

High lncRNA HULC expression is associated with poor prognosis and promotes tumor progression by regulating epithelial-mesenchymal transition in prostate cancer

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Abstract

Introduction: Recently, increasing evidence has shown that long non-coding RNAs (lncRNAs) play critical roles in tumor progression and development. However, the expression pattern and biological function of lncRNA HULC (highly upregulated in liver cancer) in prostate cancer (PCa) remain largely unclear.

Material and methods: The expression of lncRNA HULC in 53 paired PCa tissues and cell lines was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The χ^2 test was used to explore the association of lncRNA HULC expression with clinicopathologic features. Kaplan-Meier analysis was used to detect the association between HULC expression and overall survival of PCa patients. Furthermore, the function of HULC in cell growth and metastasis was detected in PCa cells.

Results: Our data showed that HULC expression was upregulated in PCa tissues and cell lines compared to adjacent non-tumor tissues and the normal prostate cell line RWPE-1 ($p < 0.05$). High HULC expression was positively associated with advanced clinicopathologic features and poor overall survival (OS) for PCa patients ($p < 0.05$). HULC inhibition suppressed PCa cell growth and metastasis both *in vitro* and *in vivo* ($p < 0.05$). Furthermore, HULC knockdown reduced N-cadherin and vimentin expression and increased E-cadherin expression in PCa cells ($p < 0.05$).

Conclusions: Our data suggested that lncRNA HULC might play oncogenic roles in PCa progression, which provided a novel therapeutic strategy for PCa patients.

Key words: long non-coding RNA, HULC, prostate cancer, epithelial-mesenchymal transition.

Introduction

Prostate cancer (PCa) is known as the most prevalent malignancy in male patients, accounting for approximately one fourth of all cancer cases, and the third leading cause of male cancer-related mortality [1]. About one third of patients with organ-confined PCa fail local therapy and progress to advanced staged or metastatic disease [2]. Metastatic PCa may finally develop into androgen-independent PCa and hormone refractory PCa, which is the major cause of death in PCa patients [3, 4]. Therefore, a good understanding of the biological molecular mechanisms involved in PCa progression is critical for the development of novel therapeutic strategies.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs which are longer than 200 nucleotides without evident protein coding function [5]. Recent evidence indicated that lncRNAs function as oncogenes or tumor suppressors, involved in the modulation of cellular processes, such as differentiation, proliferation and metastasis [6, 7]. For example, Li *et al.* showed that lncRNA HOTTIP (HOXA transcript at the distal tip) was upregulated and associated with poor prognosis in patients with osteosarcoma [8]. Li *et al.* found that lncRNA CASC2 (cancer susceptibility candidate 2) could suppress the proliferation of gastric cancer cells by regulating the mitogen-activated protein kinase (MAPK) signaling pathway [9]. Cui *et al.* indicated that upregulated lncRNA SNHG1 (small nucleolar RNA host gene 1) could contribute to progression of non-small cell lung cancer through inhibition of miR-101-3p and activation of the Wnt/β-catenin signaling pathway [10]. Those studies suggested that lncRNAs play crucial roles in tumor carcinogenesis.

In the present study, we found that the expression of HULC was upregulated in PCa tissues and cell lines. High HULC expression was positively associated with advanced clinical features and poor overall survival (OS) for PCa patients. HULC inhibition suppressed PCa cell growth and metastasis both *in vitro* and *in vivo*. Furthermore, knockdown of HULC reduced N-cadherin and vimentin expression and increased E-cadherin expression in PCa cells. Together, we demonstrated that high expression of HULC might promote tumor growth and metastasis by epithelial-mesenchymal transition in PCa.

Material and methods

Patient samples

A total of 53 PCa tissues and adjacent non-tumor tissues were obtained and histologically confirmed by a pathologist at the First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, from January 2011 to January 2012. Tissue specimens were immediately kept in RNA keeper tissue stabilizer (Vazyme) after surgery and then stored at 80°C until RNA extraction. Written informed consent was obtained from all patients. All experimental procedures were approved by the ethical board of the Henan University of Science and Technology and complied with the Declaration of Helsinki.

Cell culture and transfection

Human PCa cell lines (LNCaP, PC3 and DU145) and a normal prostate cell line (RWPE-1) were purchased from the American Type Culture Collection (ATCC). Cells were cultured in DMEM (Dulbecco's

Modified Eagle Medium) or keratinocyte (GIBICO) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified incubator at 37°C with 5% CO₂.

The shRNAs (small hairpin RNA) targeting HULC (sh-HULC) and negative control shRNA (sh-NC) were adopted and synthesized by Genechem (Shanghai). The sh-HULC sequence was as follows: AACCTCCAGAACTGTGATCCAAAATG-3'. Cells were transfected with sh-HULC or sh-NC using Lipofectamine 2000 reagents according to the manufacturer's instructions (Life Technologies).

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was isolated and reverse-transcribed to cDNA using the RNeasy system (Qiagen) according to the manufacturer's protocol. RNA was eluted with 100 µl of RNase-free water and stored at -80°C. Real-time PCR was performed in triplicate using the QuantiFast SYBR Green PCR kit (Qiagen) with a Bio-Rad C1000 Thermal Cycler. GAPDH was used as an endogenous control. The primers used are listed as follows: HULC, sense 5'-ACCTCCAGAACTGTGATCCAAAATG-3', antisense 5'-TCTTGCTTGATGCTTGGTCTG-3'; GAPDH sense 5'-CCCATCACCATCTCCAGGAG-3', antisense 5'-GT-TGTCATGGATGACCTGGC-3'.

Cell proliferation assay

Cell proliferation ability was measured with the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's protocol. Briefly, cells transfected were measured at 24, 48 or 72 h, the supernatant of each group was removed, and cells were incubated in DMEM medium containing CCK-8 for another 2 h at 37°C. The optical density (OD) value for each well was read at 450 nm using an automated microplate reader. All the experiments were repeated three times.

Flow cytometry for cell cycle

After transfection for 48 h, cells were fixed with cold absolute ethanol overnight. Cells were subsequently treated with RNase A (KeyGEN) and stained with propidium iodide (PI, Sigma) in the dark at room temperature according to the manufacturer's protocol. The cell cycle was determined by flow cytometry (Beckman). The proportion of cells in G0/G1, S and G2/M phases was calculated using FlowJo software.

Transwell invasion assay

The invasion ability was assessed using 24-well Transwells (8 µm pore size; Corning) coated

with 1 mg/ml Matrigel (BD Sciences). The cells were seeded in the upper chamber of the wells in 100 μ l of FBS-free medium, and 500 μ l of 20% FBS medium was added to the lower chambers. Following incubation for 48 h, the cells remaining on the upper membrane were removed with cotton wool. Cells that had invaded through the membrane were stained with methanol and 0.1% crystal violet and photographed with a phase-contrast inverted microscope (Olympus). Cell numbers were counted in 6 random fields per well.

In vivo tumorigenesis assay

In vivo tumorigenesis assays were performed as described previously [11]. Briefly, 1×10^6 cells were subcutaneously injected into the right flanks of nude mice. Tumor length (L) and width (W) were measured every 1 week, and tumor volume was calculated using the following equation: volume = $(W^2 \times L)/2$. After 6 weeks, the mice were sacrificed, and tumor volume and weight were measured. All animal experiments were performed with the approval of the Henan University of Science and Technology's Animal Care and Use Committee.

Western blot

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer) with protease and phosphatase inhibitors (Roche). Equal amounts of the protein were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose (NC) membranes and incubated with the primary antibodies (Abcam). The primary antibody incubation for 12 h was followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 2 h. The bound antibodies were detected using enhanced chemiluminescence reagent (Thermo Fisher Scientific).

Statistical analysis

All statistical analysis of this research was carried out using SPSS 18.0 statistical software. The result was expressed as mean \pm standard deviation. Significance of differences between groups was tested using Student's *t*-test and the χ^2 test. A log-rank test was used to analyze the statistical differences in survival based on Kaplan-Meier curves. *P*-values less than 0.05 were considered to be statistically significant.

Results

Upregulation of HULC in prostate cancer and its correlation with clinicopathologic features

The expression of HULC was examined by qRT-PCR in PCa tissues and adjacent non-tumor tissues

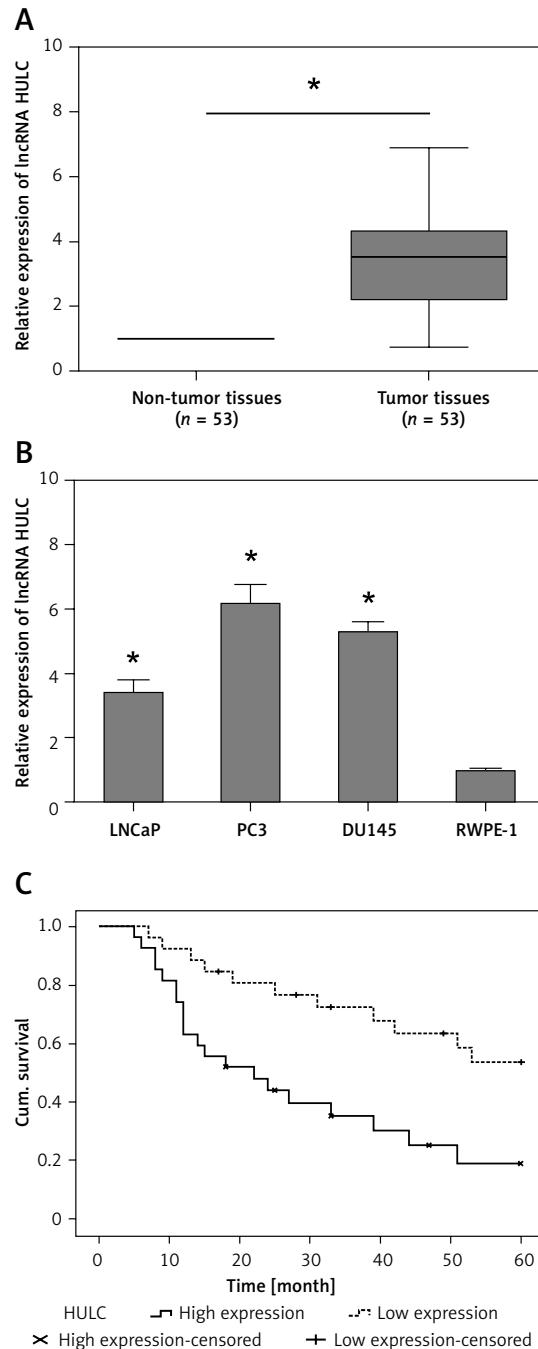


Figure 1. HULC was upregulated in PCa and associated with poor overall survival. **A** – The expression of HULC was determined in PCa tissues and adjacent non-tumor tissues by qRT-PCR. **B** – The expression of HULC was determined in PCa cell lines (LNCaP, PC3 and DU145) and a normal prostate cell line (RWPE-1) by qRT-PCR. **C** – Overall survival of PCa patients with high HULC expression was significantly lower than that in patients with low HULC expression

**P* < 0.05.

from 53 PCa patients. As shown in Figure 1 A, the HULC expression was upregulated in PCa tissues compared with adjacent non-tumor tissues

($p < 0.05$). Then, we evaluated the expression of HULC in PCa cell lines (LNCaP, PC3 and DU145) and a normal prostate cell line (RWPE-1) by qRT-PCR. As shown in Figure 1 B, the expression of HULC was upregulated in PCa cell lines compared to RWPE-1 cells ($p < 0.05$). According to the median value of HULC expression in tumor tissues, PCa patients were divided into two groups: the high HULC group ($n = 27$) and the low HULC group ($n = 26$). Correlation analysis showed that high HULC expression was correlated with advanced histological grade and lymph node metastasis (Table I, $p < 0.05$). However, HULC expression was not associated with other parameters such as age, tumor size and so on (Table I, $p > 0.05$). Moreover, Kaplan-Meier analysis showed that high HULC expression was negatively correlated with poor OS in PCa patients (Figure 1 C, $p < 0.05$). Thus, these findings suggested that HULC could play a critical role in the development and progression of PCa patients.

HULC inhibition suppresses PCa cell proliferation and invasion *in vitro*

To explore the role of HULC on PCa cell proliferation and invasion, the loss-of-function was performed by shRNA against HULC. QRT-PCR showed that HULC expression was obviously downregulat-

ed in PCa cell lines transfected with sh-HULC compared to cells transfected with sh-NC (Figure 2 A, $p < 0.05$). The CCK-8 assay showed that cell proliferation was significantly decreased when HULC was knocked down in PCa cells compared with the control group (Figure 2 B, $p < 0.05$). To determine whether the function of HULC in PCa cell proliferation involved altering cell-cycle progression, cell-cycle analysis was performed. Flow cytometric analysis showed that PCa cells transfected with sh-HULC were significantly stalled at the G1/G0 phase compared to cells transfected with sh-NC (Figure 2 C, $p < 0.05$). In addition, we detected the effect of HULC on PCa cell invasion ability. The Transwell invasion assay showed that HULC inhibition significantly reduced cell invasion ability compared with the control group (Figure 2 D, $p < 0.05$). Those results suggested that HULC could promote PCa cell proliferation and invasion *in vitro*.

HULC inhibition decreases xenograft tumor growth in mice

To explore whether HULC knockdown could inhibit tumor growth *in vivo*, PC3 cells stably transfected with sh-HULC or sh-NC were subcutaneously inoculated into nude mice. As shown in Figures 3 A and B, tumor growth in the sh-HULC

Table I. The relationship between HULC expression and clinicopathologic features of PCa patients

Clinicopathologic features	Total	lncRNA HULC expression		<i>P</i> -value
		Low	High	
Age:				
< 65	19	8	11	0.449
≥ 65	34	18	16	
Tumor diameter [cm]:				
< 2.5	26	14	12	0.494
≥ 2.5	27	12	15	
Histological grade:				
I + II	24	18	6	0.019
III + IV	29	8	21	
Gleason score:				
≤ 6	17	10	7	0.328
≥ 7	36	16	20	
Lymph node metastasis:				
Negative	40	23	17	0.031
Positive	13	3	10	
Surgical margin status:				
Negative	37	21	16	0.088
Positive	16	5	11	

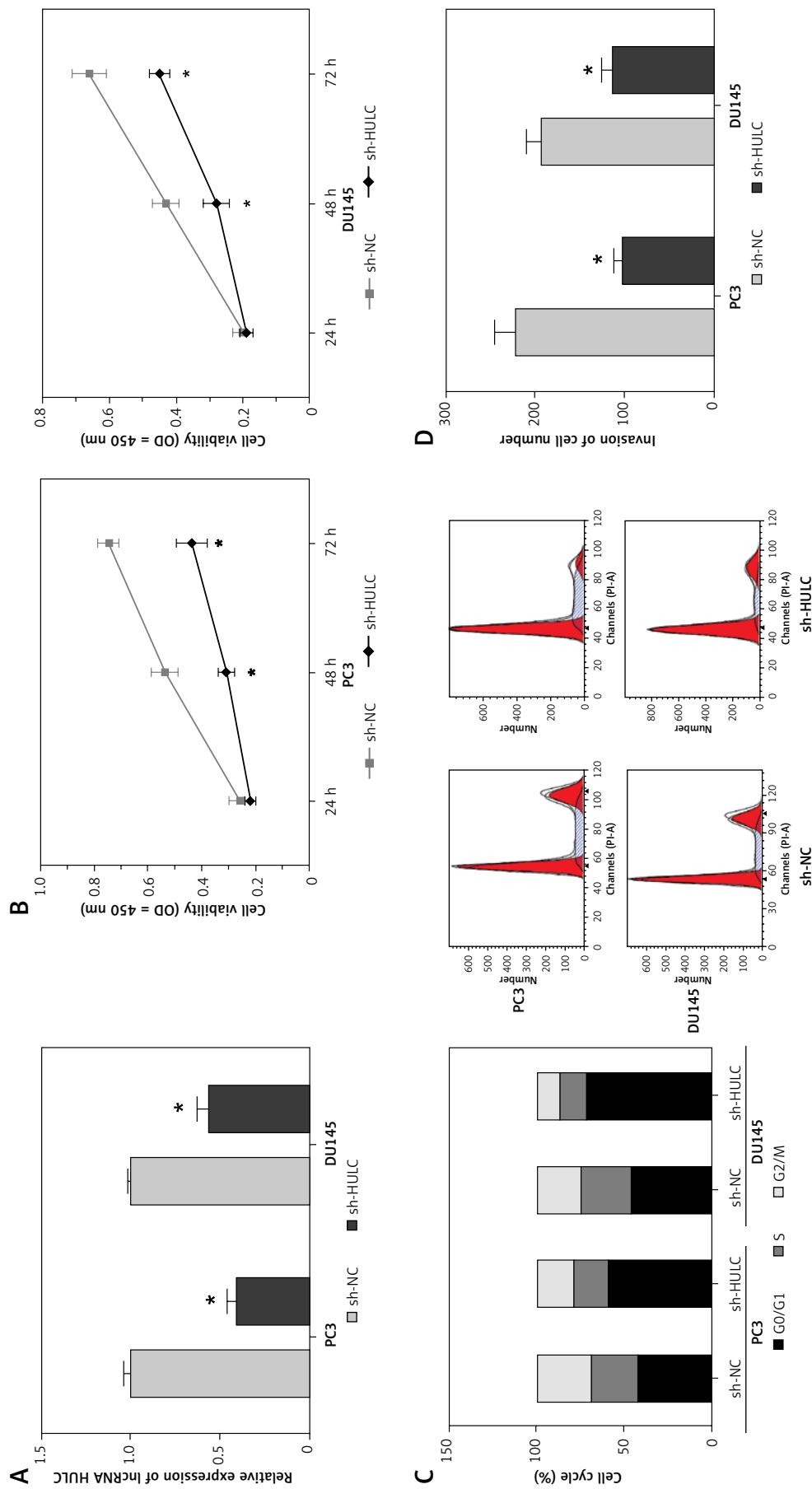


Figure 2. Knockdown of HULC suppressed PCa cell proliferation and invasion *in vitro*. **A** – The expression of HULC in PCa cells was significantly downregulated by sh-HULC compared with sh-NC. **B** – CCK-8 assay showed that HULC inhibition suppressed PCa cell proliferation. **C** – Cell cycle analysis showed that HULC knocked down arrested PCa cells in G0/G1 phase. **D** – Transwell invasion assay showed that silencing of HULC decreased the invasion ability of PCa cells
* $P < 0.05$.

group was significantly slower than that in the sh-NC group. Up to 6 weeks after injection, the average tumor weight in the sh-HULC group was obviously lower than in the sh-NC group (Figure 3 C, $p < 0.05$). Furthermore, qRT-PCR analysis showed that the average expression of HULC in tumor tissues was lower than in the sh-NC group (Figure 3 D, $p < 0.05$). These results revealed that HULC suppression could significantly reduce the growth of PCa cell xenografts *in vivo*.

HULC regulates epithelial-mesenchymal transition process in PCa

Epithelial-mesenchymal transition (EMT) is an important factor in tumor cell progression. Thus, we

next determined whether EMT markers were altered in our model. Thus, the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and vimentin were investigated by western blot. We found that the expression of N-cadherin and vimentin was decreased while E-cadherin expression was increased when HULC was knocked down in PCa cells. Therefore, we suggested that lncRNA HULC might promote PCa metastasis by inducing EMT (Figure 4).

Discussion

Although a growing number of novel treatment strategies have been developed for PCa, such as molecular targeted therapy and gene therapy, to our disappointment, satisfactory therapeutic out-

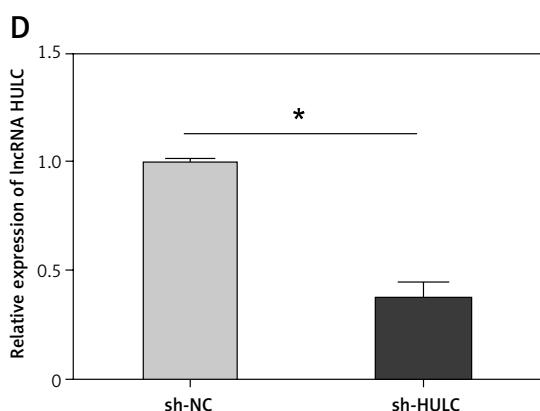
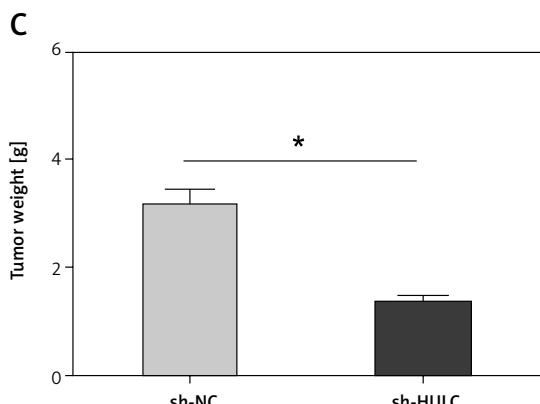
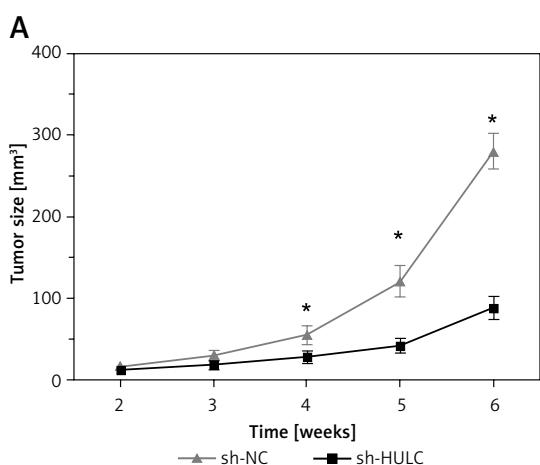


Figure 3. Knockdown of HULC inhibited tumorigenesis *in vivo*. **A** – Tumor growth curves determined after injection of PC3 cells transfected with sh-HULC or sh-NC. **B** – Representative image of tumor formation in nude mice 6 weeks after injection of sh-HULC or sh-NC cells. **C** – Tumor weight of nude mouse at the end of 6 weeks after injection. **D** – The expression of HULC in tumor tissues was detected by qRT-PCR

* $P < 0.05$.

comes have not been achieved when PCa has advanced to the castration-resistant stage [12]. Considering that the survival rate of castration-resistant prostate cancer is still low, further identification of new prognostic markers and therapeutic targets are important for the prevention and treatment of PCa. lncRNAs are a large subgroup of non-coding transcripts and are emerging as crucial regulators and prognostic markers in PCa. For example, Shukla *et al.* suggested that downregulation of lncRNA PCAT14 could predict a poor prognosis in prostate cancer [13]. Jin *et al.* found that upregulation of lncRNA PlncRNA-1 could promote proliferation and induce epithelial-mesenchymal transition in prostate cancer [14]. Zhang *et al.* showed that lncRNA UCA1 (urothelial carcinoma-associated 1) could promote cell progression by acting as a competing endogenous RNA of activating transcription factor 2 (ATF2) in prostate cancer [15].

Highly upregulated in liver cancer (HULC), located on chromosome 6p24.3 and conserved in primates, is the first identified lncRNA that is strongly overexpressed in human hepatocellular carcinoma [16]. Increasing studies have shown that HULC could be identified as a potential prognostic and therapeutic target in various cancers. For example, Sun *et al.* showed that increased expression of lncRNA HULC indicated a poor prognosis and promoted cell metastasis in osteosarcoma [17]. Lu *et al.* revealed that lncRNA HULC promoted cell proliferation by regulating the phosphoinositide-3-kinase (PI3K)/AKT signaling pathway in chronic myeloid leukemia [18]. Li *et al.* suggested that lncRNA HULC enhanced epithelial-mesenchymal transition to promote tumorigenesis and metastasis of hepatocellular carcinoma via the miR-200a-3p/ZEB1 (zinc finger E-box binding homeobox 1) signaling pathway [19]. However, the role of HULC in PCa tumorigenesis remains unclear. In the present study, we found that HULC expression was upregulated in PCa

tissues and cell lines, and high HULC expression was associated with advanced histological grade, lymph node metastasis and poor overall survival. Function assays showed that HULC inhibition significantly suppressed PCa cell proliferation and invasion and arrested PCa cells in G0/G1 phase *in vitro*. In addition, we found that HULC suppression could significantly reduce the growth of PCa cell xenografts *in vivo*. These results suggested that HULC might represent a novel indicator of poor prognosis and a potential therapeutic target in the treatment of PCa.

The EMT has been confirmed to be important in cell invasion in different cancers [20]. The EMT is closely associated with the transformation and infiltration of tumor cells. Recent studies have shown that abnormal expression of lncRNA could influence EMT in many cancers. For example, Xiong *et al.* showed that high expression of the lncRNA HEIRCC (high-expressed in renal cell carcinoma) promoted renal cell carcinoma metastasis by inducing epithelial-mesenchymal transition [21]. Zeng *et al.* suggested that knockdown of lncRNA CCAT2 (colon cancer-associated transcript 2) could inhibit cellular proliferation, invasion and EMT in glioma cells [22]. Zheng *et al.* indicated that lncRNA PVT1 (plasmacytoma variant translocation 1) could promote invasion by inducing epithelial-mesenchymal transition in esophageal cancer [23]. In our study, we found that HULC inhibition in PCa cells resulted in increased E-cadherin expression and decreased N-cadherin and vimentin expression relative to the control cells. These results indicated that HULC might promote PCa metastasis by inducing the EMT process.

In conclusion, our current study indicated that HULC overexpression was associated with PCa progression. We provided a new insight into the function of HULC in the process of PCa and suggested that HULC represented a potential prognostic and therapeutic target for PCa treatment.

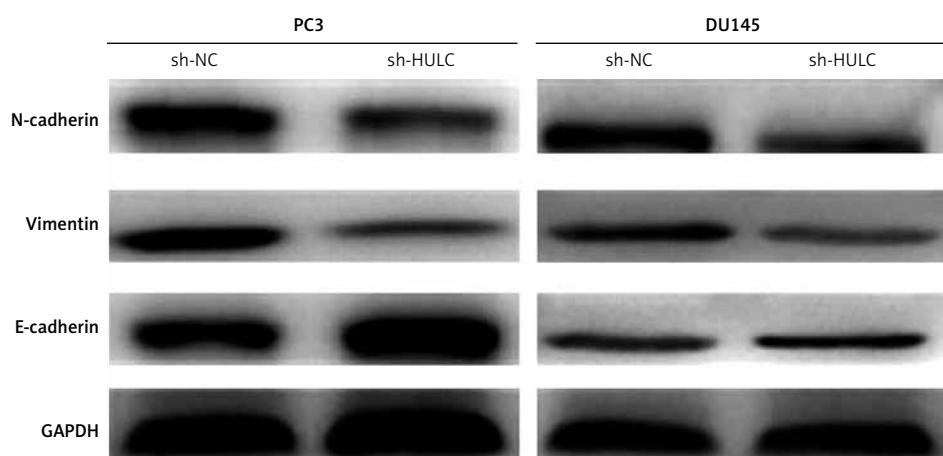


Figure 4. Effect of HULC on EMT-related gene expression in PCa cells

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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