

Original Article

Upregulation of long non-coding RNA LINC00152 by SP1 contributes to gallbladder cancer cell growth and tumor metastasis via PI3K/AKT pathway

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Abstract: Gallbladder cancer (GBC) is one of the most lethal cancers with poor prognosis. In this study, we report that the long non-coding RNA LINC00152 is significantly upregulated in GBC tissues and cell lines. The high LINC00152 levels correlated positively with tumor status progression, lymph node invasion and TNM stage advancement. Functionally, we revealed that LINC00152 dramatically promoted cell proliferation, metastasis and inhibited apoptosis *in vitro*. *In vivo*, LINC00152 overexpression significantly promoted tumor growth. Mechanistic analyses indicated that LINC00152 could participate in the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, and transcription factor specificity protein 1 (SP1) induces its overexpression. In summary, our findings suggest that LINC00152 contributes to the oncogenic potential of GBC and SP1/LINC00152/PI3K/AKT may be a potential therapeutic target for GBC.

Keywords: Long non-coding RNA, LINC00152, gallbladder cancer, PI3K/AKT, transcription factor, SP1

Introduction

Many studies on human malignant neoplasms have indicated that the abnormal expression of non-coding RNAs (ncRNAs) that lack of protein-coding potential is involved in influencing the tumor biological behaviors [1-3]. In the ncRNA family, microRNAs (miRNAs) have been most widely explored. With the advancement in whole-genome sequencing technology, long non-coding RNAs (lncRNAs) have been identified, which are a class of ncRNAs longer than 200 nucleotides [4]. lncRNAs are frequently detected to be deregulated in many malignant tumors, which may serve as prognostic markers [5-7]. Furthermore, increasing evidence has suggested that the regulatory function of lncRNAs is more extensive and complicated than that of miRNAs, such as chromatin modulation, gene transcription, post-transcriptional modulation, protein function or localization and intercellular signaling [8, 9].

LINC00152, a 828-bp lncRNA that maps to chromosome 2p11.2, was initially detected as differentially hypomethylated during hepatocarcinogenesis [10]. Then, Ji et al. found that LINC00152 was upregulated in hepatocellular carcinoma tissues and could promote cell proliferation *in vitro* and tumor growth *in vivo* by activating the mTOR signaling pathway [11]. Besides, by silencing the expression of p15 and p21 or activating EGFR-mediated phosphatidylinositol 3-kinase (PI3K)/AKT pathway, LINC00152 could promote gastric cancer progression [12, 13]. Considering its crucial role in the pathogenesis of the cancers above, we intended to explore the role of LINC00152 in gallbladder cancer (GBC) development.

GBC is one of the most lethal cancers and the fifth common gastrointestinal tract malignancy [14]. Owing to the anatomical position and poor sensitivity to chemotherapy, GBC is usually diagnosed at its advanced stage and lacking of

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effective therapies [15, 16]. In the present study, we report the novel role of LINC00152 in GBC and show that: (a) LINC00152 is upregulated in GBC tissues and cell lines; (b) the LINC00152 level is related to tumor status, lymph node status and clinical stage; (c) LINC00152 regulates cell proliferation, apoptosis and metastasis; (d) increased LINC00152 expression promotes tumorigenicity in nude mice; (e) LINC00152 activates the PI3K/AKT pathway; (f) the transcription factor specificity protein 1 (SP1) binds to the core promoter region of LINC00152 *in vitro* and *in vivo*, and regulates its expression.

Materials and methods

Tissue specimens and clinical data

This study was approved by the Human Ethics Committee of Xinhua Hospital of Shanghai Jiaotong University School of Medicine (Shanghai, China). Forty GBC tissues and neighboring non-cancerous gallbladder tissues were obtained postoperatively from Xinhua Hospital (Shanghai, China) from July 2012 to June 2014. All samples were snap-frozen and stored in liquid nitrogen prior to RNA isolation. Each sample was confirmed by two professional pathologists. None of the patients recruited to this study had received any pre-operative treatments. GBC patients were staged according to the tumor node metastasis (TNM) staging system (the 7th edition) of the American Joint Committee on Cancer. Complete clinicopathological follow-up data of the GBC patients from which the specimens were collected were available. All patients provided written informed consent.

Cell lines and culture conditions

The immortalized human non-tumorigenic biliary epithelial cell line (H69) and GBC cell lines (EH-GB2, GBC-SD, NOZ, SGC-996) were used in this study. GBC-SD, SGC-996 and H69 were purchased from Cell Bank of the Chinese Academy of Science (Shanghai, China). NOZ was purchased from the Health Science Research Resources Bank (Osaka, Japan). EH-GB2 was a generous gift from Eastern Hepatobiliary Surgical Hospital and Institute, The Second Military University, Shanghai [17]. The four cell lines (EH-GB2, GBC-SD, SGC-996, H69) were cultured in DMEM high glucose

medium (Gibco, USA), NOZ was cultured in Williams' Medium E (Genom, China) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) at 37°C in a humidified incubator with the presence of 5% CO₂.

Total RNA isolation, reverse transcription (RT) and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from tissues and cell lines using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. The RT and qRT-PCR reactions were performed as previously described [18], and the ACTIN mRNA level was used as an internal control for normalization. The PCR primers used were as follows: 5'-AAAGACCTGTACGCCAACAC-3' (forward) and 5'-GTCATACTCCTGCTTGCTGAT-3' (reverse) for ACTIN, 5'-TGGGAATGGAGGGAAAT-AAA-3' (forward) and 5'-CCAGGAAGTGTGCTGTGAAG-3' (reverse) for LINC00152, 5'-GAGGTGGTGGATTCTTGA-3' (forward) and 5'-AGCTGGATTACAGGTGTGC-3' (reverse) for SP1. The relative mRNA expression change was calculated by using 2^{-ΔΔCt} method.

Cell transfection

LINC00152 siRNA, SP1 siRNA and negative control siRNA were purchased from GenePharma, Shanghai, China. The pcDNA3.1-SP1, pcDNA3.1-LINC00152 and the empty vector were purchased from Sangon Biotech, Shanghai, China. The siRNA sequences used were as follows: 5'-GGAAUGCAGCUGAAAGAUUTT-3' (sense) and 5'-AAUCUUUCAGCUGCAUUCCTT-3' (antisense) for si-LINC00152-1, 5'-GGUGUCUGCCUGUGAUUTT-3' (sense) and 5'-AUAUCACAGGCAGACCACCTT-3' (antisense) for si-LINC00152-2, 5'-CCAACAGAUUACACAAAUTT-3' (sense) and 5'-AUUUGUGAUAAUCUGUUGGTT-3' (antisense) for si-SP1-1, 5'-GGCUGGUGUGAUGGAAUATT-3' (sense) and 5'-UAUCCAUCAACCACAGCCTT-3' (antisense) for si-SP1-2, 5'-UUCUCCGACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense) for negative control siRNA. Cells were cultured on six-well plates to confluency and transfected with siRNA or negative control using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. After 48 hours, cells were harvested for the subsequent experiments.

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Cell migration and invasion assay

Cell migration assay was performed by using 24-well transwell plates (Corning, USA). About 1×10^5 cells per well were seeded in the upper chamber with serum free medium in triplicate. For the invasion assay, the transwell plates pre-coated with Matrigel (BD, USA) were used and 2×10^5 cells were seeded in the upper chamber with serum free medium in triplicate. Medium containing 10% FBS (300 μ l) was added to the lower chamber as chemo-attractant. After incubation for 24 h, the cells above the Matrigel layer were removed by cotton swab, and the cells below the membrane were fixed by methanol, stained with 0.1% crystal violet for 10 min, and counted from five randomly chosen fields for each well.

Cell proliferation assay

After transfected with pcDNA-LINC00152/LINC00152 siRNA or negative control siRNA for 24 h, cells were seeded into 96-well plates (1×10^3 cells/well) and the absorbance (450 nm) was measured every 24 h for 96 h. Cell proliferation was assessed at least three times independently by the water-soluble tetrazolium salt assay using the Cell Counting Kit-8 (CCK8; Dojindo, Japan) according to the manufacturer's protocol.

Luciferase reporter assay

Cells were seeded on 24-well plates and cultured to confluency. Then, pcDNA-SP1 or SP1 siRNA as well as their controls were with LINC00152 promoter construct or the LINC00152 promoter that lacking of putative SP1 binding sites construct (purchased from Sangon Biotech, Shanghai, China) and internal control pRL-TK (Promega, USA) into the cells. The luciferase activities were assessed using a dual-luciferase reporter assay kit (Promega, USA) according to the manufacturer's protocol. The relative luciferase activity was normalized to Renilla luciferase activity.

Flow cytometric analysis

After transfected with pcDNA-LINC00152/LINC00152 siRNA or negative control siRNA for 48 h, 1×10^6 cells were collected and used for analysis. For cell cycle analysis, cells were fixed by pre-cold 70% ethanol for 12 h~24 h at 4°C,

and incubated in staining solution (5 U/mL RNaseA and 10 μ g/mL propidium iodide) for 30 min at 37°C. The flow cytometer (FACS Calibur; BD Biosciences, USA) was used for assessment. For cell apoptosis analysis, cells were stained with the AnnexinV/PI double staining kit (BD Biosciences, USA) and assessed by a flow cytometer. All experiments were performed in triplicate.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assays were performed using the EZ-ChIP Chromatin Immunoprecipitation Kit (Millipore, USA). After being sonicated into the genomic DNA fragments, chromatin was immunoprecipitated by anti-SP1 antibodies (Cell Signaling Technology, USA). Normal rabbit IgG antibody (Santa Cruz Biotechnology, USA) was used as the negative control. ChIP-derived DNA was quantified by PCR using specific primers for the LINC00152 promoter. The PCR primers used were as follows: 5'-ATGGATTCTGCTCACACCCAG-3' (forward) and 5'-AAGAATTGTCGCTGCCTCCTG-3' (reverse) for the putative SP1 binding sites of LINC00152 promoter.

Lentiviral transfection for stable LINC00152 expression

LV3-pGLV-H1-GFP+Puro plasmids with pcDNA-LINC00152 or control oligonucleotides, namely LV-LINC00152 and LV-NC, were purchased from GenePharma (Shanghai, China). Lentivirus transfections were performed according to the manufacturer's protocol to establish the stable LINC00152-expressing GBC-SD cells. The control clones (GBC-SD/LV-NC) were constructed similarly. The expression level of LINC00152 was assessed by qRT-PCR.

Tumor xenograft experiment and immunohistochemistry

All animal experiments were performed in animal laboratory center of Ruijin Hospital of Shanghai Jiao-tong University School of Medicine (Shanghai, China). The study protocol was approved by the Animal Care and Use committee of Ruijin Hospital. GBC-SD cells (100 μ l, 1×10^6 cells) from stable transfected lines LV-LINC00152 and LV-NC were collected and injected subcutaneously into each 4-week-old male nude mouse (4 mice for each group). Mice were monitored daily, and the tumor volumes

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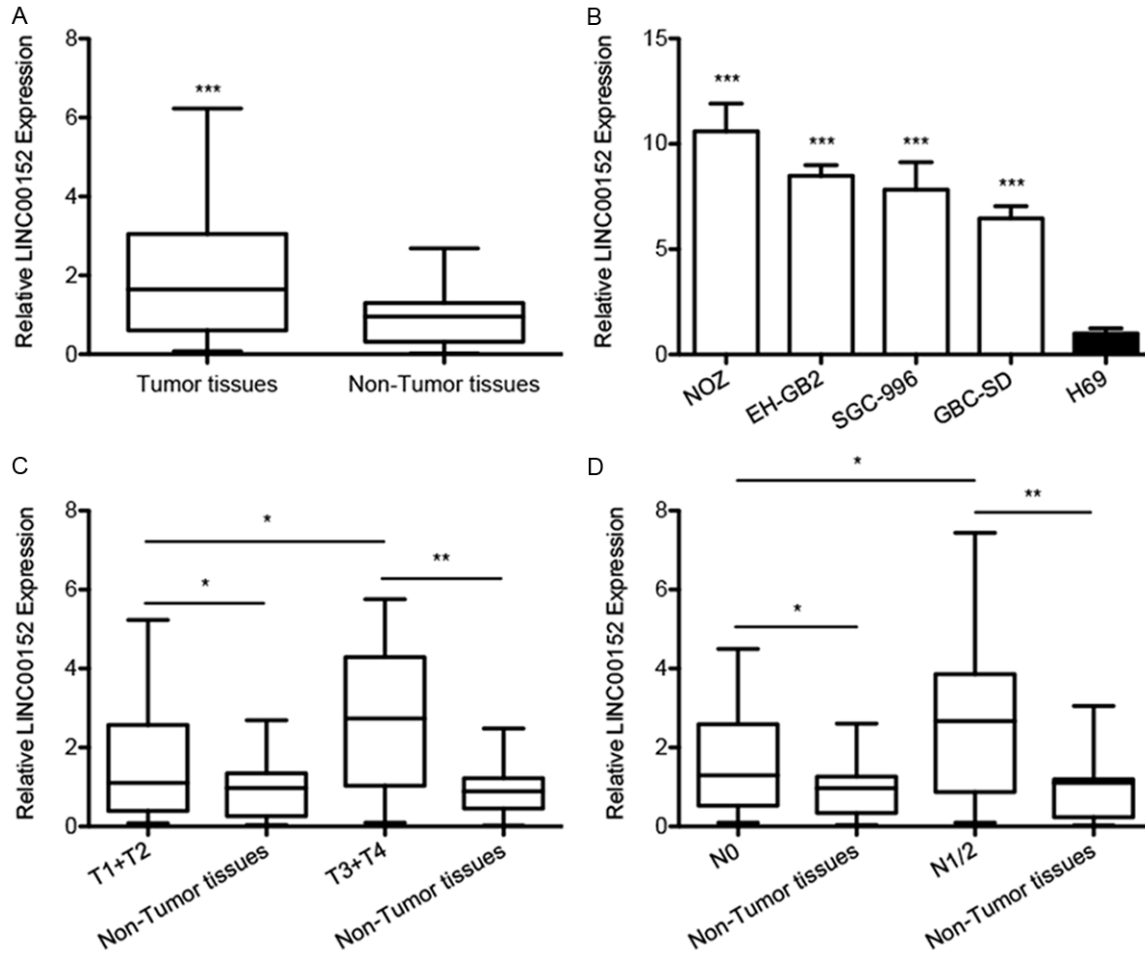


Figure 1. Upregulation of LINC00152 expression in GBC and its clinical significance. A: Relative expression of LINC00152 in 40 GBC tissues compared with matched non-tumor gallbladder tissues. *** $P < 0.001$. B: Relative expression of LINC00152 in four GBC cell lines (EH-GB2, GBC-SD, NOZ, and SGC-996) compared with the non-tumorigenic biliary epithelial cell line (H69). *** $P < 0.001$. C: Relationship between LINC00152 expression and primary tumor growth. The expression of LINC00152 in T1+T2/T3+T4 stage tumors was normalized to matched non-tumor gallbladder tissues. * $P < 0.05$, ** $P < 0.01$. D: Relationship between LINC00152 expression and lymph node metastasis. The expression of LINC00152 in N1+2/N0 stage tumors was normalized to matched non-tumor gallbladder tissues. * $P < 0.05$, ** $P < 0.01$.

were assessed ($0.5 \times \text{length} \times \text{width}^2$) per four days. After 20 days, mice were sacrificed, and all tumor grafts were excised, weighed and subjected to immunohistochemical staining of Ki-67. Anti-Ki-67 was purchased from Santa Cruz Biotechnology.

Western blot

Western blot was carried out as previously described [18]. Total protein was extracted with RIPA lysis buffer (Solarbio, China) supplemented with protease inhibitors (Roche Applied Science, Switzerland). The primary antibodies used were anti-SP1 (1:1000; Abcam, USA), anti-AKT (1:1000; Cell Signaling Technology,

USA), anti-p-AKT (1:2000; Cell Signaling Technology, USA), anti-PI3K (1:1000; Cell Signaling Technology, USA), anti-p-PI3K (1:1000; Cell Signaling Technology, USA), anti-GAPDH (1:5000; Proteintech, China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG antibody (1:1000; Beyotime, China) was used as secondary antibodies. All experiments were performed in triplicate.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 (SPSS, USA). The expression level of LINC00152 in tumor specimens was compared with adjacent non-tumor specimens using

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Table 1. Correlation between LINC00152 expression and clinicopathologic characteristics in 40 GBC patients

Characteristics	Case number	LINC00152 expression		p-value
		Low (n=17)	High (n=23)	
Gender				0.530
Male	12	6	6	
Female	28	11	17	
Age				0.218
≤60	19	10	9	
>60	21	7	14	
Histological grade				0.151
Well and moderately	16	9	7	
Poorly and others	24	8	16	
T status				0.026
T1-2	25	14	11	
T3-4	15	3	12	
N status				0.025
N0	20	12	8	
N1/2	20	5	15	
Vessel invasion				0.528
Negative	31	14	17	
Positive	9	3	6	
Clinical stage				0.015
I-II	17	11	6	
III-IV	23	6	17	

Based on the results shown in **Figure 1A**, we defined the high expression group as the ratio of the tumor/non-tumor LINC00152 expression level greater than 2 and the low expression group as less than 2.

paired samples *t*-test. The Pearson χ^2 test was applied to analyze the correlation between LINC00152 and clinicopathologic characteristics. The expression differences between high/low stages, cell lines, the expression changes after transfection, luciferase activity, cell cycle, apoptosis, migration and invasion assays were analyzed using independent samples *t*-test. All data were presented as mean \pm standard deviation. All of the *p* values were two-side and *p* values less than 0.05 were deemed statistically significant.

Results

LINC00152 is upregulated in GBC

To investigate the expression level of LINC00152 in GBC tissues, we first performed qRT-PCR in 40 pairs of tumor specimens and paired adjacent non-tumor specimens from GBC

patients. LINC00152 levels in GBC specimens were significantly higher than in paired non-tumor specimens ($P < 0.001$, **Figure 1A**). Then we assessed LINC00152 levels in four human GBC cell lines and normal biliary epithelial H69 cells, as shown in **Figure 1B**, the LINC00152 levels were generally upregulated in all GBC cell lines ($P < 0.001$). These results, taken together, indicated that LINC00152 is upregulated in GBC. In addition, we found that LINC00152 levels in tumors extending beyond the gallbladder (T3+T4) were significantly higher than tumors only detected in the gallbladder (T1+T2) ($P < 0.05$, **Figure 1C**), and LINC00152 levels in tumors spread to lymph nodes (N1/2) were also remarkably higher than tumors localized only in the gallbladder (N0) ($P < 0.05$, **Figure 1D**).

LINC00152 and clinicopathologic characteristics in GBC

To further investigate the correlation between LINC00152 and clinicopathologic characteristics in GBC patients, we divided 40 GBC patients into high LINC00152 expression group (n=23) and low LINC00152 expression group (n=17). As shown in **Table 1**, the results suggested that high LINC00152 expression correlated positively with tumor status progression ($P = 0.026$), lymph node invasion ($P = 0.025$) and TNM stage advancement ($P = 0.015$). However, there was no significant correlation between the LINC00152 expression with gender, age, histological grade and vessel invasion.

Effect of LINC00152 on GBC cell growth in vitro

To investigate the function of LINC00152 in GBC cells, plasmids containing pcDNA-LINC00152 for overexpression were transfected into GBC-SD cells which presented a relatively low level of LINC00152, LINC00152 siRNAs for knocking down its expression were transfected into NOZ cells which presented a relatively high level of LINC00152. Nearly 2700 folds LINC00152 increase in GBC-SD cells and more obvious reduction in si-LINC00152-1 NOZ cells were confirmed by qRT-PCR (**Figure 2A** and **2B**). Then we selected si-LINC00152-1 for the subsequent experiments.

As CCK8 assay indicated, LINC00152 overexpression significantly enhanced GBC-SD cells proliferation (**Figure 2C**) while LINC00152 knockdown dramatically inhibited NOZ cells proliferation (**Figure 2D**). Additionally, the per-

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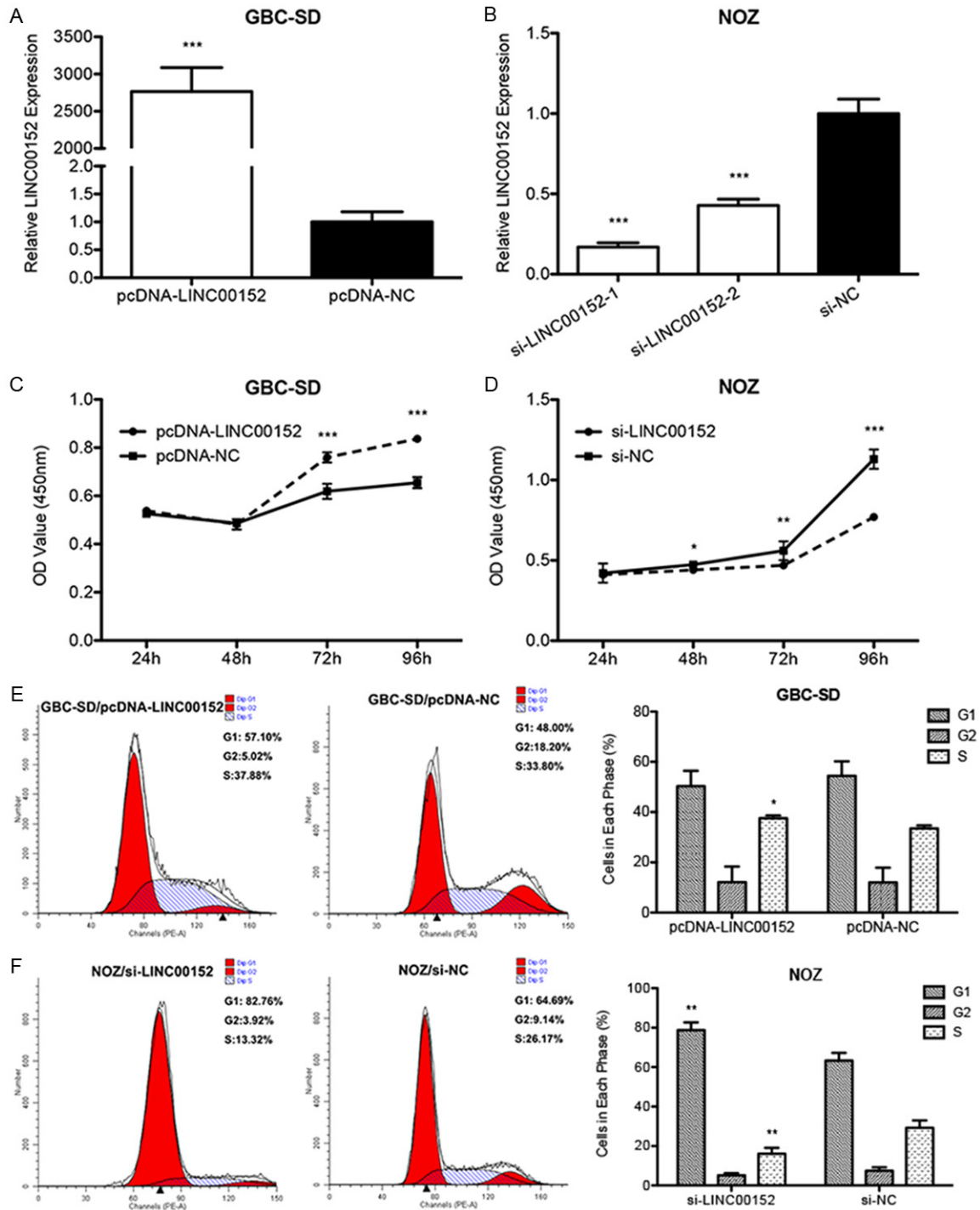


Figure 2. Effect of LINC00152 on GBC cell growth *in vitro*. **A:** The expressions of LINC00152 in cell line GBC-SD transfected with pcDNA-LINC00152 and pcDNA-NC were quantified by qRT-PCR. $***P < 0.001$. **B:** The expressions of LINC00152 in cell line NOZ transfected with si-LINC00152 and si-NC were quantified by qRT-PCR. $***P < 0.001$. **C:** Cell proliferation assay in cell line GBC-SD transfected with pcDNA-LINC00152 and pcDNA-NC were shown. $***P < 0.001$. **D:** Cell proliferation assay in cell line NOZ transfected with si-LINC00152 and si-NC were shown. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **E:** Cell cycle analyses were performed in cell line GBC-SD transfected with pcDNA-LINC00152 and pcDNA-NC. $*P < 0.05$. **F:** Cell cycle analyses were performed in cell line NOZ transfected with si-LINC00152 and si-NC. $**P < 0.01$.

centage of GBC-SD cells in S-phase increased after pcDNA-LINC00152 transfection (**Figure**

2E). On the other hand, si-LINC00152 transfection decreased the number of NOZ cells in

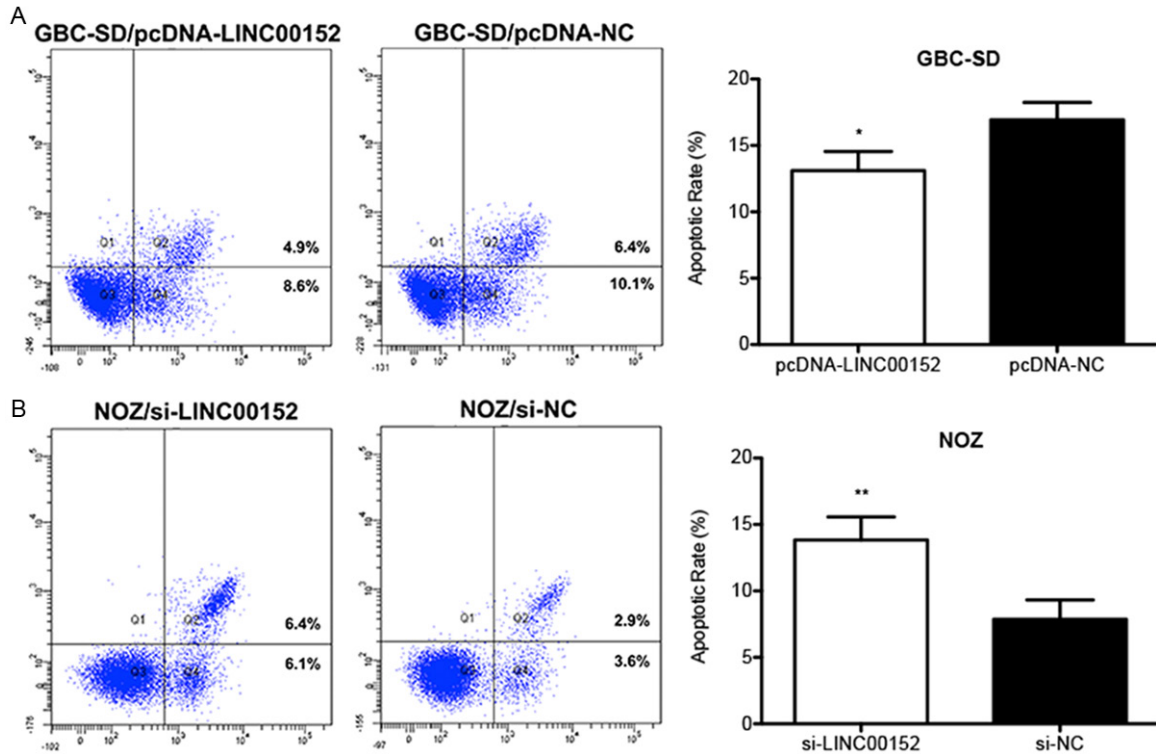


Figure 3. Effect of LINC00152 on GBC cell apoptosis *in vitro*. A: Cell apoptosis analyses were performed in cell line GBC-SD transfected with pcDNA-LINC00152 and pcDNA-NC. * $P < 0.05$. B: Cell apoptosis analyses were performed in cell line NOZ transfected with si-LINC00152 and si-NC. ** $P < 0.01$.

S-phase (Figure 2F). Taken these results together, LINC00152 is capable of promoting the tumorigenicity of GBC cells *in vitro*.

Effect of LINC00152 on GBC cell apoptosis *in vitro*

Cell apoptosis analysis was performed to elucidate whether LINC00152-mediated promotion of GBC cell growth was related the induction of apoptosis. As shown, compared with the control, pcDNA-LINC00152 transfection obviously decreased the apoptotic rate of GBC-SD cells ($13.1\% \pm 1.4\%$ vs. $16.9\% \pm 1.3\%$, $P < 0.05$, Figure 3A). Conversely, LINC00152 knockdown obviously promoted NOZ cells apoptosis ($13.8\% \pm 1.7\%$ vs. $7.9\% \pm 1.5\%$, $P < 0.01$, Figure 3B). These results suggested that LINC00152 suppresses GBC cell apoptosis *in vitro*, which may lead to the growth of GBC cell.

Effect of LINC00152 on GBC cell migration and invasion

To further explore the role of LINC00152 in GBC cell metastasis, transwell migration and invasion assays were performed. And we observed that LINC00152 overexpression both

markedly increased GBC-SD cells migration and invasion rate ($P < 0.001$, Figure 4A and 4C). However, the opposite results were obtained after LINC00152 was knocked down in NOZ cells ($P < 0.001$, Figure 4B and 4D). These results suggested a functional role for LINC00152 in promoting GBC cell metastasis.

LINC00152 promotes GBC tumor growth *in vivo*

In order to confirm the activity of LINC00152 on tumorigenesis *in vivo*, GBC-SD cells with LV-LINC00152 and LV-NC were subcutaneously injected into mice. And we observed nearly 500 folds LINC00152 increase in GBC-SD/LV-LINC00152 cells (Figure 5A). As presented in Figure 5B and 5C, we observed that tumors derived from GBC-SD/LV-LINC00152 cells grew dramatically faster than those derived from GBC-SD/LV-NC cells. The average tumor weight in LINC00152 overexpression group was remarkably heavier than the control (Figure 5D). In addition, the proliferative index of Ki-67 in tumors was examined by immunohistochemical staining. Figure 5E indicated that the Ki-67 staining positive rate was significantly higher in GBC-SD/LV-LINC00152 tumors ($P <$

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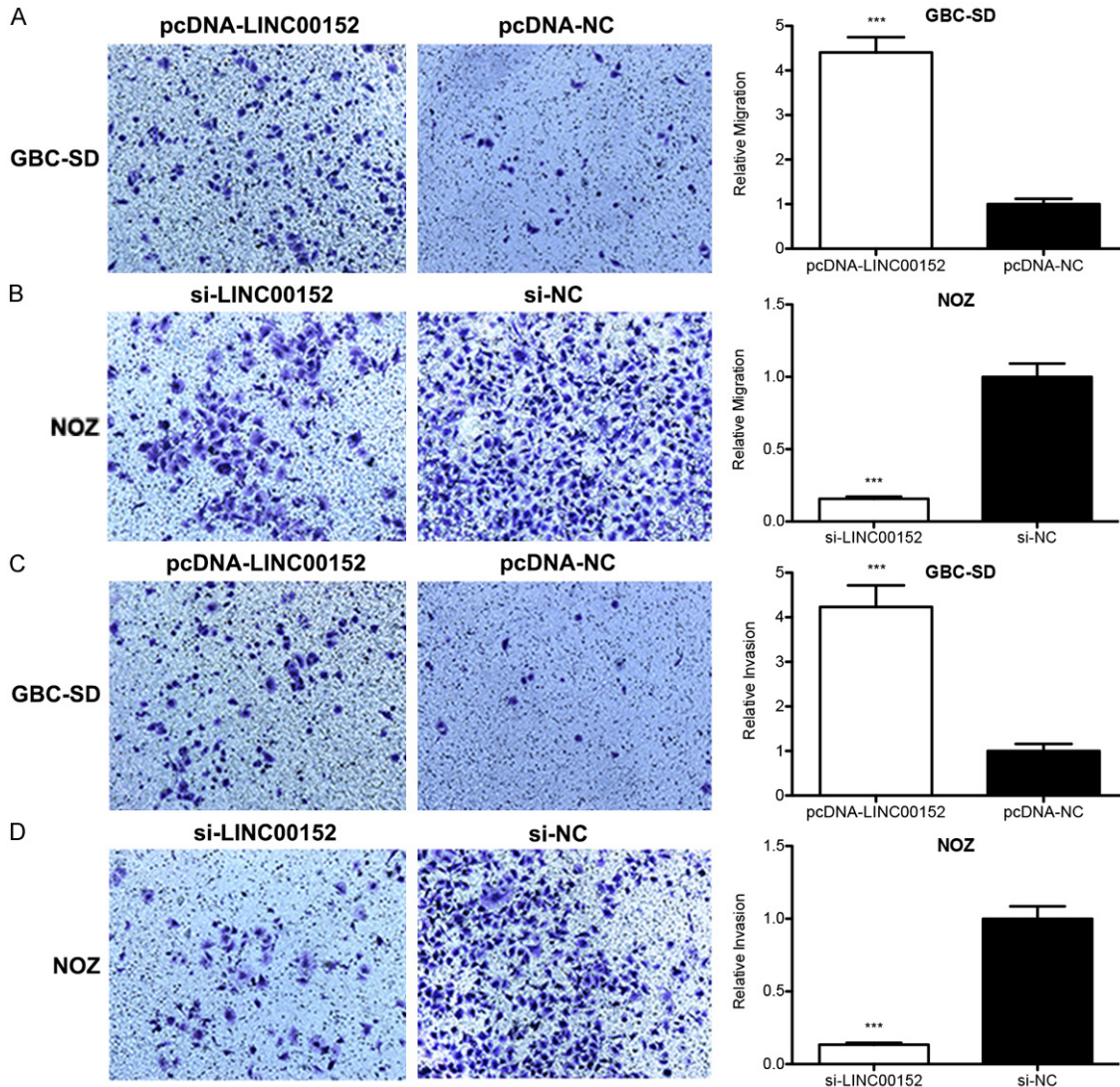


Figure 4. Effect of LINC00152 on GBC cell migration and invasion. A: Transwell migration assay in cell line GBC-SD transfected with pcDNA-LINC00152 and pcDNA-NC were shown. *** $P < 0.001$. B: Transwell migration assay in cell line NOZ transfected with si-LINC00152 and si-NC were shown. *** $P < 0.001$. C: Transwell invasion assay in cell line GBC-SD transfected with pcDNA-LINC00152 and pcDNA-NC were shown. *** $P < 0.001$. D: Transwell invasion assay in cell line NOZ transfected with si-LINC00152 and si-NC were shown. *** $P < 0.001$.

0.01). Thus, LINC00152 had capability to promote tumor growth *in vivo*.

LINC00152 participates in the PI3K/AKT signaling pathway

For some cancer cell behaviors, PI3K/AKT signaling pathway had been shown to be aberrantly activated and act as a pivotal point [19, 20]. To explore the role of PI3K/AKT signaling pathway in LINC00152 regulation, we performed western blot analysis to examine the effects of LINC00152 knockdown/overexpression on some key players in the pathway. The western blot analysis showed that both the expression

of phospho-PI3K and phospho-AKT were paralleled with LINC00152 levels, but not PI3K and AKT (**Figure 6**). These results indicated that LINC00152 might be involved in GBC cell proliferation, apoptosis and metastasis through the PI3K/AKT dependent pathway.

SP1 directly binds to the LINC00152 promoter and positively regulates its expression in GBC cell

Recently, some researchers had reported that SP1 was deregulated in cancer progression and might be an oncoprotein [21-23]. To explore whether SP1 transcriptionally regulated the

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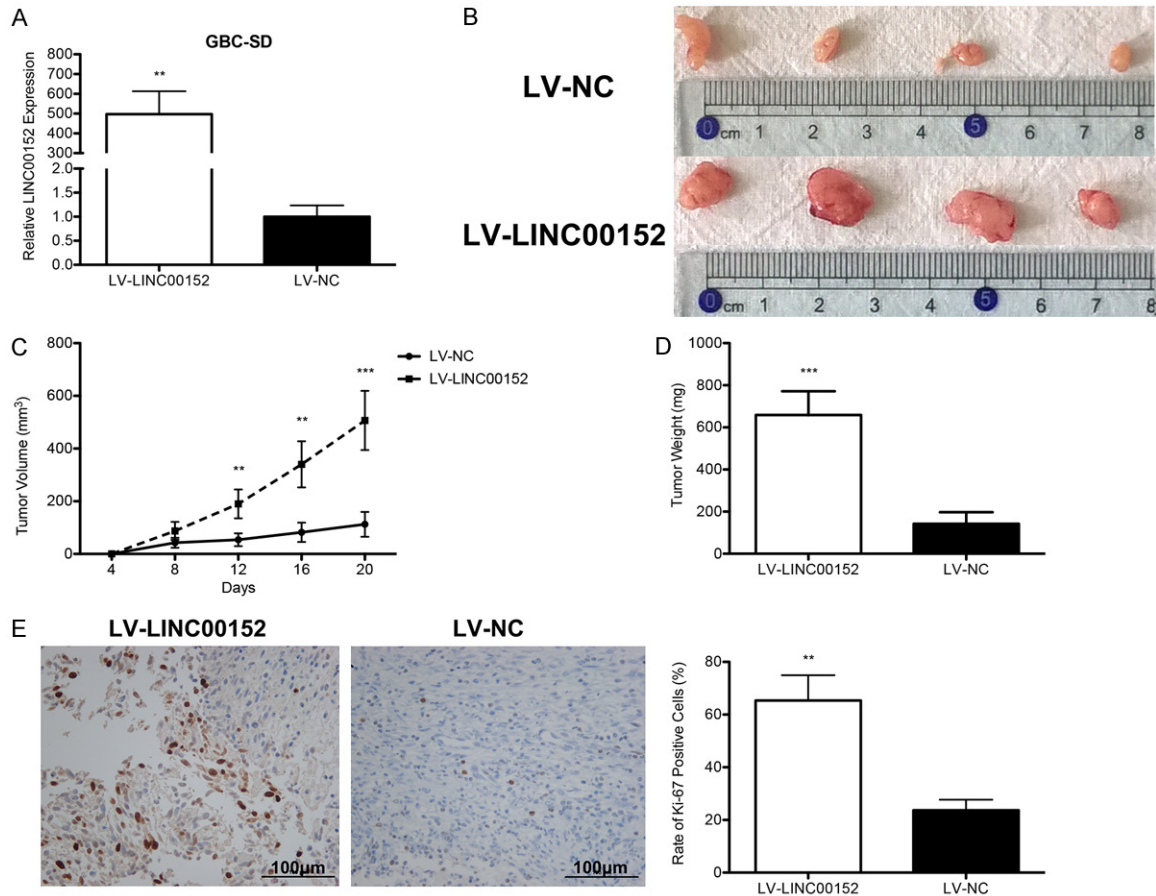


Figure 5. Elevated LINC00152 promotes GBC tumor growth *in vivo*. A: The expressions of LINC00152 in cell line GBC-SD transfected with LV-LINC00152 and LV-NC were quantified by qRT-PCR. $**P < 0.01$. B: The size of the xenograft tumor derived from GBC-SD/LV-LINC00152 and GBC-SD/LV-NC in nude mice was evaluated. C: Growth curves of tumors in nude mice. Tumor diameters were assessed per four days. $**P < 0.01$, $***P < 0.001$. D: The weight of the xenograft tumor derived from GBC-SD/LV-LINC00152 and GBC-SD/LV-NC in nude mice was shown. $***P < 0.001$. E: Representative photographs of immunohistochemical staining of Ki-67 in the xenograft tumor derived from GBC-SD/LV-LINC00152 and GBC-SD/LV-NC in nude mice. $**P < 0.01$.

expression of LINC00152, we manipulated its expression by transfecting NOZ cells with pcDNA-SP1 or SP1 siRNA. Two SP1-specific siRNAs were designed to avoid the off-target effects. Then we performed qRT-PCR and western blot assays, and confirmed the upregulation in pcDNA-SP1 cells, and more obvious knockdown in si-SP1-1 cells (Figure 7A-D). So we chose si-SP1-1 for SP1 knockdown. As to LINC00152 in NOZ cells, the results showed that ectopic SP1 enhanced its expression (Figure 7E) while si-SP1 transfection remarkably decreased its expression (Figure 7F).

To investigate the underlying mechanism of SP1-induced LINC00152 alteration in NOZ cells, we searched the sequences of LINC-

00152 promoter regions for potential SP1 binding sites from the Footer v2.0 (http://biodev.hgen.pitt.edu/footer_php/Footer_v2_0.php) and found two putative SP1 binding sites at the regions -1908 to -1903bp (CCTCCC, putative SP1 binding site-1), -1870 to -1865bp (CAGCCC, putative SP1 binding site-2) (Figure 7G). SP1 binds to GC-rich sequences that are needed for the expression and regulation of a variety of genes [24, 25]. Since the two putative SP1 binding sites were too close, we cloned LINC00152 promoter (-2000 to 0bp) or LINC00152 promoter without putative SP1 binding sites (-1750 to 0bp) into the pGL3 basic firefly luciferase reporter, and co-transfected them with pcDNA-SP1 or si-SP1 into NOZ cells respectively. Consistent with the qRT-PCR results above,

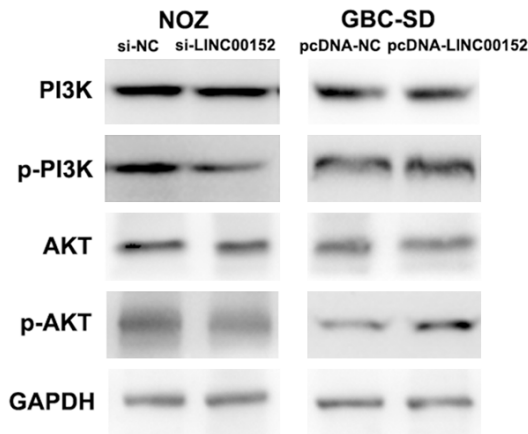


Figure 6. LINC00152 participates in the PI3K/AKT signaling pathway. Western blot was performed to assess the activation of PI3K/AKT signaling pathway by detecting the PI3K, p-PI3K, AKT and p-AKT in NOZ and GBC-SD cell lines.

Figure 7H and **7I** indicated the luciferase activity of LINC00152 promoter was paralleled with SP1 expression. While, the function of SP1 did not work in the LINC00152 promoter without the two putative SP1 binding sites (**Figure 7J** and **7K**). Furthermore, to verify a direct interaction between SP1 and the putative SP1 binding sites, the ChIP assay was carried out. In accordance with these findings, the binding of SP1 to the LINC00152 promoter region containing the two putative SP1 binding sites was observed in NOZ cells (**Figure 7L**). Taken together, these results demonstrated that SP1 acted as a direct transcriptional factor of LINC00152, and positively regulated its transcription in NOZ cells.

Discussion

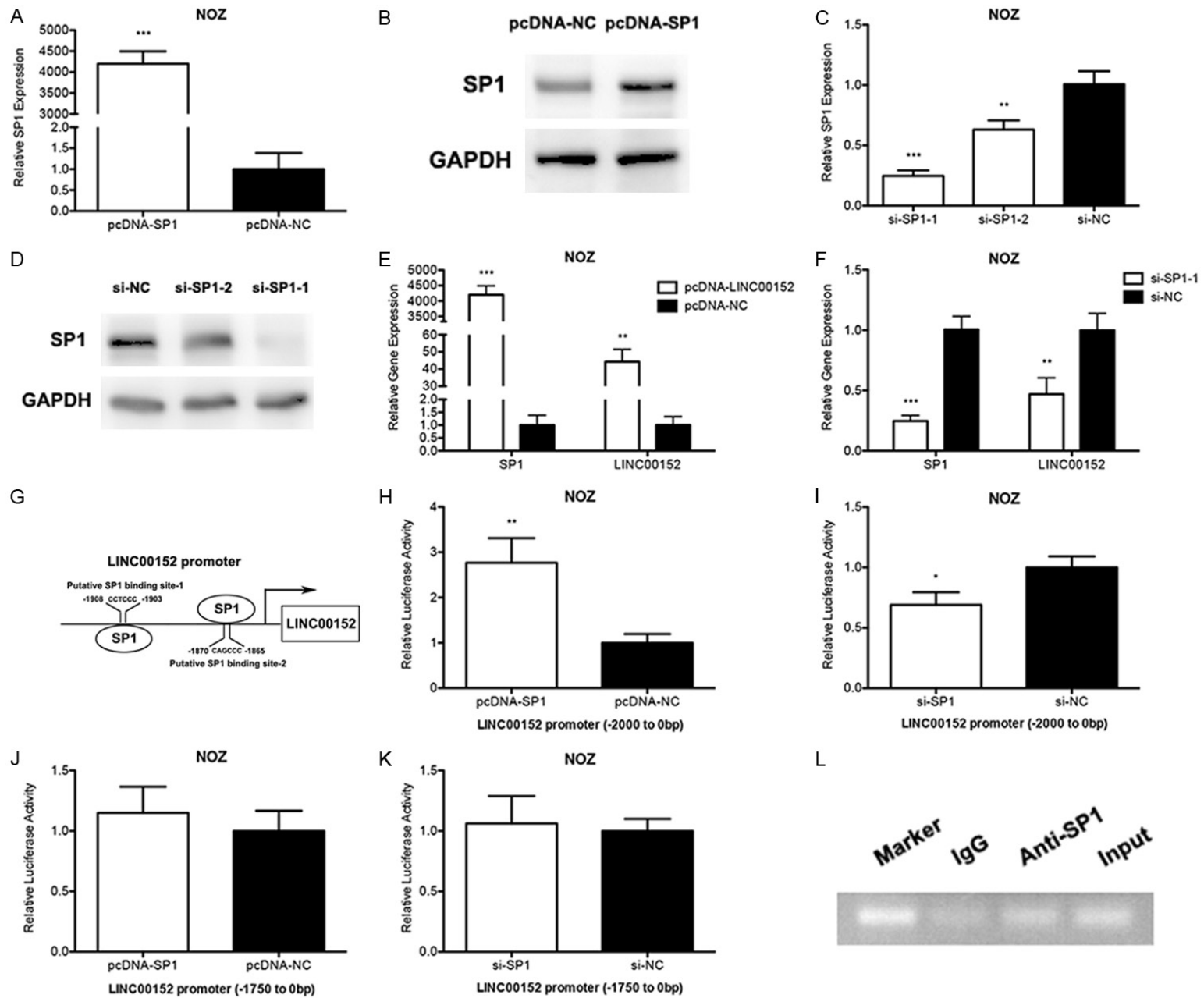
LINC00152 is a relatively novel lncRNA that has been reported to be upregulated and correlated with tumor proliferation or metastasis in hepatocellular and gastric cancer [11-13, 26-28]. However, its expression levels and functional roles in GBC are still not elucidated. By collecting a considerable number of GBC tissues and their corresponding clinical data in this study, we firstly demonstrate that LINC00152 is markedly upregulated in GBC patients and the high LINC00152 level is related to tumor status progression, lymph node invasion and TNM stage advancement. However, the survival of GBC patients could not be analyzed, on account of the cases were collect-

ed in the recent four years. By manipulating LINC00152 expression, we observed its positive effects on cell proliferation both in GBC cell and xenograft model. While, the functional studies on cell apoptosis indicated that LINC00152 might promote GBC cell growth by inhibiting cell apoptosis *in vitro*. Furthermore, we also uncovered its functional role in inducing GBC cell metastasis through transwell migration and invasion assays. Taken these results together, we claim that LINC00152 may act as a tumor-inducing lncRNA in GBC development.

Mechanically, PI3K/AKT signaling pathway is regarded as a key driver in carcinogenesis, the activation of PI3K triggers the recruitment of AKT and phosphorylates AKT to phospho-AKT (activated) [29]. The AKT, also known as protein kinase B, is a signaling hub in cancer cells: it phosphorylates a plenty of downstream targets that link it with other interrelated pathways, and therefore participates in modulating multiple processes including cell cycle progression, cell survival, migration, invasion, angiogenesis and protein synthesis [29-31]. Additionally, induced by some stimuli such as activation of FAS signaling and cell cycle discordance, the activated PI3K/AKT pathway also will inhibit cell apoptosis [32]. Zhou et al. had reported that LINC00152 could directly bind with EGFR that led to an activation of PI3K/AKT signaling pathway in gastric cancer [28]. To investigate whether LINC00152 regulates GBC cell growth through PI3K/AKT signaling pathway, we modulated LINC00152 expression in GBC-SD and NOZ cells. Interestingly, the present results showed the similar phenomenon in GBC that both the expressions of phospho-PI3K and phospho-AKT were paralleled with the expression of LINC00152. Therefore, we conclude that LINC00152 participates in PI3K/AKT signaling pathway and influences GBC cell biological features. However, a direct link between LINC00152 and PI3K/AKT pathway and the target of PI3K/AKT pathway in GBC remain unknown, and our further research will focus on these aspects.

As is known to all, lncRNAs have tumor-suppressive or oncogenic characteristics, and their dysregulation can be detected throughout the entire processes of cancer development. Activating the tumor-suppressor lncRNAs expression or blocking the oncogenic lncRNAs expression would limit the proliferous and metastatic

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Figure 7. SP1 directly binds to the LINC00152 promoter and positively regulates its expression in NOZ cells. A: The expressions of SP1 in NOZ cells transfected with pcDNA-SP1 and pcDNA-NC were quantified by qRT-PCR. *** $P < 0.001$. B: The expressions of SP1 in NOZ cells transfected with pcDNA-SP1 and pcDNA-NC were quantified by western blot. C: The expressions of SP1 in NOZ cells transfected with si-SP1 and si-NC were quantified by qRT-PCR. ** $P < 0.01$, *** $P < 0.001$. D: The expressions of SP1 in NOZ cells transfected with si-SP1 and si-NC were quantified by western blot. E: The expression of LINC00152 was significantly upregulated in NOZ cells transfected with pcDNA-SP1. ** $P < 0.01$, *** $P < 0.001$. F: The expression of LINC00152 was significantly down regulated in NOZ cells transfected with si-SP1. ** $P < 0.01$, *** $P < 0.001$. G: Schematic representation of two putative SP1 binding sites in the promoter of LINC00152. H: Luciferase reporter assay on NOZ cells co-transfected with firefly luciferase constructs containing the LINC00152 promoter (-2000 to 0bp) and pcDNA-SP1 or pcDNA-NC. ** $P < 0.01$. I: Luciferase reporter assay on NOZ cells co-transfected with firefly luciferase constructs containing the LINC00152 promoter (-2000 to 0bp) and si-SP1 or si-NC. * $P < 0.05$. J: Luciferase reporter assay on NOZ cells co-transfected with firefly luciferase constructs containing the LINC00152 promoter (-1750 to 0bp) and pcDNA-SP1 or pcDNA-NC. K: Luciferase reporter assay on NOZ cells co-transfected with firefly luciferase constructs containing the LINC00152 promoter (-1750 to 0bp) and si-SP1 or si-NC. L: The binding of SP1 to the LINC00152 promoter in NOZ cells was assessed by ChIP assay using an anti-SP1 antibody. SP1-enriched the promoter of LINC00152 containing the putative SP1 binding sites was amplified by PCR. Isotype IgG was used as a negative control, and the total input was used as a positive control.

characteristics of cancer cells [33]. LINC00152 had been reported for its oncogenic characteristics several times in hepatocellular and gastric cancer. However, the underlying mechanism for its overexpression has not been clarified yet. Our present research firstly points out that the transcription factor SP1 directly binds to the core promoter region of LINC00152 *in vitro* and *in vivo*, and regulates its expression. Accumulating evidence has suggested that the SP-family of proteins determines genes expression that relating to various cancers cell proliferation and metastasis [21, 23]. In patients with kinds of tumors, higher SP1 levels are considered worse prognosis [34, 35]. Hence, SP1 could be regarded as a target for GBC therapy.

In conclusion, our study has suggested that LINC00152 was upregulated in GBC and involved in tumor growth and metastasis by targeting PI3K/AKT signaling pathway. And LINC00152 overexpression is induced by the transcription factor SP1. Therefore, SP1, its targeted gene LINC00152 and PI3K/AKT signaling pathway are potential to be prognostic markers or therapeutic targets for the novel management of GBC.

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Disclosure of conflict of interest

None.

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