Cancer Management and Research

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ORIGINAL RESEARCH

RETRACTED ARTICLE: LINC-PINT Inhibited Malignant Progression of Bladder Cancer by Targeting miR-155-5p

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Background: This study mainly explored the expression level of LINC-PINT in bladder cancer and its relationship with prognosis. Meanwhile, the effect of LINC-PINT on the biological function of bladder cancer was also explored.

Methods: The expression levels of LINC-PINT and miR-155-5p were detected by qRT-PCR. The prognostic significance of LINC-PINT in bladder cancer was studied by the Kaplan–Meier curve and Log rank test. CCK-8 and Transwell assays were used to analyze the proliferation, migration, and invasion ability. The targeting relationship between LINC-PINT and miR-155-5p was analyzed using bioinformatics and dual-luciferase reporter assays.

Results: The expression of LINC-PINT was down-regulated in bladder cancer tissues and cell lines, and miR-155-5p showed the opposite trend in bladder cancer tissues. Kaplan–Meier curve proved that the patients with low LINC-PINT expression had a lower five-year survival rate and the Log rank test displayed that LINC-PINT was a prognostic factor of BC. CCK-8 and Transwell results showed that LINC-PINT could inhibit the ability of proliferation, migration, and invasion. LINC-PINT was proved to target miR-155-5p in bladder cancer. Dual-luciferase reporter gene assay showed that the relative luciferase activity of overexpressed miR-155-5p co-transfected with LINC-PINT-wt was significantly lower. LINC-PINT was negatively correlated with miR-155-5p.

Conclusion: LINC-PINT is a potential prognostic marker of bladder cancer, and the up-regulation of Lin-PINT can inhibit the proliferation, invasion, and migration of bladder cancer cells by targeting miR-155-5p.

Keywords: LINC-PINT, bladder cancer, miR-155-5p, inhibited, progression

Bladder cancer (BC) is one of the most common urinary tract tumors in the clinic, and its incidence is increasing significantly. Especially in males, its incidence was the fourth with 62,100 new cases in 2020, while its incidence remains the fourth with 64,280 new cases in 2020.[1,2] It is characterized by a high recurrence rate, rapid metastasis, and poor prognosis.[3,4] The metastatic cell carcinoma accounts for the majority of the pathological types in BC, while other types of urothelial carcinoma are relatively rare. In bladder urothelial carcinoma, most of the muscle layer of invasive BC usually adopts transurethral resection for bladder tumor treatment, but it is easy to relapse and progress.[5,6] Radical cystectomy combined with pelvic lymph node dissection was the gold standard treatment for a small percentage of muscular invasive BC, but metastatic rates and mortality were generally high.[3,7] Although a variety of clinical features or molecular biomarkers have been used to predict the prognosis of BC, they all have their limitations. Therefore, the construction of new predictive models and the discovery of new prognostic markers are still helpful for patients' prognosis and the selection of treatment methods.[8–10]

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Received: 19 February 2021
Accepted: 12 April 2021
Published: 1 June 2021

Long non-coding RNAs (LncRNAs) are a class of RNAs with transcriptional quantities ranging from 20 nt to 100 kb, which do not encode proteins and regulate gene expression in the form of RNA at multiple levels, such as splicing, transcription, and genome imprinting.[11–13] Studies have found that lncRNA is involved in the physiological and pathological processes of the human body, and its abnormal expression can lead to the occurrence of a variety of diseases.[14,15] Among them, mutations of specific lncRNAs may promote the formation, progression and metastasis of tumors in many human malignancies including BC.[16,17] Based on these characteristics, we believe that lncRNA plays an important role in the occurrence, development, treatment, and prognosis of tumors. However, the function of most lncRNAs remains unclear.

LINC-PINT is a widely expressed transcription product induced by p53 in the human body.[18,19] LINC-PINT has been shown to regulate tumor cell proliferation by inducing apoptosis and DNA damage.[20,21] In addition, LINC-PINT has been found to be involved in the development of melanoma, pancreatic ductal adenocarcinoma, and ovarian cancer, among other tumors.[22–24] However, the role of LINC-PINT in BC has not been investigated. Therefore, this study first detected the expression level in BC tissu[illegible] then test its effect on the ability of proliferation, invasion and migration, the molecular mechanism of its [illegible]ulation of BC progress was also explored. LINC[illegible]INT c[illegible] be used as a prognostic indicator of BC and [illegible]n mon[illegible] the prognosis of BC patients, providin[illegible]a theo[illegible]cal basis for BC targeted therapy.

Materials and M[illegible]thods

Patients and Tis[illegible]ues C[illegible]llection

In the current study, 113 [illegible]ents wit[illegible]BC were collected at the Affiliat[illegible] Hos[illegible]tal of [illegible]eifang Medical University from Febru[illegible]y 2013 [illegible] February 2015. The BC tissues and correspondin[illegible]nal tissues were obtained by surgery and were confir[illegible]d by histopathology. All tissues were immediately placed [illegible] liquid nitrogen and stored at −80°C. None of the patients received any antitumor treatment, including radiotherapy or chemotherapy before surgery. Clinical information on enrolled subjects was collected for subsequent studies. The patients were followed up by telephone or medical record system from the first day after surgery. This study was approved by the Ethics Committee of Affiliated Hospital of Weifang Medical University and was conducted in accordance with the Declaration of Helsinki. The follow-up period was 5 years, mainly including patient review results and survival.

Cell Culture and Transient Transfection

Human BC cell lines T24, J82, SW780, 5637, and normal urothelial cell-line SV-HUC-1 were purchased from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All the cells were cultured in Dulbecco's Modified Essential Medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, NY, USA), 100 μg/mL penicillin (Sigma Aldrich, US[illegible]d 100 μg/mL streptomycin (Sigma Aldrich, US[illegible]), and incu[illegible]ted at 37° C with 5% CO_2. The medium wa[illegible]replaced on[illegible] every 2 days. The transfection exp[illegible]iment w[illegible] carrie[illegible]out when the cells grew to 80% f[illegible]ion deg[illegible]ee.

The LINC-PINT was[illegible]trod[illegible]ed into vector pcDNA3.1 to construct pcDNA[illegible]INC-PI[illegible] and th[illegible]empty vector pcDNA 3.1 was used a[illegible]a n[illegible]ative contr[illegible]C). pcDNA-LINC-PINT, NC, wildtype LINC[illegible]INT (WT-LINC-PINT) or mutant LINC[illegible]NT (MUT-LIN[illegible]PINT) were purchased from Gen[illegible]Pharma (Shanghai, China). Then, NC or pcDNA-LINC-PIN[illegible] were trans[illegible]cted into 5367 and T24 in the use of Lipof[illegible]amine [illegible]000 kit (Invitrogen, Thermo Fisher [illegible]ientific, Inc., Waltham, MA, USA), respectively.

RNA Isolation and Quantitative [illegible]eal-Time Polymerase Chain Reaction (qRT-PCR) for RNA Expression Level

Total RNA was extracted from frozen BC tissues, paracancerous tissues, and BC cell lines by TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was then performed using PrimeScript RT Master Mix (Takara Bio Inc.) or PrimeScript Reverse Transcriptase (Takara Bio Inc.), respectively. Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted at a Thermal Cycler Dice™ Real-Time System (Takara Bio Inc.) with SYBR® Premix Ex Taq™ II (Takara Bio Inc.). The internal control was GAPDH expression for LINC-PINT or U6 mRNA for miR-155. The sequences for qPCR were as follows: LINC-PINT, 5′-GAACGTGAAACTGTGGGCAC-3′ (forward) and 5′-TGCTGAGCCTCCTACCTCAT-3′ (reverse); GAPDH, 5′-GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-TTGATTTTGGAGGCATCTCG-3′ (reverse); miR-155, 5′-GCCGAGAACCCCTA-3′ (forward) and 5′-CTCAACTGGTGTCGTGGA-3′ (reverse); and U6, 5′-GCTTCGGCAGCACATATACTAAAAT-3′ (forward) and 5′-CGCTTCACGAATTTGCGTGTCAT-3′ (reverse). The

$2^{-\Delta\Delta Ct}$ method was used to quantify the expression level of the target gene.

Cell Proliferation Assay

For cell proliferation, Cell Counting Kit-8 (CCK-8) assay was conducted. T24 and 5637 cells at the logarithmic stage were collected and seeded into 96-well cell culture plates, and 3 duplicate wells were set in each group. 10 μL CCK-8 solution was added to the cells at 0, 24, 48, and 72 h. After 2 h incubation, the absorbance values of the cells in each well at 450 nm were detected.

Cell Migration and Invasion Assays

All cells were prepared into suspension with a serum-free cell culture medium. 100 μL cell suspension (1×10^5 cells/chamber) was added to the upper chamber of the Transwell chamber, and a cell culture medium containing 10% FBS was added to the lower chamber. The cells were incubated at 37°C for 24 h; then, the cells without membrane penetration were wiped off with cotton swabs and stained with crystal violet. Five fields were randomly selected under the microscope to analyze the number of cell migration. For invasion assay, before the experiment, the cells were humidified with matrix glue.

Targeted Prediction and Identification of Dual-Luciferase Reporting System

Bioinformatics software Starbase 3.0 was used to analyze the complementary binding sites of LINC-PINT and miR-155-5p. In brief, the fragment of LINC-PINT possessing the assumptive miR-155-5p binding sites was cloned into a pmirGLO Dual-Luciferase Vector (GenePharma, Shanghai, China). MUT-LINC-PINT was constructed by conjugating sequence mutation of Linc-PINT, and a luciferase reporter vector without the mutation (WT-Linc-PINT) was constructed. Mut-LINC-PINT or WT-LINC-PINT were co-transfected with miR-155 mimics (5′-UUAAUGCUAAUCGUGAUAGGGGUU-3′), miR-155 inhibitor (5′-AACCCCUAUCACGAUUAGCAUUAA-3′), mimic NC (5′-UUCUCCGAACGUGUCACGUTT-3′) or inhibitor NC (5′-CAGUACUUUUGUGUAGUACAA-3′) into T24 and 5637 cells, respectively. After 48 h of culture, luciferase activity was detected with a luciferase activity detection kit. The expression level of miR-155 in BC tissues and normal tissues was determined according to the above qRT-PCR method. Meanwhile, qRT-PCR was used to detect the changes of miR-155 expression in T24 cells transfected with control, NC, and pcDNA-LINC-PINT.

Statistical Analyses

The experimental data were analyzed using SPSS 21.0 software (SPSS Inc., Chicago, IL, USA) or GraphPad 5.0 (GraphPad Software, Inc., La Jolla, CA, USA), and the measurement data were expressed by mean ± SD. *T*-test was used to compare the data between the two groups, and one-way analysis of variance was used to compare the differences between the multiple groups. Kaplan–Meier curve and Log rank test were utilized to analyze the effect of LINC-PINT on the prognosis of patients with BC. The correlation analysis of LINC-PINT and miR-155 in BC tissue was carried out by the Spearman method.

Results

Expression of LINC-PINT in BC Tissues and Cell Lines

The average relative expression level of LINC-PINT in 131 BC tumor tissues was 0.519 ± 0.204, which was significantly lower than that in corresponding normal tissues (0.968 ± 0.2[illegible]0) ($P < 0.05$, Figure 1A). Similarly, the relative expression levels of LINC-PINT in T24, J82, SW780, and 5637 BC cell lines were markedly lower than those in normal human BC cell lines SV-HUC-1, and the differences were statistically significant. It is worth noting that the relative expression levels of LINC-PINT in 5637 and T24 cells were lower than those in the other two ($P < 0.05$, Figure 1B).

Correlation Between Expression of LINC-PINT and Clinical Data of Patients with BC

Based on the median expression level of LINC-PINT in BC tissues, the high expression group (n = 57) was greater than the median, and the low expression group (n =56) was less than the median. The low expression of LINC-PINT was positively correlated with TNM stage ($P = 0.001$) and lymph node metastasis ($P = 0.011$), but not with age, gender, tumor size and differentiation ($P > 0.05$, Table 1).

Downregulation of LINC-PINT is Associated with Poor Prognosis in BC Patients

Figure 2 shows the five-year survival curve of BC patients. The results showed that the five-year survival rate of

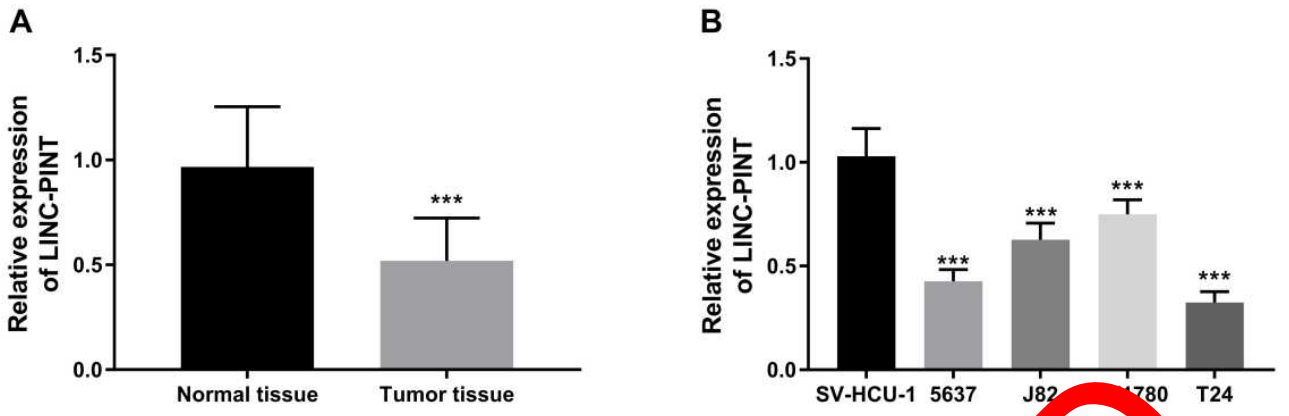

Figure 1 Relative LINC-PINT expression in BC tissues and cell lines. (**A**) The qRT-PCR analysis detected the expression level of LINC-PINT in the BC tissues and the corresponding normal tissues. (**B**) qRT-PCR analysis of LINC-PINT in BC cell lines and human normal lung cell lines. ***$P < 0.001$.

patients in the low expression group was signally lower than that in the high expression group ($P < 0.05$). In Table 2, the Cox regression analysis showed that LINC-PINT (HR = 3.327, $P = 0.029$), TNM stage (HR = 2.640, $P = 0.042$) and lymph node metastasis (HR =2.874, $P = 0.030$) were independent prognostic factors for BC.

Table 1 Correlation of the LINC-PINT Expression with Clinical Characteristics in BC

Parameters	Cases (n = 113)	LINC-PINT Expression		P
		Low (n = 57)	High (n = 56)	
Age				0.222
< 60	50	22	28	
≥ 60	63	35	28	
Gender				0.606
Male	74	37	37	
Female	39	22	19	
Tumor size				0.109
< 3 cm	48	20	28	
≥ 3 cm	65	37	28	
Differentiation				0.636
Well, Moderate	58	28	30	
Poor	55	29	26	
Lymph node metastasis				0.001
Negative	81	33	48	
Positive	32	24	8	
TNM stage				0.011
I, II	76	32	44	
III, IV	37	25	12	

The Effect of LINC-PINT on Cell Proliferation, Migration, and Invasion

CCK-8 assay was used to detect the effect of LINC-PINT on cell proliferation. Firstly, qRT-PCR results showed that pcDNA-LINC-PINT significantly increased the relative expression of LINC-PINT ($P < 0.001$, Figure 3A and B). Then the CCK-8 assay displayed that the enforced expression of LINC-PINT promoted the proliferation ability of BC cells ($P < 0.001$, Figure 3C and D). Moreover, Transwell assays were conducted for migration and invasion of BC cells. The results indicated that the number of cells that migrated and invaded decreased after overexpression of LINC-PINT. It is concluded that LINC-PINT could inhibit the migration and invasion capabilities of BC cells, compared with that of untreated cells and cells transfected si-NC ($P < 0.001$, Figure 3E and F).

LINC-PINT Targets miR-155-5p

Bioinformatics online website analysis found that miR-155-5p and LINC-PINT have multiple complementary bases, suggesting that miR-155-5p may be a potential target of LINC-PINT (Figure 4A). The dual-luciferase reporter gene experiment further proved that the relative luciferase activity of WT-LINC-PINT co-transfection with miR-155-5p mimic was significantly decreased, while the relative luciferase activity of WT-LINC-PINT co-transfection with miR-155-5p inhibitor group was increased, compared with the control group ($P < 0.001$). However, in the cells transfected with MUT-LINC-PINT, there was no significant difference in the relative luciferase activity among all groups ($P > 0.001$), as shown in

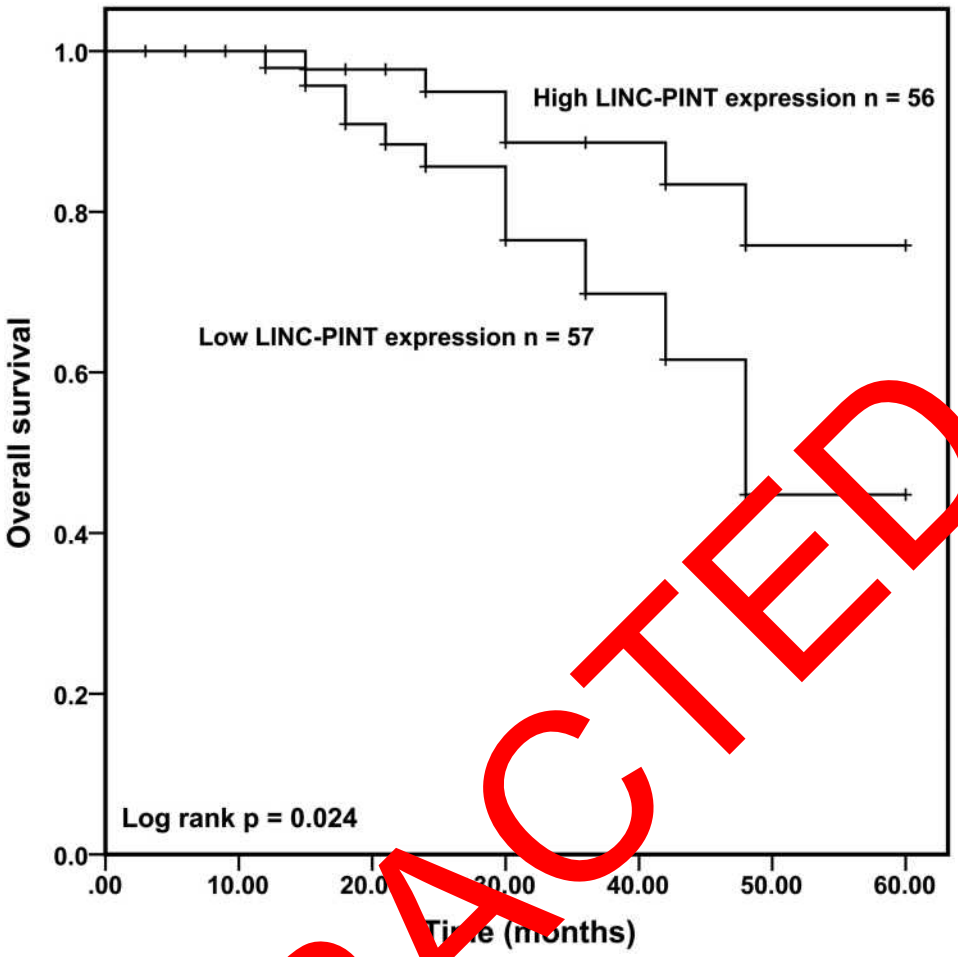

Figure 2 Kaplan–Meier curves for BC patients with high and low levels of LINC-PINT (Log rank test: $P = 0.024$).

Figure 4B. The differences in the expression level of miR-155-5p in normal tissues and BC tissues were verified by qRT-PCR, and the results showed that miR-155-5p expression was up-regulated in BC tissues ($P < 0.001$, Figure 4C). Furthermore, Spearman correlation analysis showed that the LINC-PINT was negatively correlated with the miR-155-5p in BC tumor tissues (r = −0.65, $P < 0.0001$, Figure 4D). Then, the expression level of miR-155-5p in T24 cells transfected with pcDNA-LINC-PINT, NC, and control were detected by qRT-PCR. Figure 4E shows that the miR-155-5p expression was declined when Linc-Pint was overexpressed, while there was no significant change in the control and NC groups ($P < 0.001$).

Table 2 Multivariate Cox Analysis of Clinical Characteristics in Relation to Overall Survival

Characteristics	Multivariate Analysis		
	HR	95% CI	*P*-value
LINC-PINT	3.327	1.129–9.805	0.029
Age	1.682	0.677–4.179	0.263
Gender	1.989	0.680–5.818	0.209
Tumor size	0.438	0.145–1.327	0.144
Differentiation	2.136	0.790–5.775	0.135
Lymph node metastasis	2.874	1.107–7.460	0.030
TNM stage	2.640	1.034–6.738	0.042

Abbreviations: HR, hazard ratio; CI, confidence interval.

Discussion

In this paper, the regulatory role of LINC-PINT by sponging miR-155-3p in BC was gradually confirmed. LINC-PINT could be used as a suppressor to retard BC progression. LINC-PINT has been explored in other tumors and diseases, but in BC, this is the first report to comprehensively investigate the prognostic significance of LINC-PINT and its effect on biological function.

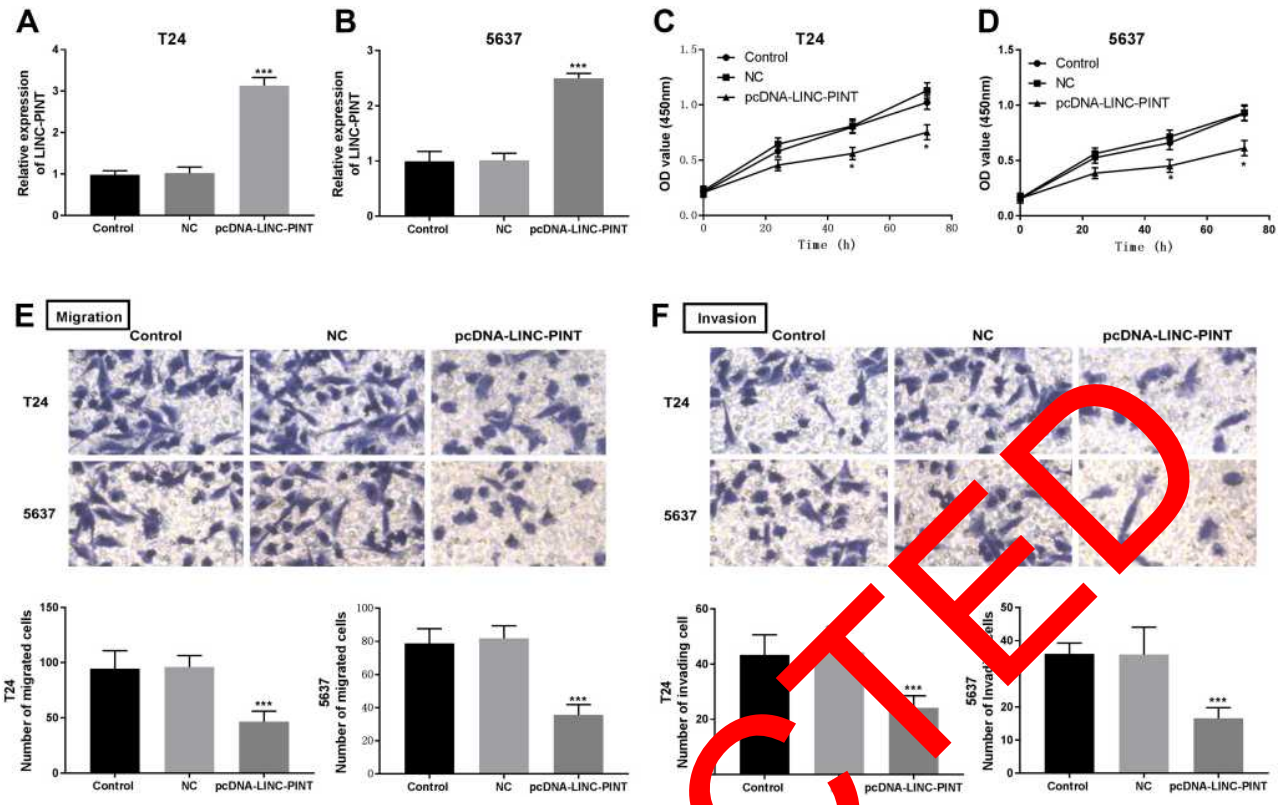

Figure 3 Effects of LINC-PINT expression levels on cell proliferation, migration and invasion abilities in T24 and 5637 cells. (**A** and **B**) The expression level of LINC-PINT was verified by qRT-PCR after transient transfection with si-NC/pc-DNA-LINC-PINT. (**C** and **D**) CCK-8 assay was performed to examine cell proliferation. (**E**) The migration abilities of T24 and 5637 cells were assessed with Transwell assay (magnification ×200). (**F**) The invasive abilities of T24 and 5637 cells were assessed with Transwell assay (magnification ×200). ***$P < 0.001$, *$P < 0.05$.

Specifically, the expression levels of LINC-PINT were dramatically decreased in BC tissues and cell lines, suggesting that LINC-PINT may play an important role in the inhibition of BC progression. Furthermore, analysis of clinicopathological features, Kaplan–Meier curve, and the Log rank test revealed that LINC-PINT was an independent prognostic factor for BC patients with low expression of LINC-PINT usually have a poor 5-year survival rate and a poor prognosis. Previous studies revealed that LINC-PINT was a tumor suppressor in gastric cancer; it participates in cellular behavior by targeting miR-21 and predicts poor prognosis in patients.[25] In pancreatic cancer, there are similar conclusions. Downregulated LINC-PINT can be used as a minimally invasive biomarker for early screening of pancreatic cancer and can also be used to predict poor prognosis in patients.[26] Therefore, combining previous studies with our present conclusion, we speculate that LINC-PINT may be a novel prognostic indicator and potential therapeutic target in BC.

In order to further study the role of LINC-PINT in BC, the effect of LINC-PINT on the proliferation, migration, and invasion of BC was further tested in this study. First, we chose to overexpress Linc-PINT in T24 and 5637 cells for cell proliferation assays. CCK-8 results showed that the proliferation ability of BC cells was significantly inhibited by overexpression of LINC-PINT. Transwell assays showed that the ability of cell migration and invasion was weaker when the expression of LINC-PINT in BC was enhanced. Therefore, these observations and data from cell assays strongly suggest that LINC-PINT might inhibit the occurrence and progression of BC tumors. The regulation results of LINC-PINT on BC cells in this study were similar to those of previous studies. In a study of LINC-PINT and ovarian cancer, scientists found that Linc-PINT inhibits the proliferation, migration, and invasion of ovarian cancer cells via sponging miR-374a-5p and is a potential marker for the treatment of ovarian cancer.[27] Besides, LINC-PINT has also been proved to inhibit the invasion through the miR-767-5p/TET2 axis in thyroid cancer.[28]

It is well known that the biological functions of lncRNAs are related to the regulation of the expression

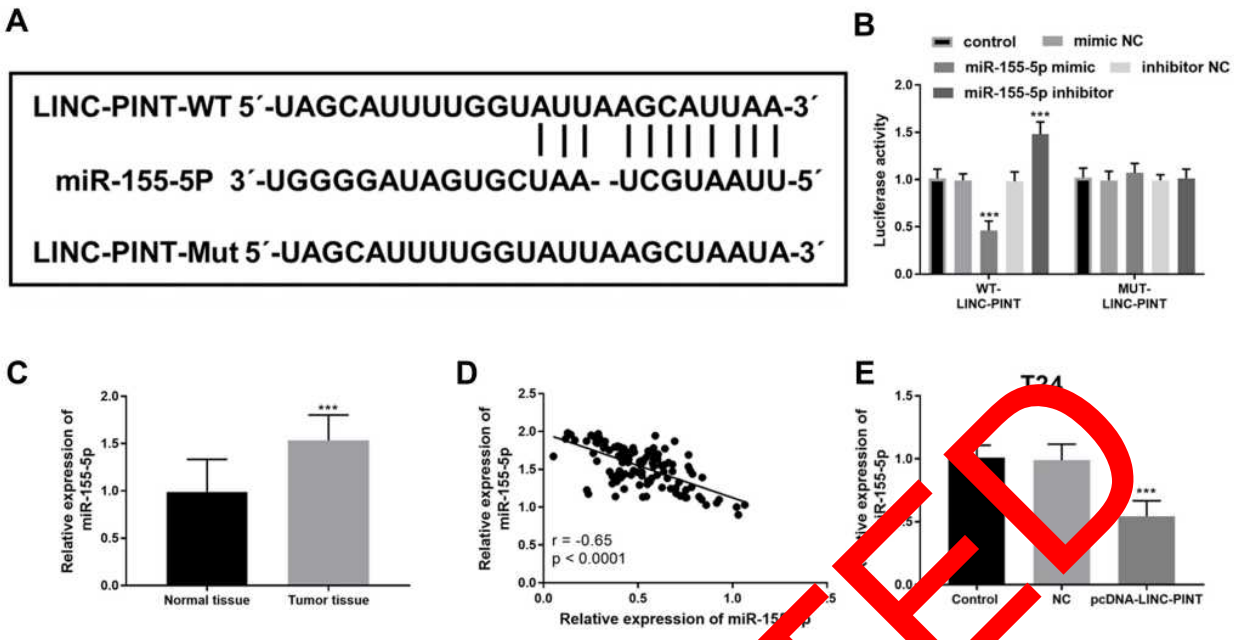

Figure 4 miR-155-5p acted as the target of LINC-PINT. (**A**) The binding sites between LINC-PINT and miR-155-5p. (**B**) Luciferase reporter assay was used to confirm the interaction between miR-155-5p and LINC-PINT. (**C**) The qRT-PCR analysis detected the expression level of miR-155-5p in the BC tissues and the corresponding normal tissues. (**D**) Correlation analysis between LINC-PINT and miR-155-5p. r = −0.65, $P < 0.0001$. (**E**) qRT-PCR was used to determine the expression of miR-155-5p, and the groups were divided into control, NC, and pc-DNA-LINC-PINT. ***$P < 0.001$.

of downstream target genes. LncRNAs have been found to play a suppressor or oncogene role by targeting the expression of miRNAs in different tumors, which could regulate various biological processes through mediating several molecular pathways.[29,30] Our experiment found that LINC-PINT had complementary binding sites with miR-155-5p, and luciferase reporter assays directly verified that miR-155-5p was the target of LINC-PINT. miR-155-5p is upregulated in a variety of tumors, including oral cancer, nasopharyngeal carcinoma, and colonic cancer.[31–33] Z-C Dong et al found that the expression of miR-155 was elevated in BC tissues, and confirmed that miR-155 increased BC cell apoptosis and decreased BC cell proliferation by inhibiting the activity of GSK-3β/β-catenin.[34] In the present study, we also confirmed that miR-155-5p was up-regulated in BC tissues, and a markedly negative correlation between miR-155-5p and LINC-PINT was also observed. LINC-PINT positively regulated the expression of miR-155-5p in T24 cells. Combining the previous study by Z-C Dong and our present results, LINC-PINT might play an inhibitory role in BC progression by sponging miR-155-5p. Numerous studies demonstrated that lncRNAs and miRNAs have a significant association with different signaling pathways, such as PI3K/Akt and STAT3 signaling, in different cancers.[29,35] STAT3 pathway plays a tumor-promoting role in BC, which contributes to the metastasis of BC cells via upregulating of MMP-2 and MMP-9, as well as genes in the EMT pathway.[36] However, LINC-PINT plays a regulatory role in BC through which pathway remains need to be investigated in future studies.

However, this study still has some limitations, such as a small sample size and no animal model validation. Aiming at the shortcomings of this study, we will expand the sample size and verify it through subcutaneous tumor formation in nude mice, to further explore the detailed mechanism of Linc-Pint regulation of BC.

In conclusion, current studies have shown that LINC-PINT is significantly down-regulated in BC tissues and cell lines, and the low expression of LINC-PINT is associated with poor prognosis of patients. LINC-PINT can target miR-155-5p to inhibit the proliferation, migration, and invasion of BC cells. LINC-PINT plays an important role in the development of BC, by providing a candidate biomarker for monitoring the prognosis of BC patients and a new idea and theoretical basis for the treatment of BC. In recent years, although a growing number of lncRNAs have been annotated, few lncRNAs were used in clinical. This study preliminarily explored the potential clinical prognostic significance of LINC-PINT in BC, which provides a theoretical basis and potential therapeutic target for

clinical treatment of BC. However, there is still a long way to go to apply this marker to clinical use.

Disclosure

The authors report no conflicts of interest in this work.

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