

Downregulation of HDGF inhibits the tumorigenesis of bladder cancer cells by inactivating the PI3K-AKT signaling pathway

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Background: Hepatoma-derived growth factor (HDGF) is a heparin-binding protein that has been observed to be abnormally expressed in numerous malignancies, but the definite role of HDGF in bladder cancer (BCa) has not been clarified. Here, we conduct the present study to evaluate correlations between HDGF and BCa.

Methods: Bioinformatics analysis was used to evaluate HDGF expression levels in BCa tissues. The effect of HDGF on cell proliferation, migration, invasion, cell cycle and apoptosis was analyzed utilizing CCK-8, clone formation, Transwell assays and flow cytometry, respectively. In addition, the xenograft tumor model was established.

Results: Based on bioinformatics analysis, we noticed that HDGF was highly expressed in BCa tissues and was positively correlated with poor prognosis in patients. Knockdown of HDGF markedly reduced tumorigenesis in BCa cells. Furthermore, the results of flow cytometry showed that HDGF deletion enhanced apoptosis in T24 and 253J cells and led to cell cycle arrest in G1 phase. In further studies, we found that tumor growth was inhibited in xenograft nude mouse models with HDGF deletion. The results of RNA-seq analysis revealed that the PI3K-AKT signaling pathway-related genes were obviously changed in HDGF-deficient 253J cells, and this result was further confirmed by Western blot analysis.

Conclusion: In summary, we suggest that HDGF plays a substantial role in BCa and promotes tumor development and progression by regulating the PI3K-AKT signaling pathway, which provides a promising target for BCa treatment.

Keywords: HDGF, bladder cancer, tumorigenesis, PI3K/AKT signaling

Introduction

Bladder cancer (BCa) is one of the most common malignant tumors in humans, and it is ranked first among all urologic tumors worldwide.¹ The number of patients newly diagnosed with BCa is approximately 549,393, and 199,922 patients died from tumor progression in the last year.² BCa is generally classified into muscle-invasive bladder cancer (MIBC) and non-muscle-invasive bladder cancer (NMIBC).³ The current standard treatments for MIBC and NMIBC are transurethral resection of bladder tumor (TURBT) and radical cystectomy, respectively, with or without postoperative cisplatin-based combination chemotherapy.⁴⁻⁷ However, due to high recurrence,⁸ the cost and curative effect of BCa are still unsatisfactory. Considering early diagnosis and effective treatment, it remains urgent to identify novel cytogenetic molecules for BCa.

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HDGF is an acidic heparin-binding protein that was first identified from the cell line Hun-7.^{9,10} HDGF belongs to a new family of growth factors called HDGF-related proteins¹¹ and can transport to the nucleus, where it functions as a transcription factor, via nuclear localization signals.^{12,13} Overexpression of HDGF has been found to be associated with many human cancers, such as lung cancer, gastrointestinal stromal cancer, pancreatic cancer, and gastric carcinoma,^{14–17} but the correlation between HDGF and BCa remains unknown.

We conducted the present study to assess HDGF expression levels in BCa patients. The relationship between HDGF level and patient prognosis was analyzed utilizing a public gene expression database - ONCOMINE microarray datasets (<https://www.oncomine.org>). We further silenced the expression of HDGF using lentiviral shRNA to investigate the function and mechanism of HDGF in vitro and in vivo. We hypothesize that HDGF is strongly correlated with BCa carcinogenesis in vitro and in vivo and that HDGF may be a potential therapeutic target for the diagnosis and prognosis of BCa.

Materials and methods

Bioinformatic analysis of the HDGF in BCa

The mRNA level of HDGF in BCa was analyzed via ONCOMINE microarray datasets (<https://www.oncomine.org>). The datasets play a powerful role in screening differentially expressed genes (DEGs) in tumor and normal tissues. By searching “HDGF”, “BCa”, “Cancer vs Normal Analysis” and “mRNA”, 3 datasets with 7 sub-units were included in the present analysis. Based on the above datasets, a meta-analysis was performed to compare HDGF expression between cancer and normal tissues. In addition, we further compared the level of HDGF between MIBC and NMIBC tissues based on DB3 and Lee bladder datasets. IHC results of HDGF in BCa and normal tissue are available in public database-The Human Protein Atlas portal (www.proteinatlas.org).¹⁸ The survival information of patients was obtained from the Lee bladder dataset.

Cell culture

The human bladder cancer cell lines T24 and 253J were purchased from the American Type Culture Collection (Manassas, VA, USA). The two cell lines, T24 and 253J, were cultured in PRMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco,

Australia). The cells were maintained at 37 °C in a humidified incubator with a constant air flow of 5% CO₂.

Recombinant lentivirus

The recombinant lentivirus short hairpin RNA targeted hume HDGF sequence (Lv-shHDGF) or non-silencing control sequence (Lv-shCon) were purchased from Bio-link-gene Inc. (Shanghai, China). The target sequences of Lv-shHDGF and Lv-shCon were 5'- AACCGGCAGAAGGAGTACAAA-3' and 5'-TTCTCCGAACGTGTACAGT-3', respectively.

Cellular infection of lentivirus

In brief, T24 and 253J cells were cultured in complete medium containing recombinant lentivirus (Lv-shHDGF or Lv-shCon) for 12 hrs; then, the medium was replaced with normal medium. As observed by a fluorescence microscope, the proportion of GFP-positive cells represented the infection efficiency. The culture medium containing puromycin (Solarbio, Beijing, China) was used to kill cells that were unsuccessfully transfected with virus.

Quantitative real-time PCR

Total RNA was extracted from the cells using an RNA-Quick purification Kit (ES Science, China), and the RNA was reverse transcribed into complementary DNA (cDNA) using a qPCR RT kit (TOYOBO, Japan). The cDNA was used for RT-PCR assays (SYBR Green Master Mix; YESEN, Shanghai, China) according to the manufacturer's instructions. Relevant data were analyzed with the $2^{-\Delta\Delta Ct}$ method normalized to β -actin. The primers for HDGF were 5'-CTCTTCCCTTACGAGGAATCCA-3' (forward) and 5'-CCTTGACAGTAGGGTTGTTCTC-3' (reverse).

Western blot analysis

T24 and 253J cells with stable HDGF knockdown and control plasmid expression were collected and lysed in RIPA buffer (Beyotime, Shanghai, China), and Western blot assay of lysates of equal mass was performed as described previously.¹⁹ The following antibodies were used: anti-HDGF, E-cadherin, N-cadherin, P53, Bax, Bcl-2, PI3K, AKT, p-AKT, β -actin and GAPDH (all 1:2000) (CST, USA).

CCK-8 cell proliferation assays

T24 and 253J cells infected with Lv-shHDGF or Lv-shCon were seeded in 96-well plates at a density of 2000 cells/well and incubated in a culture chamber at 37 °C for 1, 2, 3, 4 or 5 days. Then, the optical density (OD) was measured at

450 nm using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each group consisted of five duplicates, and the experiment was performed in triplicate.

Colony formation assays

Cells infected with Lv-shHDGF or Lv-shCon lentivirus were seeded in six plants (T24: 600/well; 253J: 1000/well, respectively). After 14 days of culture, the cells were fixed with 4% paraformaldehyde for 30 mins. After washing with PBS again, the colonies were stained with 5% crystal violet for 30 mins. Colonies exceeding 50 cells were counted using ImageJ. Each group consisted of three duplicates, and the experiment was performed three times.

Cell migration assays

T24 and 253J cells were digested with trypsin (Gibco, USA) and dissociated repeatedly to prepare a single cell suspension. Migration assays were performed as described previously.²⁰ After rubbing away the cells failed to through the membrane and washing with PBS three times, these chambers were photographed using an upright fluorescence microscope. Three random fields of vision were adopted for counting, and each experiment was performed in triplicate.

Cell invasion assays

The invasion assay procedure was similar to the migration assay procedure. However, the chambers used in the invasion assay were coated with diluted Matrigel (BD Bioscience, USA). The incubation times for the T24 and 253J cells were 36 h and 72 h, respectively.

Cell cycle analysis

Cells were harvested by trypsinization and fixed with 75% cold ethanol for 24 h at 4 °C. The next day, the cells were resuspended in 100 μL RNaseA (Solarbio, Beijing, China) at 37 °C for 30 mins. Then, the cells were stained with 400 μL propidium iodide (Solarbio, Beijing, China) at 4 °C for 30 mins in the dark. Cell cycle distribution was recorded with a flow cytometer (BD Bioscience, USA).

Cell apoptosis analysis

Cells were harvested, washed with cold PBS, and resuspended in 400 μL 1× binding buffer (BD Bioscience, USA). Next, 100 μL of the cell solution was transferred into a 5 mL culture tube, and 5 μL of PE Annexin V and 5 μL of 7-AAD (BD Bioscience, USA) were added to the culture tube. Cells were incubated for 15 mins at room temperature

in the dark, and 400 μL of 1× binding buffer was added to each tube. The apoptosis of cells was analyzed in triplicate by flow cytometry (BECKMAN COULTER, USA) according to the manufacturer's instructions.

In vivo xenograft assays

Female nude mice were purchased from the Model Animal Research Center of Nanjing University and housed in specific pathogen-free barrier facilities. Twelve four-week-old female nude mice were randomly divided into two groups. A total of 5×10^6 T24 cells infected with Lv-shHDGF were injected into the right axilla of six mice, and six other mice were injected with equal concentration of T24 cells infected with Lv-shCon. The diameters of the xenografts were measured using a slide caliper every 3 days for 27 days. The xenograft tumor volume was calculated using the formula: $V = (\text{length} \times \text{width}^2) / 2$. At the end of the experiment, mice were euthanized and imaged. The xenograft tumors were removed from each mouse, imaged, weighed, and fixed in 4% paraformaldehyde.

All animal studies were approved by The Institutional Animal Care and Use Committee of the Shandong University and all animal studies were carried out in accordance with the UK Animal (Scientific Procedures) Act, 1986 and associated guidelines.

Immunohistochemistry (IHC) staining

The nude mouse xenograft tumors were paraffin embedded and sliced into 4 μm thick paraffin sections. Then, these tumor tissues were subjected to IHC staining as previously described.²¹ HDGF primary antibody (1:100, Abcam, USA) and Ki-67 antibody (1:200, ZhongShan Bio-Tech, China) were used. The signal intensity was scored using the following criteria: 0= negative, 1= weak, 2= moderate, and 3= strong. The percentage of stained cells was scored as follows: 1=0%, 2=25–50%, 3=51–75%, and 4=76–100%. The final staining scores were calculated as the intensity × the staining percentage.

RNA sequencing analysis

The 253J cells infected with Lv-shHDGF or Lv-shCon were collected, and the total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). Library construction and sequencing were performed by Annoroad Gene Technology Inc. (Beijing, China). The libraries were sequenced using the Illumina platform. DEGs were screened according to the following criteria: a $p < 0.05$ and an absolute fold change > 2.0 . The intracellular signaling

pathway was determined using KEGG based on DEGs, and protein-protein interaction analysis (PPI) was performed.

Statistical analysis

The data presented as the mean \pm SD were analyzed by Student's *t*-test. The correlations between HDGF expression level and the clinicopathological features of BCa patients were analyzed using the independent-sample T test. In addition, the CCS analysis was performed by Kaplan-Meier analysis. The Statistical Package for Social Science version 21 (SPSS Inc. Chicago, IL, USA) and GraphPad Prim 5 software (GraphPad Software Inc., San Diego, CA, USA) were used, and $p < 0.05$ was considered statistically significant.

Results

HDGF was elevated in BCa tissues

To evaluate the potential role of HDGF in BCa, the ONCOMINE microarray database was utilized. Three databases were included, and a meta-analysis was performed based on these data. As shown in [Figure 1A](#), it was obvious that the expression level of HDGF was higher in BCa tissues than in normal tissues ($p < 0.001$). The information acquired from the DB3 and Lee Bladder databases indicated that MIBC tissues showed elevated HDGF levels compared to NMIBC and normal tissues ([Figure 1B and C](#)). IHC results acquired from The Human Protein Atlas portal indicated that HDGF was highly expressed in BCa compared to normal ([Figure S1](#)). BCa patients with HDGF overexpression were found to be correlated with poor Cancer-specific survival (CSS) but not overall survival (OS) ([Figure 1D and E](#)).

Associations between HDGF expression level and clinicopathological features of BCa patients

A total of 165 BCa patients and their essential information, such as age, sex, pathological stage, pathological grade, and survival time, were included in the present study. The correlations between HDGF level and clinical characteristics of the 165 BCa patients were further evaluated. We found that high HDGF levels were strongly associated with muscular invasion, lymph node metastasis, pathological grading, progression and CCS (all $p \leq 0.05$). Although patients with older age, recurrence and poor OS also showed higher HDGF levels, these

differences were not statistically significant ($p > 0.05$). All results are shown in [Table 1](#).

HDGF expression levels were downregulated by lentivirus-mediated shRNA in BCa cells

The infection efficiency of bladder cancer cells $>90\%$ identified by GFP expression was considered to be satisfactory ([Figure 2A](#)). The mRNA and protein levels of HDGF in T24 and 253J cells infected with Lv-shHDGF were obviously decreased ([Figure 2B–E](#)). These results indicated that the expression of HDGF was knocked down successfully.

Knockdown of HDGF suppressed BCa cell proliferation and clone formation in vitro

The proliferation of T24 cells infected with Lv-shHDGF was significantly suppressed compared to that of control cells according to the CCK-8 assay, and similar results were found in 253J cells ([Figure 3A](#)). Moreover, a cloning assay was performed to analyze the role of HDGF in BCa cell colony formation. As shown in [Figure 3B and C](#), the clonogenic capacity in Lv-shHDGF-infected cells was dramatically impaired. Indeed, our results demonstrated that HDGF was important for T24 and 253J cell proliferation and colony formation.

HDGF depletion reduced migration and invasion in BCa cell lines

Transwell assays indicated that HDGF knockdown significantly reduced the migration and invasion capability of T24 and 253J cell lines ([Figure 3D–G](#)). Furthermore, key proteins related to epithelial-mesenchymal transition (EMT) were analyzed by Western blot, and the results indicated that HDGF knockdown increased the expression of E-cadherin but decreased the levels of N-cadherin and vimentin ([Figure 3H and I](#)). These findings demonstrated that HDGF deletion was significantly associated with the impairment of BCa cell migration and invasion abilities.

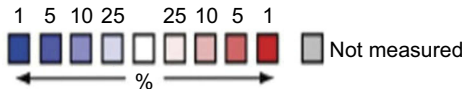
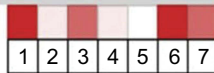
Disruption of HDGF expression inhibited the cell cycle in BCa cells

The results described above indicate that HDGF plays a substantial role in the proliferation of BCa cells; therefore, flow cytometry was conducted to identify whether

Comparison of HDGF across 7 analyses
Over-expression

A

Median rank	P-value	Gene
569.0	4.90E-7	HDGF

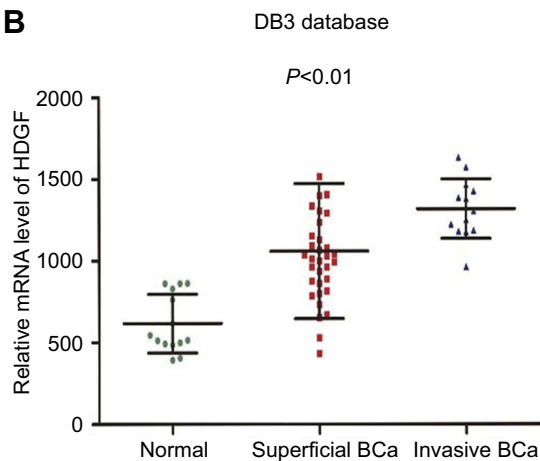


The rank for a gene is the median rank for that gene across each of the analyses. The P-value for a gene is its P-value for the median-ranked analysis.

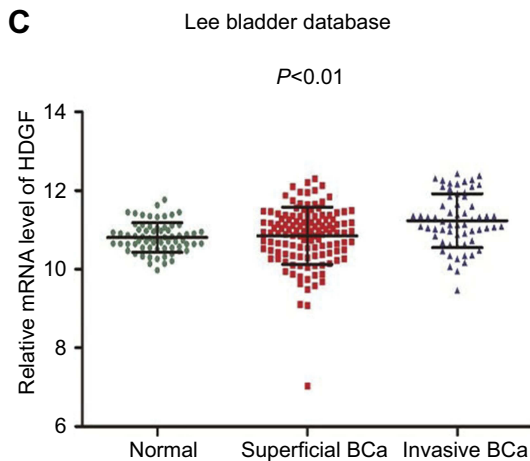
Legend

1. Infiltrating bladder urothelial carcinoma vs. normal
Dyrskjot bladder 3, Cancer res, 2004
2. Stage ois bladder urothelial cancer vs. normal
Dyrskjot bladder 3, Cancer res, 2004
3. Superficial bladder cancer vs. normal
Dyrskjot bladder 3, Cancer res, 2004
4. Infiltrating bladder urothelial carcinoma vs. normal
Lee bladder, J Clin Oncol,2010
5. Superficial bladder cancer vs. normal
Lee bladder, J Clin Oncol,2010
6. Infiltrating bladder urothelial carcinoma vs. normal
Sanchez carbayo bladder 2, J Clin Oncol, 2006
7. Superficial bladder cancer vs. normal
Sanchez carbayo bladder 2, J Clin Oncol, 2006

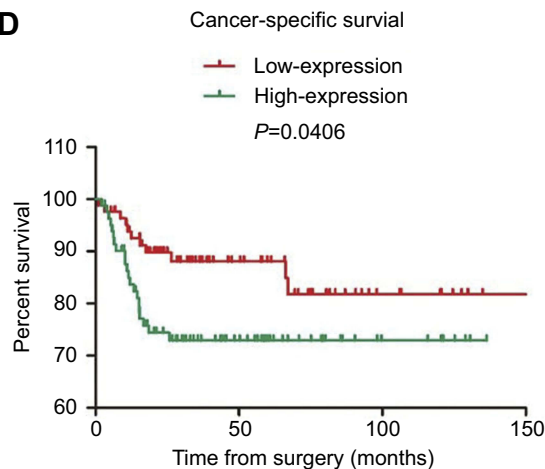
B



C



D



E

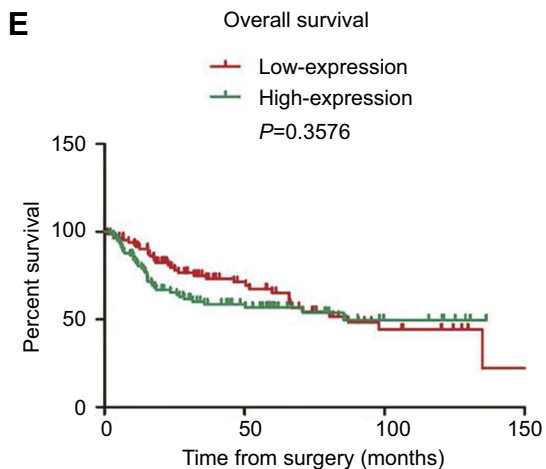


Figure 1 Expression of HDGF is higher in BCa tissue and appears to be closely related to muscle invasion and CCS (All the data from ONCOMINE database). **(A)** Meta-analysis of the HDGF mRNA expression between the BCa and normal urothelial tissue ($p < 0.001$). **(B and C)** HDGF expression is elevated in MIBC compared to NMIBC and normal tissues according to DB3 and Lee Bladder databases ($p < 0.01$). **(D and E)** The Kaplan-Meier analysis indicates overexpression of HDGF predicts poor CCS but not OS using Lee Bladder database.

Abbreviations: HDGF, Hepatoma-derived growth factor; BCa, Bladder cancer; CCS, Cancer-specific survival; OS, Overall survival.

HDGF plays a role in cell cycle distribution. Interestingly, T24 cells infected with Lv-shHDGF were dramatically arrested in G1 phase compared to control

cells, and the number of cells in S and G2 phase decreased. Similar results were observed in 253J cells (Figure 4A and B). Western blotting showed that the

Table 1 Clinicopathological parameters and Hepatoma-derived growth factor (HDGF) expression level in 165 BCa patients based on Lee Bladder database

Characteristics	No. of patients	HDGF expression (Mean \pm SD)	p-value
Patients	165	10.96 \pm 0.75	
Age			0.610
\leq 65 y	73	10.77 \pm 0.76	
$>$ 65 y	92	11.11 \pm 0.72	
Sex			0.974
Man	135	10.96 \pm 0.68	
Female	30	10.95 \pm 1.03	
Muscular invasion			0.000*
Negative	103	10.79 \pm 0.74	
Positive	62	11.24 \pm 0.68	
Lymph node metastasis			0.049*
Negative	149	10.92 \pm 0.76	
Positive	16	11.31 \pm 0.59	
Distant metastasis			0.753
Negative	158	10.96 \pm 0.75	
Positive	7	10.87 \pm 0.77	
Grade			0.000*
Low	105	10.72 \pm 0.72	
High	60	11.37 \pm 0.63	
Recurrence			0.598
No	67	10.81 \pm 0.81	
Yes	36	11.27 \pm 0.60	
Missing information	62	—	
Progression			0.010*
No	134	10.88 \pm 0.76	
Yes	31	11.27 \pm 0.66	
Overall survival			0.122
Alive	96	10.88 \pm 0.76	
Death	69	11.06 \pm 0.74	
Cancer-specific survival			0.002*
Alive	133	10.87 \pm 0.74	
Death	32	11.33 \pm 0.71	

Note: * $P < 0.05$ is considered significant (the independent-sample T test).

expression levels of the G1 checkpoint proteins cyclin D1 and cyclin E were obviously downregulated with HDGF deletion (Figure 4C). These results suggested that HDGF knockdown inhibited proliferation by inducing T24 and 253J cell cycle arrest in G1 phase.

HDGF downregulation promoted apoptosis in BCa cells

As shown in Figure 4D and E, the distribution of early and late apoptosis was obviously increased in T24 and 253J cells infected with Lv-shHDGF. In addition, checkpoint proteins of apoptosis were detected by Western blot. The levels of P53 and Bax were higher in T24 and 253J cells with downregulated HDGF, but the expression of Bcl-2 was lower (Figure 4F). These results suggest that HDGF silencing induces apoptosis in BCa cells.

Downregulation of HDGF suppressed BCa cell growth in vivo

As shown in Figure 5A–C, the tumor size and weight of nude mice injected with Lv-shHDGF-infected cells were significantly lower than those of control mice. Furthermore, IHC staining indicated that HDGF and Ki-67 levels in HDGF knock-out tumors were decreased (Figure 5D and E). These results demonstrated that HDGF deletion inhibited BCa cell tumorigenicity in vivo.

HDGF depression regulated the PI3K-AKT pathways

To further illuminate the mechanism of HDGF in BCa tumorigenesis and progression, RNA-seq was performed. With the cut-off criteria of absolute fold-change ≥ 2.0 and $q < 0.05$, we found that 66 genes were upregulated and 96 genes were downregulated after HDGF deletion (Figure 6A). Then, GO enrichment analysis was performed, and a total of 162 DEGs were categorized into 42 functional groups. Three main GO classification categories, cellular component, biological process and molecular function, contained 13, 20 and 9 functional groups, respectively (Figure 6B). Based on the KEGG database, pathway enrichment was further analyzed. We found that the PI3K-AKT signaling pathway passed the filtering criteria ($p < 0.05$, Figure 6C). PPI network analysis demonstrated that HDGF with an 11-gene signature coordinately drove tumor progression in BCa (Figure 6D).

The RNA-seq results indicated that the mRNA levels of key genes in the PI3K-AKT pathway were obviously changed with HDGF deletion (Figure 7A). Then, the checkpoint protein level of the PI3K-AKT was further verified by Western blotting. As shown in Figure 7B, we found that the levels of PI3K and p-AKT were significantly decreased in cells with downregulated HDGF.

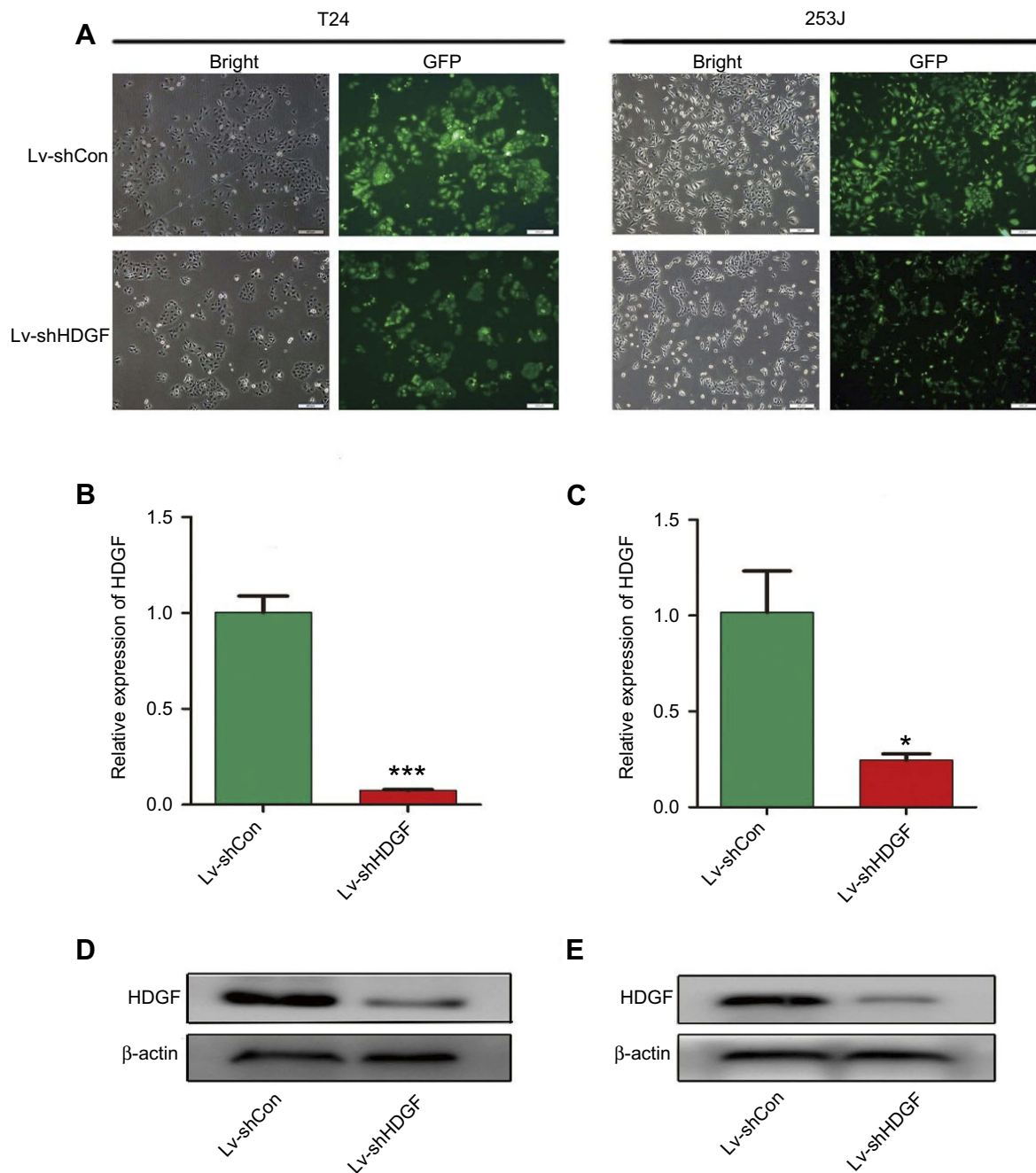


Figure 2 Lentivirus-mediated interference downregulates the expression of Hepatoma-derived growth factor (HDGF) in BCa cells. **(A)** The bladder cancer cells are examined by fluorescence microscopy after lentivirus infection for 72 h. **(B–C)** Real-time qPCR indicates mRNA levels are lower in T24 and 253J cells infected with Lv-shHDGF than Lv-shCon. **(D–E)** The protein levels of HDGF are downregulated in T24 and 253J cells infected with Lv-shHDGF. Every result is verified at least three times. (* $p < 0.05$; *** $p < 0.001$).

Discussion

Despite the fact that many recent studies have focused on BCa, BCa treatment has seen little progress.²² Over the past three decades, the five-year survival rate of BCa has been maintained, with a limited range of clinical therapeutics.²² In addition, due to complicated pathological characteristics, inconspicuous early symptoms and

high rates of recurrence, the treatment and surveillance of BCa is still costly.^{23–25} Therefore, identifying critical molecular mechanisms and more effective therapeutic targets for BCa is extremely important.

HDGF is a vital regulator of cancer cell activities, and it plays a broad role in tumorigenesis, such as in cancer cell transformation, apoptosis, angiogenesis and metastasis.²⁶

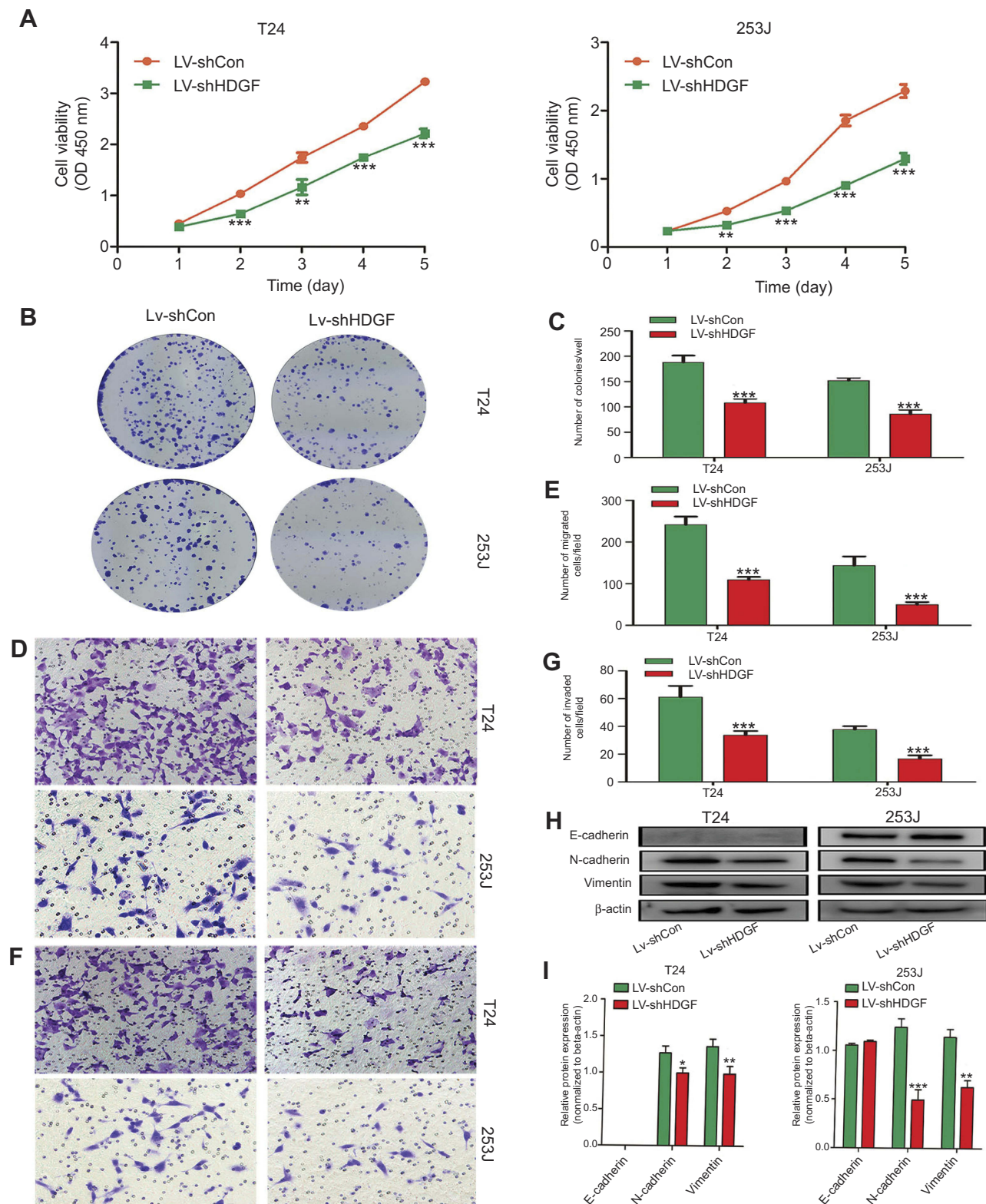


Figure 3 Knockdown of HDGF dramatically inhibits the proliferation, migration and invasion of BCa cells. **(A)** The growth curves of BCa cells infected with Lv-shCon or Lv-shHDGF were determined by CCK-8 assay. **(B–C)** Representative images of T24 and 253J cells colony information after infection with Lv-shCon or Lv-shHDGF. **(D–E)** Transwell migration assay in T24 and 253J cells with stable HDGF knockdown and control plasmid expression; **(F–G)** Transwell invasion assay in BCa cells with stable HDGF down-regulated and control. **(H)** Representative Western blot results of proteins related to EMT in T24 and 253J cells with stable HDGF knockdown and control plasmid. **(I)** The gray intensity quantification of EMT of T24 and 253J cells. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Abbreviation: EMT, epithelial-mesenchymal transition.

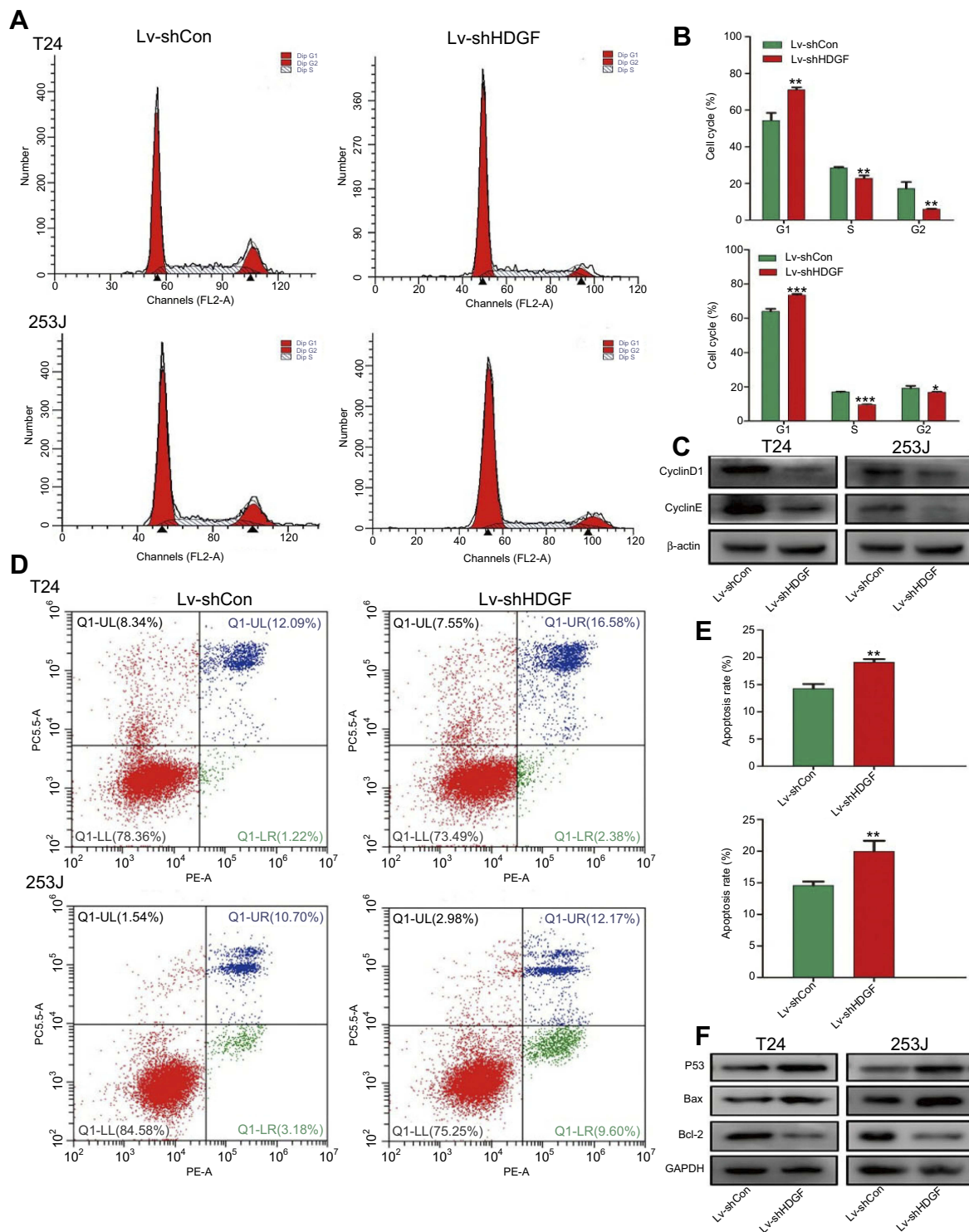


Figure 4 HDGF deletion represses cell cycle and induces apoptosis of BCa cells. **(A–B)** Representative cell cycle data was measured by flow cytometry in BCa cells infected with Lv-shHDGF or Lv-shCon. **(C)** Western blot results of proteins related to cell cycle in T24 and 253J cells with stable Hepatoma-derived growth factor (HDGF) knockdown or control expression. **(D–E)** Apoptosis of BCa cells infected with Lv-shHDGF or Lv-shCon were analyzed by flow cytometry. **(F)** Proteins involved in apoptosis was analyzed by Western blot. (* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$).

Although a previous study found that HDGF had the capacity to translocate to the nucleus, where it binds to DNA through its PWWP domain and acts as an oncogene, the

concrete mechanism by which HDGF promotes tumor progression remains unclear.²⁷ In the present study, by utilizing the ONCOMINE database, we found that the mRNA level

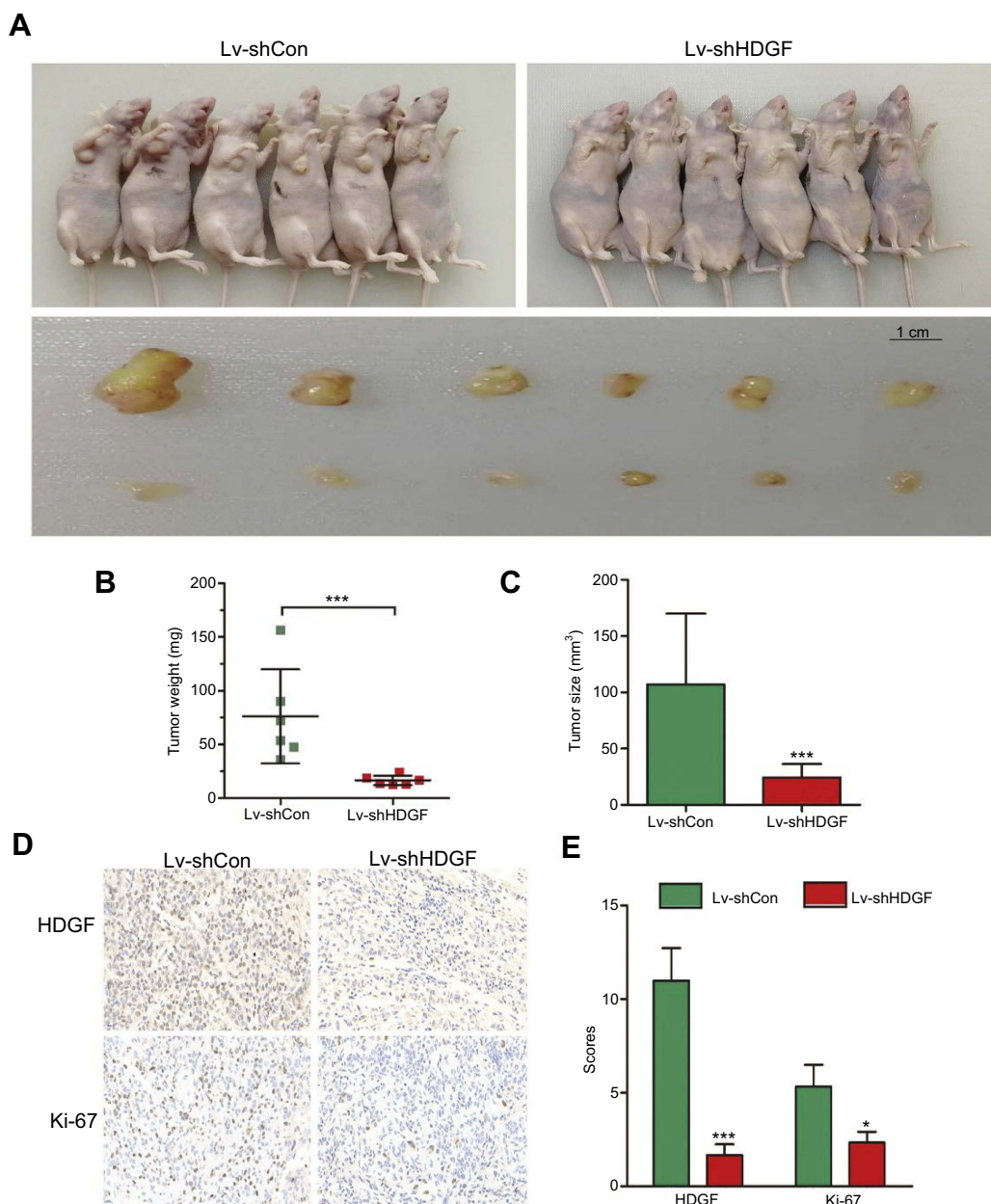


Figure 5 HDGF deletion suppresses T24 cell tumorigenicity in vivo. **(A)** Representative picture of animals and xenograft tumors. **(B)** Weights of xenograft tumors derived from T24 cells with HDGF deletion and control. **(C)** Tumors sizes were measured after tumors were dissected. **(D)** IHC examination of HDGF and Ki-67 in tumor sections (magnifications $\times 400$). **(E)** Histogram shows IHC scores in control and Hepatoma-derived growth factor (HDGF) deletion group. (* $p < 0.05$; *** $p < 0.001$).

of HDGF was significantly higher in BCa tissues than in normal tissues and that its level was elevated in MIBC compared to NMIBC tissues. Furthermore, the survival data from the ONCOMINE datasets indicated that HDGF overexpression predicted poor CSS. The above results indicate that elevated levels of HDGF are closely related to the occurrence and progression of BCa. However, until now, no study has focused on the relationship between HDGF and BCa. Therefore, we carried out the present study to verify whether HDGF is a credible candidate biomarker of BCa.

In this study, the mRNA level of HDGF in T24 and 253J cells was downregulated by the lentivirus-mediated RNA interference system. As is well known, lentiviruses have the capacity to integrate into the genome of host cells; thus, they are extensively used as vectors for shRNA expression.²⁸ Some studies showed that HDGF depletion significantly reduced cancer cell proliferation, migration and invasion, including in gallbladder cancer, gastric cancer, and human glioma cells,^{29–31} but in contrast to the above results, Guo, Z., et al found that

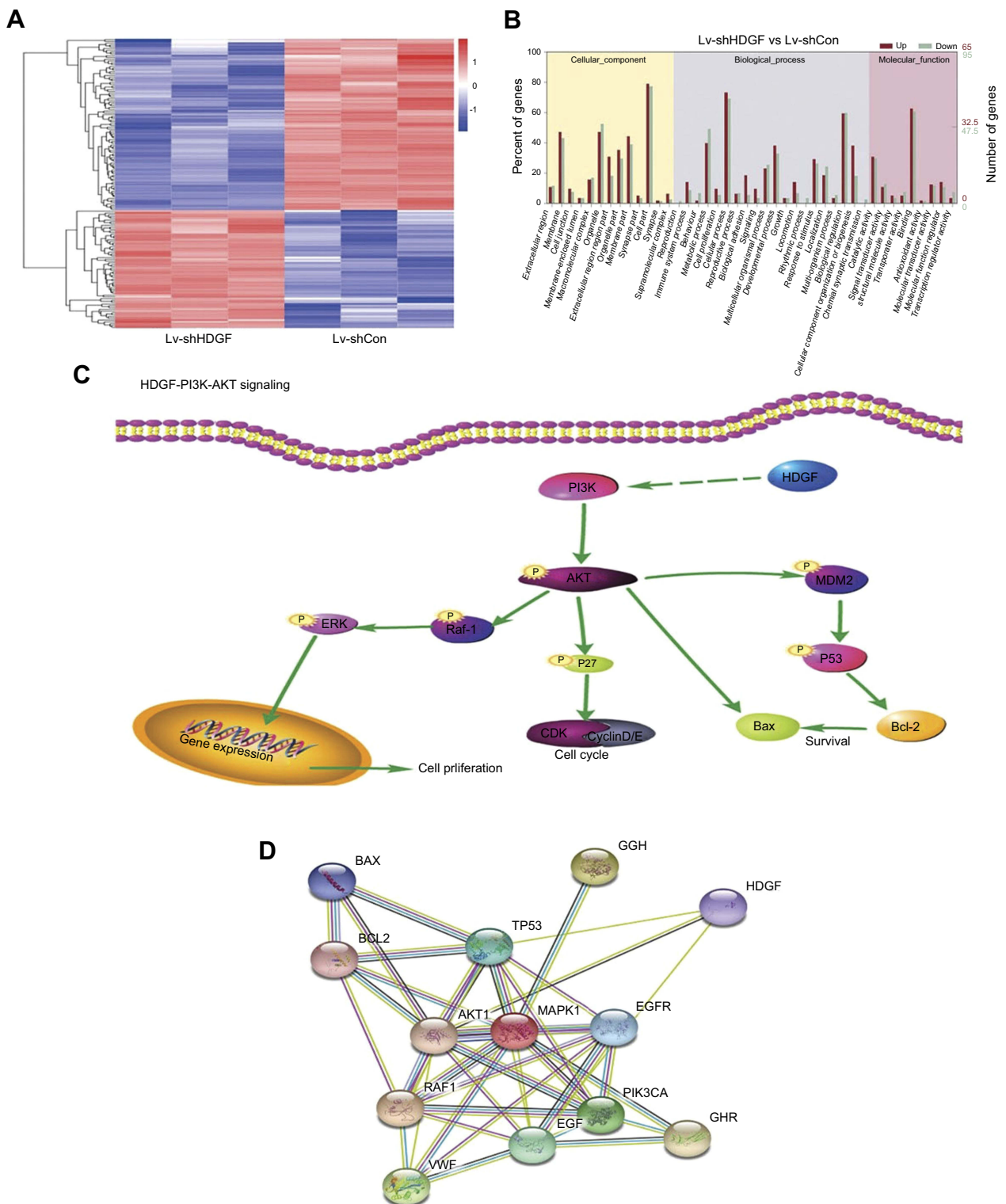


Figure 6 HDGF downregulated affects PI3K-AKT pathway. **(A)** Heat map representing unsupervised hierarchical clustering of mRNA expression levels in 253J cells infected with Lv-shHDGF or Lv-shCon. Red and Blue indicate high and low expression respectively. **(B)** Go analysis including the cellular component, biological process and molecular function was performed on DEGs. **(C)** Hepatoma-derived growth factor (HDGF) positively regulates PI3K-AKT signaling pathway based on KEGG analysis. **(D)** PPI analysis of HDGF and the PI3K-AKT pathway related genes.

downregulation of HDGF stimulated cell migration and invasion in breast cancer.³² We first investigated the correlations between HDGF expression and BCa, and we

found that HDGF deletion significantly inhibited the malignant phenotype of T24 and 253J cells, including proliferation, colony formation, migration and invasion.

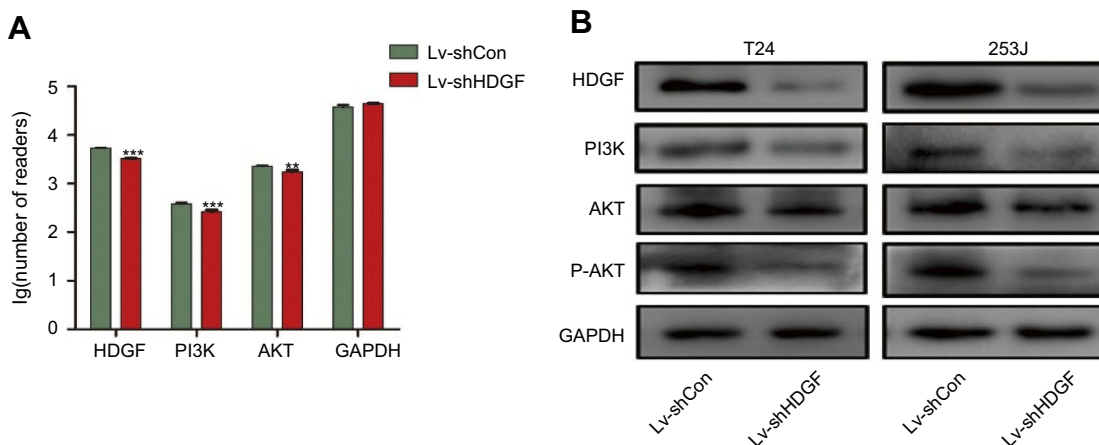


Figure 7 The expression level of key genes related to the PI3K-AKT pathway. **(A)** RAN-seq analysis indicates mRNA level of HDGF, PI3K and AKT in 253J cells with stable Hepatoma-derived growth factor (HDGF) knockdown and control expression. **(B)** Representative Western blot results of proteins related to PI3K-AKT pathway in 253J cells infected with Lv-shHDGF or Lv-shCon. (* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$).

Similar to our findings, the results based on the ONCOMINE database also indicated that HDGF overexpression was closely related to muscular invasion and lymph node metastasis in BCa. It has been well established that EMT is the initiation of tumor invasion and metastasis,³³ therefore, key protein levels of EMT were analyzed. In accordance with the previous study, E-cadherin was found to be negatively expressed in T24 cells.^{34,35} Western blot analysis showed that the protein levels of N-cadherin and vimentin were decreased but that E-cadherin was increased in HDGF-downregulated cells. These results provide evidence for a strong relationship between HDGF and cell migration and invasion.

The proliferation and viability of cells were closely related to the cell cycle and apoptosis,³⁶ therefore, flow cytometry was performed to assess BCa cell cycle distribution and apoptosis. It was found that knockdown of HDGF significantly increased the component of G1 phase and promoted apoptosis in T24 and 253J cells. The checkpoint protein expression levels of the cell cycle (cyclin D1 and cyclin E) and apoptosis (P53, Bax and Bcl-2) were also changed with HDGF deletion. Similarly, previous reports demonstrated that downregulated HDGF cells arrested in G1 phase and had increased percentages of apoptotic cells in Ewing's sarcoma and human glioma cells,^{37,38} which is in full support of our results.

In addition, many studies have indicated that HDGF is closely related to tumor growth in xenograft models,³⁹⁻⁴¹ therefore, xenograft tumor models were constructed in the present study. Our results indicated that HDGF downregulation effectively inhibited tumor growth. IHC staining showed

that Ki-67 was obviously downregulated in xenograft tumors with HDGF deletion. Ki-67 is strongly associated with tumor cell growth and has been embraced as a classic proliferation marker in diagnostic surgical pathology.⁴² We first found that HDGF downregulation could inhibit tumorigenicity of BCa in vivo.

To explore the molecular mechanism of HDGF regulation in cell fate, RNA sequencing was conducted. The results showed that 162 genes were differentially expressed between HDGF downregulated 253J cells and control cells. Of 162 genes, 66 were overexpressed, and others were downregulated. GO analysis indicated that DEGs were involved in cell proliferation, growth, cellular process, signaling and biological regulation. KEGG analysis demonstrated that HDGF deletion was likely to regulate cell proliferation, survival and cell cycle through the PI3K-AKT axis. Previous studies indicated that PI3 kinase was involved in regulating agonist-stimulated membrane trafficking events and that the PI3K-AKT pathway was involved in many instances of human cancer occurrence and progression, including prostate cancer, colorectal cancer and breast cancer.⁴³⁻⁴⁶ In the present study, Western blotting showed that the expression levels of PI3K and p-AKT were downregulated in HDGF deletion cancer cells, and gene expression levels of their downstream regulators were also changed, including Cyclin D1, Cyclin E, P53, Bax, and Bcl-2. Although our results strongly support the promoting effect of HDGF on BCa formation, the underlying molecular mechanism of the potential interaction between HDGF and PI3K-AKT activation needs to be further explored.

Conclusion

In conclusion, our study indicates that HDGF deletion inhibits cell proliferation and viability by arresting cell cycle progression and promoting cell apoptosis. We also verify that HDGF is closely related to the migration and invasion of BCa cells. The RNA-sequencing analysis shows that HDGF affects BCa cell carcinogenesis associated with the PI3K-AKT pathway. These findings suggest that HDGF may be a promising prognostic indicator and a novel therapeutic candidate in BCa.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects for the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

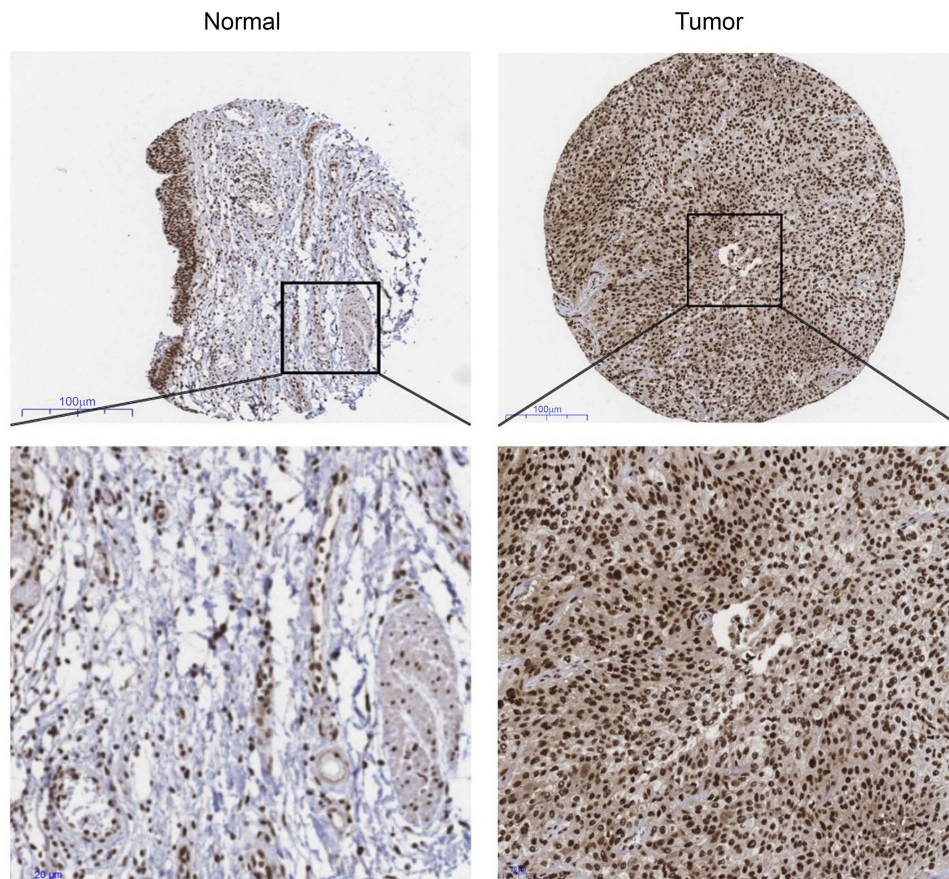


Figure S1 Representative image of IHC staining of HDGF in normal bladder tissues and BCa tissues (upper: $\times 40$; lower: $\times 400$) (Date comes from public database-The Human Protein Atlas portal (www.proteinatlas.org)).

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