

Upregulated GAPLINC predicts a poor prognosis in bladder cancer patients and promotes tumor proliferation and invasion

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Abstract. Previous studies have demonstrated that long noncoding RNAs (lncRNAs) exhibit critical regulatory roles in cancer biology. However, few lncRNAs have been well characterized in bladder cancer. In the previous study, we demonstrated that gastric adenocarcinoma associated, positive CD44 regulator, long intergenic noncoding RNA (GAPLINC) was significantly upregulated in bladder cancer tissues compared with normal tissues in The Cancer Genome Atlas (TCGA) cohort (P=0.039) and a validated cohort of 80 patients with bladder cancer (P=0.021). Statistical analysis revealed that GAPLINC expression level was associated with tumor stage in the validated cohort (P=0.017). Kaplan-Meier analysis demonstrated that patients in the high GAPLINC expression group had a worse overall survival (P=0.0386), indicating that GAPLINC may be a sensitive prognostic biomarker for patients with bladder cancer. Furthermore, knockdown of GAPLINC inhibited cell proliferation and colony formation, promoted cells cycle arrest at G1 phase and suppressed cells migration and invasion. The findings of the present study suggest that GAPLINC exhibits an oncogenic role in bladder cancer and may be a potential prognostic biomarker and therapeutic target.

Introduction

Bladder cancer is one of the most common malignant tumors (1). It is estimated that bladder cancer accounts for 38,600 new cases and causes ~15,000 mortalities worldwide annually (2). Approximately 75% of the cases are non-muscle-invasive bladder cancer (NMIBC), which commonly recurs but rarely progresses. However, the remaining cases are muscle-invasive bladder cancer (MIBC), which have a worse prognosis and ultimately endanger the lives of patients (3,4). At present, little is known regarding the molecular mechanisms of bladder cancer, and no sensitive prognostic biomarker has been identified (5). Therefore, it is imperative to investigate the underlying mechanism and identify novel prognostic biomarkers of bladder cancer.

Long noncoding RNAs (lncRNAs) are an important type of noncoding RNA (ncRNA); they are >200 nucleotides in length but lack protein-coding capacity (6). An increasing amount of evidence has demonstrated that lncRNAs exhibit crucial regulatory roles in the biological processes of cancer, including genomic imprinting, chromatin modification and post-transcriptional processing (7-10). Increasingly, studies have indicated that lncRNAs may be used as potential prognostic biomarkers and therapeutic targets (11,12). Gastric adenocarcinoma-associated, positive CD44 regulator, long intergenic noncoding RNA (GAPLINC) has been demonstrated to be aberrantly expressed in gastric and colorectal cancer (13,14). However, the clinical significance and biological functions of GAPLINC in bladder cancer remain unknown.

In the present study, GAPLINC expression level in bladder cancer tissues was investigated using RNA sequencing data from The Cancer Genome Atlas (TCGA) database the result was validated in a cohort of 80 pairs of bladder cancer tissues and normal adjacent tissues. The present study aimed to determine the relationship between GAPLINC expression and the prognosis for patients with bladder cancer, and explore the role of GAPLINC in bladder cancer cell proliferation, cell cycle, migration and invasion.

Materials and methods

Patients and tissue samples. A total of 80 paired bladder cancer and adjacent normal tissue samples were collected from patients

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Abbreviations: lncRNAs, long noncoding RNAs; GAPLINC, gastric adenocarcinoma associated, positive CD44 regulator, long intergenic noncoding RNA; NMIBC, non-muscle invasive bladder cancer; MIBC, muscle-invasive bladder cancer; TCGA, The Cancer Genome Atlas; TANRIC, The Atlas of Noncoding RNAs in Cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNAs, small interfering RNAs

Key words: long noncoding RNA, bladder cancer, prognosis, proliferation, invasion

who underwent cystectomy in Sun Yat-sen Memorial Hospital of Sun Yat-sen University (Guangzhou, China). All tissue samples were collected with written consent from the patients and were approved by the Hospital Ethics Review Committee. All tissues samples were stored in RNA later (Ambion; Thermo Fisher Scientific, Waltham, MA, USA) at -80°C until extraction. All tissue samples were graded according to the World Health Organization (WHO) grading system and were staged according to the tumor-node-metastasis (TNM) classification system (15). The patients were divided into a high (n=40) and low (n=40) GAPLINC expression groups (n=40) according to GAPLINC expression. The median value of GAPLINC expression was used as the cut-off value.

TCGA bladder cancer RNA sequencing data. TCGA database contains RNA sequencing data for multiple types of cancer. The RNA sequences of 251 bladder cancer tissue and 19 normal tissues were downloaded from the Atlas of Noncoding RNAs in Cancer (TANRIC) database (http://ibl.mdanderson.org/tanric/_design/basic/index.html) (16).

Cell culture and small interfering RNA (siRNA) transfection. The human bladder cancer cell lines UM-UC-3, T24, J82, 5637, RT4 and the normal urothelium cell line SV-HUC-1 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). T24 and 5637 were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) in a humidified incubator with 5% at 37°C. UM-UC-3, J82 and RT4 were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Little Chalfont, UK) in a humidified incubator with 5% CO₂ at 37°C. siRNAs were obtained from Shanghai GenePharma Co., Ltd. (Shanghai GenePharma Co., Ltd., Shanghai, China). siRNA transfection was conducted using Lipofectamine® RNAiMAX Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. In total, 5 µl siRNA per well was used in each transfection in a 6-well plate. The siRNA sequences were as follows: Si#1 for GAPLINC, 5'-GCAGGGUAUGCACAGAUGUTT-3', and Si#2 for GAPLINC 5'-GGACAGAGGCCAGAACAATT-3'. Negative control siRNA for GAPLINC 5'-UUCUCCGAA CGUGUCACGUTT-3'.

RNA extraction and reverse transcription-quantitative polymerase chain reaction RT-qPCR. Total RNA of UM-UC-3, T24, J82, 5637 and RT4 cancer cells was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using PrimeScript RT Master Mix (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. RT was performed with the following protocol: 37°C for 15 min, 85°C for 5 sec. With the cDNA of cancer cells (UM-UC-3, T24, J82, 5637, RT4), we performed qPCR to examine the gene expression using SYBR-Green PCR Master mix (Roche Diagnostics, Basel, Switzerland) in a LightCycler 96 Real-Time PCR instrument (Roche Diagnostics, Basel, Switzerland). Relative gene expression level was determined using β-actin as a normalizer. The qPCR reaction included a preincubation step (95°C for 300 sec), 40 cycles of amplification

step (95°C for 15 sec, 56°C for 15 sec and 72°C for 15 sec), a melting step (95°C for 10 sec, 65°C for 60 sec, 97°C for 1 sec) and a cooling step (37°C for 30 sec). The sequences of primers were as follows, β-actin forward, 5'-ACTGGAACGGTGAAG GTGAC-3'; β-actin reverse, 5'-AGAGAAGTGGGGTGG CTTTT-3'. GAPLINC forward, 5'-TGGACTCAGGCACGT TTACAG-3'; and GAPLINC reverse, 5'-TCATTGTTCTGG CCTCTGTCC-3'. All fold changes were calculated using the comparative Cq (2^{-ΔΔCq}) method (17).

Protein extraction and western blot analysis. Cells were washed and lysed in RIPA buffer containing fresh protease and phosphatase inhibitor cocktails (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total protein was extracted according to the manufacturer's protocol. The Bio-Rad assay system (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure the total protein concentration. For western blot analysis, equal amounts of protein (30 µg/well) was separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Blotted membranes were blocked in 5% skimmed milk in TBST, followed by incubation with following primary antibodies overnight at 4°C: Cyclin D1 (1:1,000; cat. no. 2922; Cell Signaling Technology, Inc., Danvers, MA, USA), CDK4 (1:1,000; cat. no. 12790; Cell Signaling Technology, Inc.), p18 (1:1,000; cat. no. 2896; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.). Membranes were then washed with TBST and were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (catalog no., sc-2004, 1:5,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h. The bound antibodies were detected using enhanced chemiluminescence kit (EMD Millipore, Billerica, MA, USA) with Genesys 2.0 (Syngene Europe, Cambridge, UK). GAPDH and β-tubulin were used as internal controls.

Flow cytometric analysis of cell cycle. A flow cytometric assay was conducted to analyze the cell cycle of bladder cancer cells. At 48 h after siRNA transfection, cells were collected, washed with PBS, fixed with 7% ice-cold ethanol overnight, and stained with propidium iodide (PI). The cell cycle was detected by FACSVerse™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell proliferation assay. One Solution Cell Proliferation assay (Promega Corporation, Madison, WI, USA) and a colony formation assay were conducted to determine the cell proliferation. For One Solution Cell Proliferation assay, 1x10³ cells were planted in 96-well plates with >5 replicate wells. Absorbance was measured with the multifunctional microplate reader SpectraMax M5 (Molecular Devices, LLC, Sunnyvale, CA, USA) at 490 nm. The measurement of absorbance was conducted every 24 h for a total of 5 days. For the colony formation assay, 1x10³ cells were plated in 6-well plates at 48 h after RNA transfection and cultured in corresponding medium. After 1 week, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 30 min, and then stained with 0.1% crystal violet for 30 min for visualization and counting.

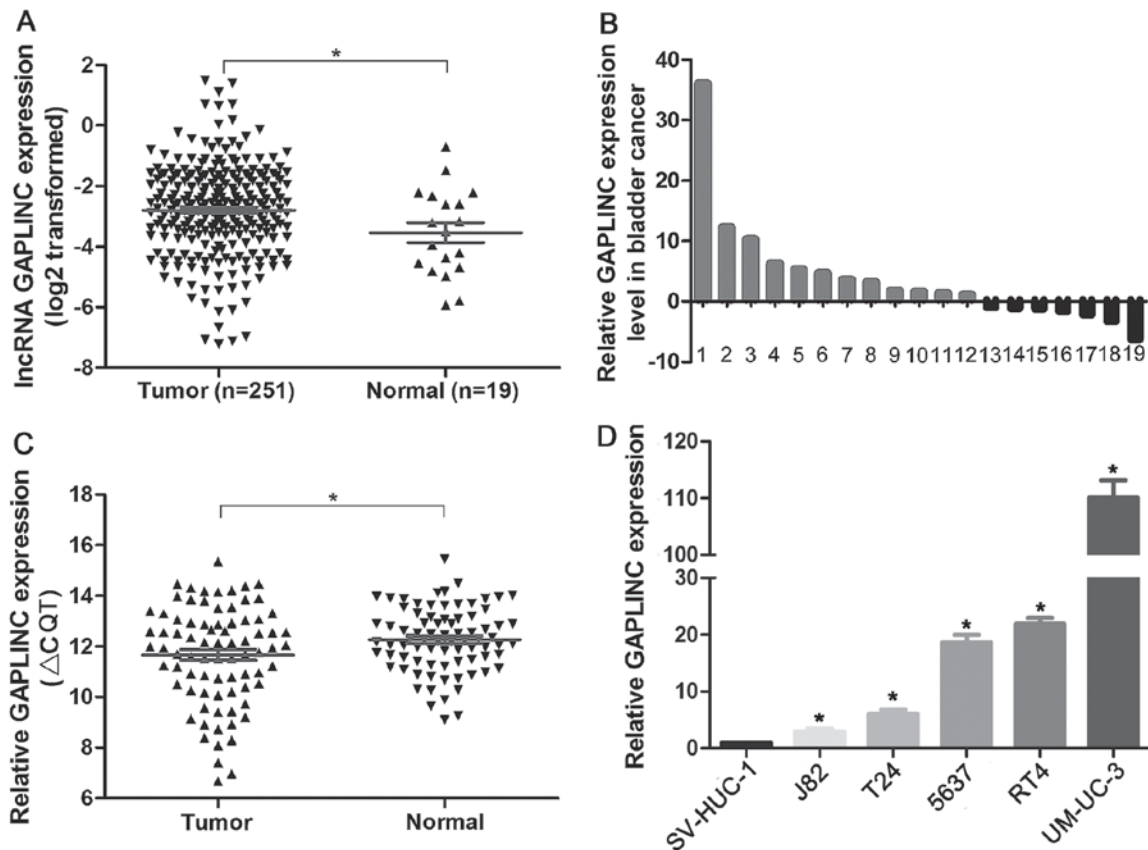


Figure 1. GAPLINC is upregulated in bladder cancer tissues and cell lines. (A) GAPLINC expression in bladder cancer tissues in TCGA cohort (* $P < 0.05$ vs. normal). (B) GAPLINC expression in 19 bladder cancer tissues in TCGA cohort. Data are represented as fold changes (cancer vs. normal tissues) (C) GAPLINC expression in bladder cancer tissues in the validated cohort (* $P < 0.05$ vs. normal). (D) GAPLINC expression in bladder cancer cell lines. (* $P < 0.05$ vs. SV-HUC-1), data are presented as means \pm SD. Statistical analysis was conducted by Student's t-test in Figs. 1A-C and by one-way ANOVA in Fig. 1D. GAPLINC, gastric adenocarcinoma associated, positive CD44 regulator, long intergenic noncoding RNA; TCGA, The Cancer Genome Atlas; SD, standard deviation; IncRNA, long noncoding RNA; Cq, quantification cycle.

Wound healing assay. The cells were plated into 6-well plates at 48 h after RNA transfection. After 24 h, the transfected cells were wounded with a 200 μ l pipette tip. Following scratching, the cells were cultured in serum-free medium (Gibco; Thermo Fisher Scientific, Inc.). Images of the wound were captured at 0 and 24 h with inverted microscope (x100). The wound closure rate (%) = migrated cell surface area/total surface area x100.

Cell migration and invasion assay. The migration assay was performed using 24-well Transwell insert chambers (Corning Incorporated, Corning, NY, USA) with 8- μ m pore size polycarbonate filters. Approximately 1×10^4 cells were seeded into the upper chamber with serum-free medium and the lower chamber was filled with 600 μ l medium with 10% FBS. For the invasion assay, Matrigel Invasion Chambers in the 24-well plates (BD Biosciences, Franklin Lakes, NJ, USA) were used. A total of 1×10^5 cells were seeded into the upper chamber with serum-free medium and the lower chamber was filled with 600 μ l medium with 10% FBS. After 24 h, cells in upper chambers were removed, and migratory or invasive cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The number of migratory or invasive cells was counted in 5 randomly selected fields under an inverted fluorescence microscope (x100, magnification).

Statistical analysis. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference. The significance of the differences was analyzed by the Student's t-test or χ^2 test when only two groups were compared. The significance of the differences was analyzed by one-way analysis of variance (ANOVA) using Bonferroni post hoc test in the case of multiple comparisons. Kaplan-Meier analysis was used to analyze the patients' survival. All the experiments were performed a minimum of three times.

Results

Upregulation of GAPLINC in bladder cancer and cell lines. To investigate the GAPLINC expression level in bladder cancer tissues and normal tissues, we first analyzed the RNA sequencing data from TCGA database was initially analyzed. The results demonstrated that GAPLINC was significantly upregulated in 251 bladder cancer tissues compared with 19 normal tissues (Fig. 1A and B; $P = 0.039$). To validate the findings from TCGA database, the present study further examined the GAPLINC expression level in 80 pairs of bladder cancer tissues and adjacent normal tissues by RT-qPCR, using β -actin as a reference gene (Fig. 1C; tumor vs. normal $P = 0.021$). The

Table I. Association between GAPLINC expression and clinical characteristics (n=80).

Characteristics	Cases	GAPLINC expression		χ^2	P-value
		Low	High		
Age, years				0.058	0.809
<60	25	12	13		
>60	55	28	27		
Sex				0.032	0.858
Female	15	7	8		
Male	65	33	32		
Tumor size (cm)				0.056	0.813
<3	27	14	13		
>3	53	26	27		
T stage				5.689	0.017 ^a
T1	26	18	8		
T2-4	54	22	32		
N stage				2.451	0.117
N0	68	37	31		
N1	12	3	9		
Grade				0.069	0.793
Low	19	10	9		
High	61	30	31		

^aDenotes statistical significance (P<0.05). GAPLINC, gastric adenocarcinoma associated, positive CD44 regulator, long intergenic noncoding RNA; T, tumor; N, node.

results supported a strong association between the RT-qPCR results and the TCGA database analysis. Furthermore, the expression pattern of GAPLINC in bladder cancer cell lines (UM-UC-3, T24, J82, RT4 and 5637) and a normal urothelium cell line SV-HUC-1 were determined. Similar to the changes in tissue samples, the results indicated the GAPLINC expression level was significantly higher in bladder cancer cell lines than in normal urothelial cells (Fig. 1D). Based on the expression pattern of GAPLINC, we hypothesized that GAPLINC may be a potential oncogene in bladder cancer.

Association between GAPLINC expression and clinical characteristics. The association between GAPLINC expression and clinical characteristics of patients with bladder cancer was analyzed (Table I). According to the cut-off value of GAPLINC expression, bladder cancer patients were classified into the high (n=40) or the low GAPLINC expression groups (n=40). Statistical analysis revealed that GAPLINC expression level was associated with tumor stage. The GAPLINC expression level was significantly higher in MIBC tissues compared with that in NMIBC tissues (Fig. 2A; P=0.017). However, no significant association was identified between GAPLINC expression and age, sex, tumor size, N stage or tumor grade. Additionally, Kaplan-Meier analysis demonstrated that patients in the high GAPLINC expression group had significantly shorter overall survival times than patients in the low GAPLINC expression group (Fig. 2B; P=0.0386).

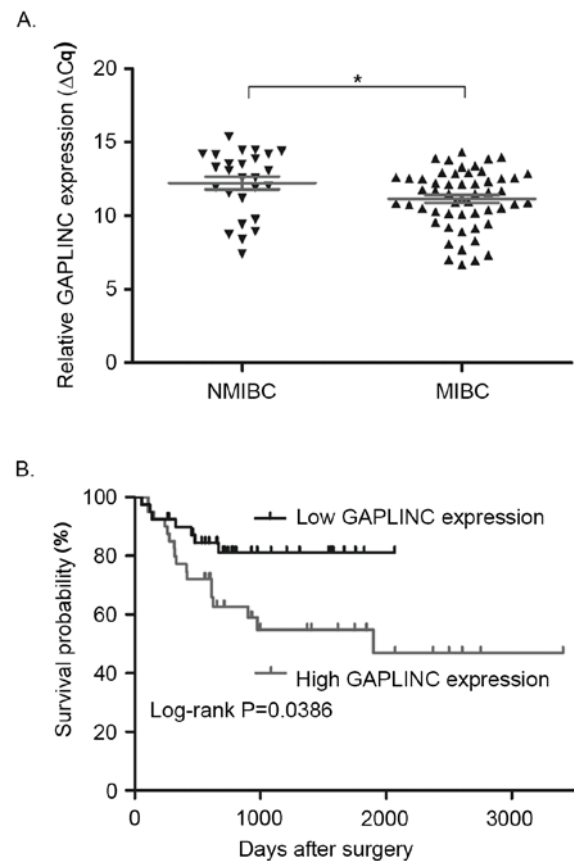


Figure 2. Association between GAPLINC expression and clinical characteristics. (A) The GAPLINC expression level was significantly higher in MIBC tissues compared with that in NMIBC tissues (*P<0.05 vs. NMIBC). (B) Kaplan-Meier analysis demonstrated that patients in the high GAPLINC expression group had significantly shorter overall survival time. Data are presented as the mean \pm SD. Statistical analysis was conducted by Student's t-test in Fig. 2A and by Log-rank test in Fig. 2B. GAPLINC, gastric adenocarcinoma associated, positive CD44 regulator, long intergenic noncoding RNA; SD, standard deviation; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer; Cq, quantification cycle.

Knockdown of GAPLINC inhibits bladder cancer cell proliferation. Due to the upregulation of GAPLINC in bladder cancer tissues and cell lines, we hypothesized that GAPLINC may be involved in a number of critical biology processes, including proliferation and invasion. To determine the role of GAPLINC on bladder cancer biological behavior, siRNAs were designed to knock down GAPLINC. The RT-qPCR results demonstrated that GAPLINC was sufficiently silenced in UM-UC-3 and T24 cells (Fig. 3A). The One Solution Cell Proliferation assay and colony formation assay were employed to evaluate the effect of GAPLINC on the proliferation of bladder cancer cells. Evident inhibitory effects on cell proliferation were observed in the GAPLINC knockdown group (Fig. 3B and C). The colony formation assay demonstrated that the colonies in the GAPLINC knockdown group were smaller and fewer compared with the normal control group (Fig. 3D and E).

GAPLINC silencing promotes cell cycle arrest at G1 phase by regulating cell cycle-associated genes. Flow cytometric analysis was performed to evaluate the effects of GAPLINC on the cell cycle. A significant cell cycle arrest was

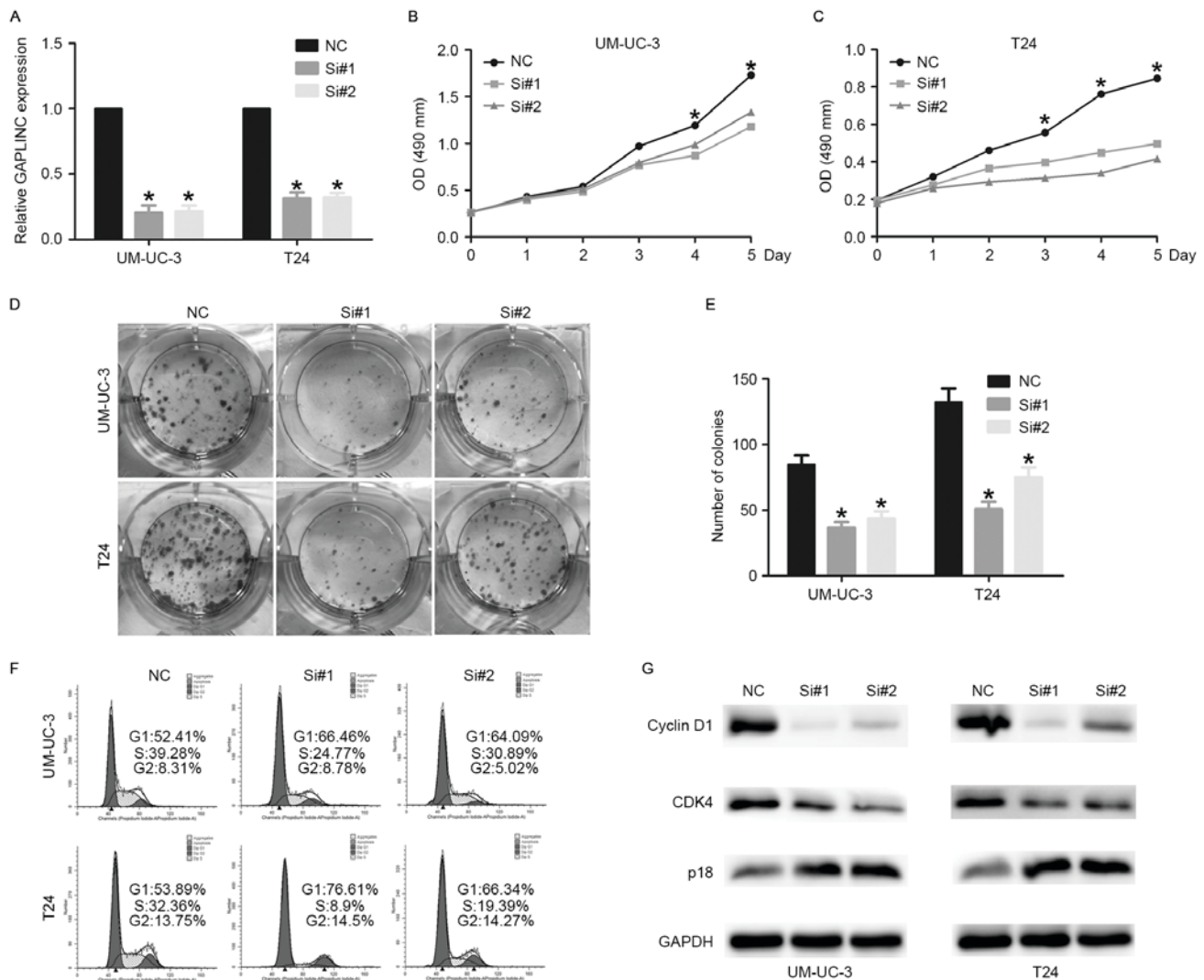


Figure 3. Knockdown of GAPLINC inhibits bladder cancer cell proliferation. (A) GAPLINC was sufficiently silenced by siRNAs ($P < 0.05$ vs. NC). (B and C) The One Solution Cell Proliferation assay indicated that knockdown of GAPLINC inhibited bladder cancer cell proliferation ($P < 0.05$ vs. NC). (D and E) Colony formation assay indicated that knockdown of GAPLINC inhibited bladder cancer cell proliferation ($P < 0.05$ vs. NC). (F) Cell cycle analysis revealed that GAPLINC influenced the proliferation of UM-UC-3 and T24 cells by regulating the cell cycle. (G) Western blotting revealed that GAPLINC silencing promotes cell cycle arrest through regulating the expression of cell cycle-associated proteins. Data are shown as means \pm SD are shown. Statistical analysis was conducted by one-way ANOVA. GAPLINC, gastric adenocarcinoma associated, positive CD44 regulator, long intergenic noncoding RNA; siRNA, short interfering RNA; si#1, short interfering RNA 1; si#2, short interfering RNA 2; SD, standard deviation; OD, optical density; NC, negative control; CDK4, cyclin dependent kinase 4.

observed at G1 phase, with an evident decrease in S phase following GAPLINC silencing (Fig. 3F). To validate the flow cytometry results, western blot analysis was performed to determine the alteration of G1/S checkpoint-associated proteins. The results identified that the expression of cyclin D1 and cyclin-dependent kinase 4 (CDK4) were decreased in the GAPLINC knockdown group. Furthermore, the expression of p18, one of the inhibitors of CDK4, was increased following GAPLINC knockdown (Fig. 3G). Taken together, the results suggest that GAPLINC silencing promotes cell cycle arrest, potentially through regulating the expression of cell cycle-associated proteins.

Knockdown of GAPLINC inhibits bladder cancer cell migration and invasion. A previous study identified that GAPLINC promoted migration and invasion in gastric cancer and colorectal cancer (13,14). However, the role of GAPLINC in

migration and invasion of bladder cancer remains unknown. The effect of GAPLINC on migratory and invasive ability of bladder cancer cells was investigated using wound-healing assays and Transwell assays. The wound-healing assay indicated that the GAPLINC knockdown inhibited the healing of bladder cancer cells compared with the normal control group (Fig. 4A and B). The Transwell assays revealed that the GAPLINC knockdown group exhibited a significantly reduced number of migratory and invasive cells (Fig. 4C-F). Overall, these results indicated that GAPLINC serves an important role in the migration and invasion of bladder cancer cells.

Discussion

It has been demonstrated that the human transcriptome comprises not only many types of protein-coding RNAs, but

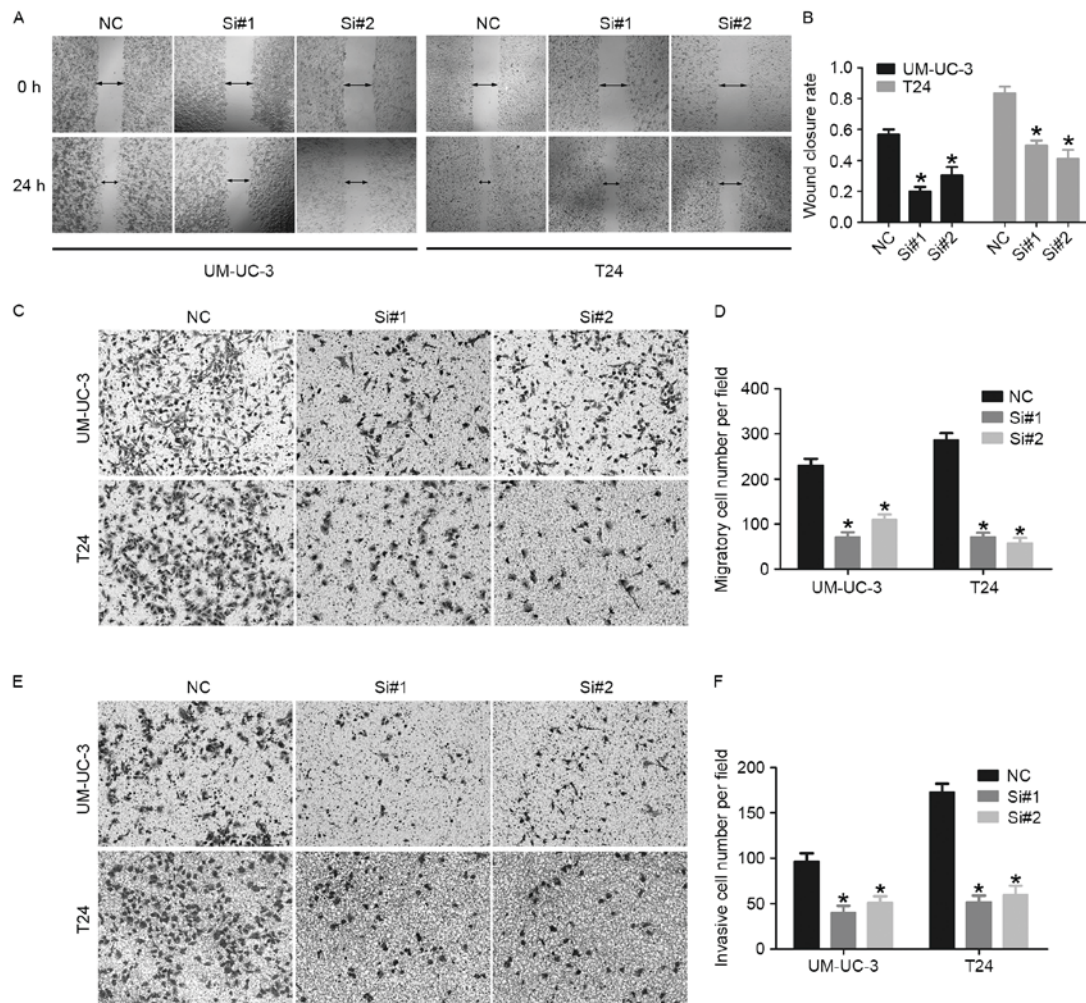


Figure 4. Knockdown of GAPLINC inhibits bladder cancer cells migration and invasion. (A and B) The wound-healing assay indicated that knockdown of GAPLINC inhibited the healing of bladder cancer cells (* $P < 0.05$ vs. NC). (C and D) Transwell migration assays revealed that knockdown of GAPLINC inhibited bladder cancer cell migration (* $P < 0.05$ vs. NC). (E and F) Transwell invasion assays revealed that knockdown of GAPLINC inhibited bladder cancer cell invasion (* $P < 0.05$ vs. NC). Data are shown as means \pm SD. Statistical analysis was conducted by one-way ANOVA. GAPLINC, gastric adenocarcinoma associated, positive CD44 regulator, long intergenic noncoding RNA; NC, negative control; si#1, short interfering RNA 1; si#2, short interfering RNA 2.

also a large number of ncRNAs, including lncRNAs (18,19). An increasing amount of evidence has indicated that certain lncRNAs exhibit crucial roles in human cancers, acting as oncogenes or tumor suppressors (20,21). The lncRNA GAPLINC was previously reported to be overexpressed in gastric cancer and colorectal cancer. GAPLINC is associated with poor prognosis of gastric cancer and regulates gastric cancer cells invasiveness (13,22). GAPLINC also interacts with PTB-associated splicing factor (PSF) and non-POU domain-containing octamer-binding (NNO) protein, and ultimately promotes invasion in colorectal cancer (14). However, little is known about the association between GAPLINC and bladder cancer.

To the best of our knowledge, this is the first report of GAPLINC being involved in the progression of bladder cancer. In the present study, GAPLINC was demonstrated to be frequently upregulated in bladder cancer tissues and its upregulation was associated with poor prognosis of bladder cancer patients. The GAPLINC expression level was significantly higher in MIBC tissues compared with that in NMIBC tissues. These results imply that GAPLINC might be a potential diagnostic biomarker and act as an oncogene in bladder

cancer, and may also be able to distinguish NMIBC from MIBC, which requires more complementary therapy.

The dysregulation of cell proliferation is one of the causative factors of bladder cancer (23,24). In the present study, the One Solution Cell Proliferation assay and colony formation assay demonstrated that GAPLINC knockdown significantly inhibited the proliferation of UM-UC-3 and T24 cells. The flow cytometric analysis indicated that GAPLINC knockdown inhibited cells proliferation through inducing cell cycle arrest at the G1 phase. Western blot analysis was performed to determine the alteration of G1/S checkpoint-associated proteins and identified that the expression of cyclin D1 and CDK4 were decreased and the expression of p18 was increased following GAPLINC knockdown. Previous studies have identified that the complex of cyclin D1-CDK4 serves an important role in cell cycle regulation (25,26) and p18 is one of the inhibitors of CDK4 (27). Therefore, the most likely mechanism underlying the dysregulation of proliferation involves a decrease in p18 expression, which leads to an increase of the complex of cyclin D1-CDK4, and then ultimately promotes bladder cancer cells to go progress through the G1 phase to the S phase of the cell cycle.

Due to the importance of invasiveness and metastasis on prognosis of bladder cancer patients (28,29), the present study further explored the role of GAPLINC on the invasive ability of bladder cancer cells. The wound healing assay and Transwell assays demonstrated that GAPLINC knockdown significantly inhibited the migratory and invasive abilities of bladder cancer cells. These observations may explain why GAPLINC was upregulated in MIBC tissues compared with NMIBC tissues, and were in agreement with the results of other studies (13,14) on gastric cancer and colorectal cancer.

To conclude, the present study demonstrated that GAPLINC expression level was markedly increased in bladder cancer tissues and cell lines, and was associated with the tumor stage and prognosis in bladder cancer patients. Furthermore, knockdown of GAPLINC significantly suppressed the bladder cancer cells proliferation, migration and invasion. The findings of the present study indicate that GAPLINC serves an important function in bladder cancer and may serve a novel therapeutic target.

Acknowledgements

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Competing interests

The authors declare that they have no competing interests.

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