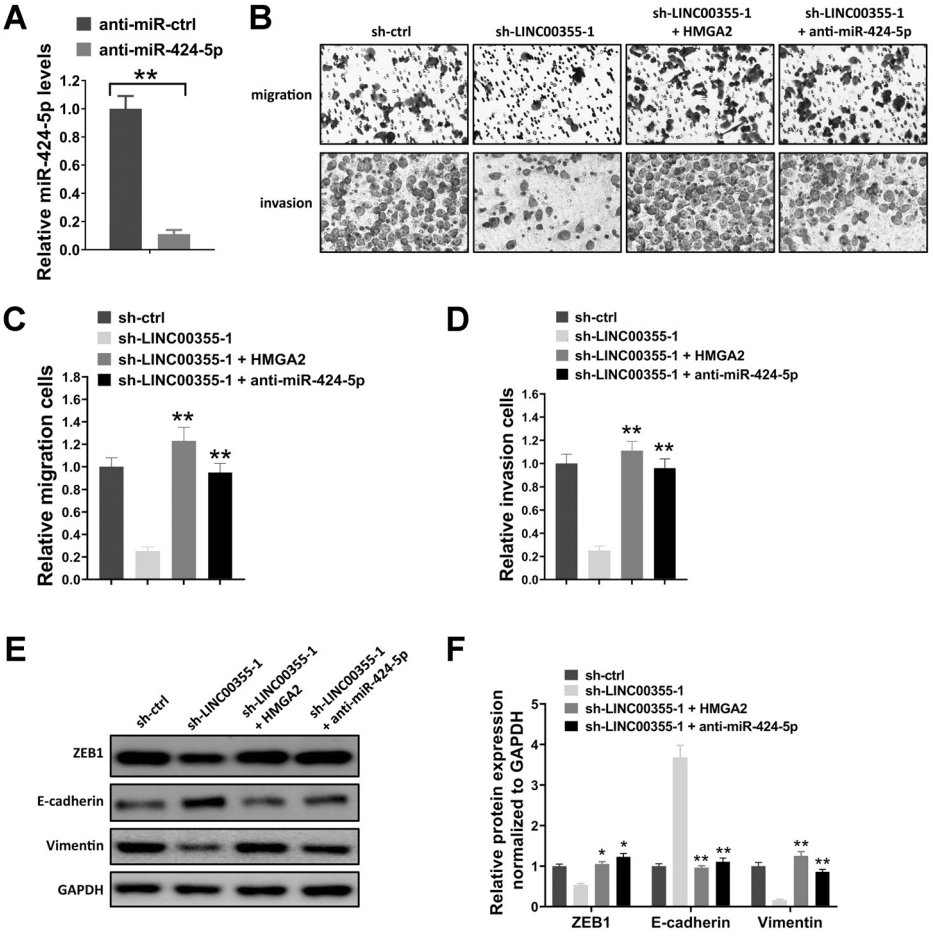

Figure 6. HMGA2 overexpression or miR-424-5p knockdown abolishes the effects caused by silencing LINC00355 in bladder cancer cells. A) The transfection efficacy of miR-424-5p inhibitor in HT-1376 cells was confirmed using qRT-PCR. B, C) The migration and invasion ability of HT-1376 cells with silencing LINC00355 and overexpressed HMGA2 or decreased miR-424-5p was determined using Transwell migration and invasion assay. D) The mRNA expression levels of EMT markers ZEB1, E-cadherin, and Vimentin in HT-1376 cells with silencing LINC00355 and overexpressed HMGA2 or decreased miR-424-5p were detected by qRT-PCR. E) The protein levels of EMT markers ZEB1, E-cadherin, and Vimentin in HT-1376 cells with silencing LINC00355 and overexpressed HMGA2 or decreased miR-424-5p were detected by western blot. *p<0.05; **p<0.01

The aberrant expression of LINC00355 in bladder cancer indicated that LINC00355 might modulate cancer progression in bladder cancer. Therefore, the expression of LINC00355 was changed in bladder cancer cells to further investigate the role of LINC00355 in bladder cancer development and metastasis. Gain-and-loss-of-function results revealed that overexpression of LINC00355 promoted migration, invasion, and EMT of bladder cancer cells, and downregulation of LINC00355 suppressed migration, invasion, EMT, and lung metastasis of bladder cancer cells. These results were consistent with the previous studies where elevated LINC00355 induced malignant phenotypes, including proliferation, migration, and invasion of gastric cancer cells [21]. In LSCC, knockdown of LINC00355 suppressed proliferation, migration, and invasion ability *in vitro* and tumor growth *in vivo* [31]. Yan et al. showed that cancer-associated fibroblasts (CAFs) exosome-mediated transfer of LINC00355 modulated bladder cancer cell proliferation and invasion [32]. However, the role of LINC00355 in regulating EMT and lung metastasis of bladder cancer was not reported in the study performed by Yan et al. Collectively, these results indicated that LINC00355 regulated migration, invasion, EMT, and lung metastasis of bladder cancer cells.

LncRNAs usually modulated disease development through the ceRNA mechanism [18]. Therefore, to explore the underlying mechanism of LINC00355 on bladder cancer, the putative miRNAs bound by LINC00355 were explored. Results identified that LINC00355 could target miR-424-5p, and LINC00355 could inhibit the expression of miR-424-5p. Up to now, this study was the first that reported that LINC00355 could target miR-424-5p. Besides, given miRNAs exerted functions in diseases through suppressing the mRNA gene expression by binding to 3'-UTR of the target gene [33]. Thus, the potential target genes bound by miR-424-5p were studied. Results identified that HMGA2 was a target of miR-424-5p, and miR-424-5p negatively regulated the expression of HMGA2. The study performed by Li et al. also revealed that miR-424-5p could target HMGA2 in mesangial cells [34]. Furthermore, our findings also revealed that LINC00355 regulated the expression levels of HMGA2, and miR-424-5p could abolish the regulation effects. These results suggested that LINC00355 regulates HMGA2 expression by acting as a molecular sponge for miR-424-5p.

To better investigate the potential mechanism of LINC00355 on bladder cancer, the role of HMGA2 in bladder cancer was first explored. Results indicated that overexpression of HMGA2 induced EMT of bladder cancer cells. These results were in agreement with the findings obtained by Hawsawi et al. [35]. They reported that overexpressed HMGA2 promoted EMT through regulating the MAPK pathway in prostate cancer [35]. HMGA2 also modulated EMT in gastric cancer through regulating TWIST1 expression [36]. Furthermore, we found that HMGA2 overexpression or miR-424-5p knockdown abolished the effects of silencing LINC00355 on bladder cancer cells. In other words, LINC00355 modulated bladder cancer cell aggressive behaviors via the miR-424-5p/HMGA2 axis. Besides, these results also indicated that miR-424-5p functioned as a tumor suppressor and HMGA2 acted as an oncogene in bladder cancer. In previous studies, miR-424-5p was proven to exert the function of a tumor suppressor [37]. For example, miR-424-5p suppressed cell proliferation and cell cycle via targeting PD-L1 and modulating the PTEN/PI3K/AKT/mTOR signaling pathway in breast cancer [37]. Besides, miR-424-5p suppressed the metastasis and invasion ability of intrahepatic cholangiocarcinoma via binding to ARK5 [38]. HMGA2 is a non-histone architectural transcription factor, and accumulating evidence reported that HMGA2 was elevated in tumor tissues, and it could function as an oncogene to promote cancer progression [39–41]. Mansoori et al. demonstrated that HMGA2 was overexpressed in breast cancer tissues, and elevated HMGA2 promoted proliferation, migration, invasion, and stemness capacity of breast cancer cells [42]. Sun et al. suggested that HMGA2 regulated CD44 expression levels to aggravate gastric cancer cell motility and sphere formation ability [43]. Especially, HMGA2 was highly expressed in bladder cancer tissues, which correlated with poor survival in chemo-treated bladder cancer patients [44]. Hence, these results indicated that LINC00355 promoted migration, invasion, and EMT ability of bladder cancer through elevating HMGA2 expression via acting as a sponge for miR-424-5p.

In conclusion, this study revealed that LINC00355 was upregulated in bladder cancer. Besides, LINC00355 regulated HMGA2 expression by acting as a molecular sponge for miR-424-5p. LINC00355 regulated migration, invasion, and EMT ability of bladder cancer through modulating HMGA2 expression via functioning as the miR-424-5p sponge. Therefore, LINC00355 may be a promising target for bladder cancer therapy.

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