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LncRNA LINP1 promotes proliferation and inhibits apoptosis of gastric cancer cells by repressing RBM5

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Abstract. – OBJECTIVE: Recent studies have revealed that long non-coding RNAs (lncRNAs) play a crucial role in tumor progression. Gastric cancer (GC) is one of the common types of malignancies worldwide. This study aimed to identify the exact function of lncRNA LINP1 in the progression of GC.

PATIENTS AND METHODS: LINP1 expression in paired cancer tissues and adjacent normal tissues of GC patients was detected by Real-Time quantitative Polymerase Chain Reaction (RT-qPCR). The effect of LINP1 silence on proliferation and apoptosis of GC cells was detected. Meanwhile, the underlying mechanism of LINP1 function was explored by RT-qPCR and Western blot assay. Furthermore, tumor formation assay was performed to examine the ability of LINP1 in tumor formation *in vivo*.

RESULTS: LINP1 expression was remarkably up-regulated in GC tissues compared with adjacent normal tissues. The growth ability of GC cells was significantly inhibited after silencing of LINP1 *in vitro*. Besides, the apoptosis of GC cells was markedly increased after silencing of LINP1. The silence of LINP1 significantly up-regulated the expression of RBM5 in GC cells. Meanwhile, RBM5 expression in GC tissues was remarkably lower than that of the adjacent normal tissues. Furthermore, tumor formation assay showed that knockdown of LINP1 markedly inhibited tumor formation *in vivo*.

CONCLUSIONS: These results suggested that LINP1 could down-regulate RBM5. Meanwhile, LINP1 remarkably promoted growth ability and suppressed apoptosis of GC *in vitro* and *in vivo*. Our findings might provide a novel regulator and therapeutic strategy for GC patients.

Key Words:
Long non-coding RNA, LINP1, Gastric cancer (GC), RBM5.

Introduction

Gastric cancer (GC) is the fourth most prevalent malignant disease in the world. High morbidity of GC leads to almost 730,000 deaths annually[2]. Due to atypical and absence symptoms at the early stage, GC is usually diagnosed at an advanced stage. By the time, GC is characterized by malignant proliferation and distant metastasis. Therefore, it is urgently required to identify potential biomarkers and therapeutic targets for GC patients.

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides without protein-coding function. Recent studies have indicated that the aberrant expression of lncRNAs is closely related to biological behaviors in human cancers. LncRNA LUCAT1 contributes to the proliferation and invasion of esophageal squamous cell carcinoma[3]. LncRNA LINC00052 inhibits metastasis of hepatocellular carcinoma cells by up-regulating EPB41L3[4]. LncRNA AFAP1-AS1 inhibits apoptosis and enhances proliferation of lung adenocarcinoma cells[5]. Meanwhile, the association between lncRNA PVT1 and miR-497/HK2 axis has been found in glucose metabolism, cell motility and tumor progression of osteosarcoma[6]. In addition, lncARSR activates the PTEN-PI3K/Akt signal pathway and facilitates doxorubicin resistance of hepatocellular carcinoma. This may serve as a potential therapeutic target and prognostic biomarker[7].

The present study revealed that lncRNA LINP1 expression was significantly up-regulated in GC tissues. Moreover, *in vitro* experiments indicated that LINP1 regulated the apoptosis and proliferation of GC cells. RBM5 acts as a tumor suppressor in tumor development. Furthermore,

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we discovered that lncRNA LINP1 played its function in GC cells by regulating RBM5.

Patients and Methods

GC Patients and Cells

Human GC tissues were collected from 50 GC patients who received surgery at the Affiliated Wujiang Hospital of Nantong University. All fresh tissues were preserved at -80°C for subsequent use. Signed informed consents were obtained from all participants before the study, which was approved by the Ethics Committee of the Affiliated Wujiang Hospital of Nantong University. Shanghai Model Cell Bank (Shanghai, China) offered us HGC-27, MKN-45, SGC-7901, BGC-823 GC cell lines and normal human gastric epithelial cell line (GES). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in an incubator.

Cell Transfection

Lentivirus expressing short-hairpin R (shRNA; Biosettia Inc., San Diego, CA, US against LINP1 was synthesized by epharma (Shanghai, China). When the de e cells reached 70%, LINP1 shRNA NP RNA) and empty vector (EV) were fecte cells according to the instr ybrene (Genepharma, Shanghai, Chin sfection efficiency was detected Real-Ti ita-tive Polymerase Chai action (RT-q 48 h later.

RNA Extraction and Real Time-quantita e Polymerase Chain React

Total RN tiss and cells was extracted by the ol gent (itrogen, Carlsbad, CA, U equen extracted RNA was reverse trans omplementary deoxyrib leic ac NA) as a template. SYB Real-Time olymerase Chain Reac (RT- Ra, Dalian, China) was c ted 00 system (Applied Biosys ter City, CA, USA). Primers used for RT ere as follows: LINP1, forward: AGCCGG TACACCTTT-3′ and reverse: 5′-GGAAAGC CGTCTGTTGTT-3′; glyceraldehyde 3-phosphate dehydro PDH), forward: 5′-CCAAAATC AT-GCTGG-3′ and reverse -TGATGC G-GACTGTGGTCATTCA ecific therm cycle was as follows: pre-de n at 95 or 5 min, denaturation at 95°C fo aling at 60°C for 30 s, fo otal of 35 c

Cell Proliferat Ass

According ction Cell Proliferation Reagen MT Roche, Basel, Switzerlan e proli ransfected cells in 96-we ates was mo d every 24 h by MTT a absorbance at 490 nm was measu by yme-linked immunosorbent assay (ELISA) re tem (Multiskan Ascent, La tems, Helsink land).

lony Formation Assay

GC cells (1.5 cells/well) were first seeded 6-well pl followed by culture for 10 y, formed colonies were fixed wi aldehyde for 30 min and stained with 0.5% crystal violet for 5 min. The number f colonies containing more than 50 cells was y CANNON camera. Image-Pro Plus er Springs, MD, USA) was used for data alysis.

Flow Cytometric Analysis

Annexin-V-FITC (fluorescein isothiocyanate) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) was used for detecting the apoptosis of GC cells. Briefly, harvested cells were washed twice with ice-cold Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA), and 100 μL flow cytometry binding buffer was added. After 5 μl of Annexin V/FITC and 5 μL of Propidium Iodide (PI) were mixed, the cells were stained for 15 min in the dark at the room temperature. 400 μL of binding buffer was added in each tube. FACSCalibur flow cytometer was used to analyze the apoptosis of cells (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blot Analysis

Cells were first washed with pre-cooled PBS and lysed with cell lysis solution (RIPA; Beyotime, Shanghai, China). The concentration of extracted protein was determined by the bicinchoninic acid (BCA) protein assay kit (TaKaRa, Dalian, China). Target proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Biller-

ica, MA, USA). The membranes were incubated with primary antibodies of rabbit anti-GAPDH and rabbit anti-RBM5 provided by Abcam (Cambridge, MA, USA) overnight. On the next day, the membranes were incubated with goat anti-rabbit secondary antibody. Immunoreactive bands were analyzed by Image J software (NIH, Bethesda, MD, USA).

Xenograft Model

The research was approved by the Animal Ethics Committee of Nantong University. Transfected BGC-823 cells (6×10^5/mL) were implanted into NOD/SCID mice (6-week-old) subcutaneously. Tumor diameters were detected every 5 days. Tumor volume was calculated as the formula: volume = length × width 2 × 1/2. Tumors were extracted after 4 weeks.

Statistical Analysis

GraphPad Prism 5.0 (La Jolla, CA, USA) was used for all statistical analyses. Student's *t*-test was selected when appropriate. Experimental results were expressed as mean ± Standard Deviation (SD). *p*-value less than 0.05 was cons statistically significant.

Results

Expression Level of LINP1 i GC Tissues and Cells

First, RT-qPCR was co ted LINP1 expression in 52 pa and

GC cell lines. As a result, L ignificantly up-regulated in GC es th of the adjacent normal tissu Figure 1A) m-pared with the expressi GES cells, P1 expression was significa r in G ells (Figure 1B).

LINP1 Knockd Inh ited Cel Proliferation itro

According 1 exp ion in GC cells, BGC-823 G ere en for knockdown of L 1 *in* 1 shRNA (sh-LINP1) a mpty vector (were synthesized and tra to BGC-823 cells. Then, LINP1 expre mined by RT-qPCR (Figure 2A). Subsequent liferation assay found th ockdown of L significantly inhibited cell proliferation (Figure 2B). Furthermore, results of the colony formation assay showed the number rmed colonies was markedly ed after L 1 knockdown (Figure 2C).

LI down Promoted Apoptosis of GC Cells

Cell apoptosis assay revealed that after LINP1 ed down *in vitro*, the apoptosis rate of GC cells increased significantly (Fig- e 3).

INP1 Promoted GC Tumorigenesis a RBM5

RT-qPCR results demonstrated that the mRNA expression of RBM5 was significantly up-regulated in GC cells transfected with LINP1 shRNA

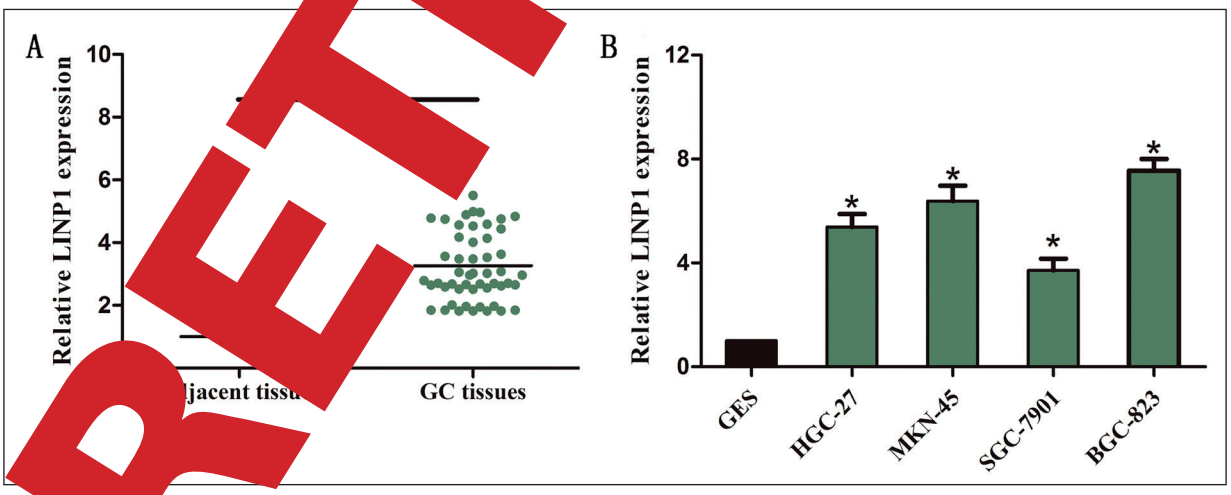

Figure sion level of LINP1 was up-regulated in GC tissues and cell lines. **A,** LINP1 expression significantly increased i s compared with the adjacent tissues. **B,** The expression levels of LINP1 relative to GAPDH were determined in i GC cell lines and GES (a normal human gastric epithelial cell line) by RT-qPCR. Data were presented as mean ± standard error of the mean. *$p<0.05$.

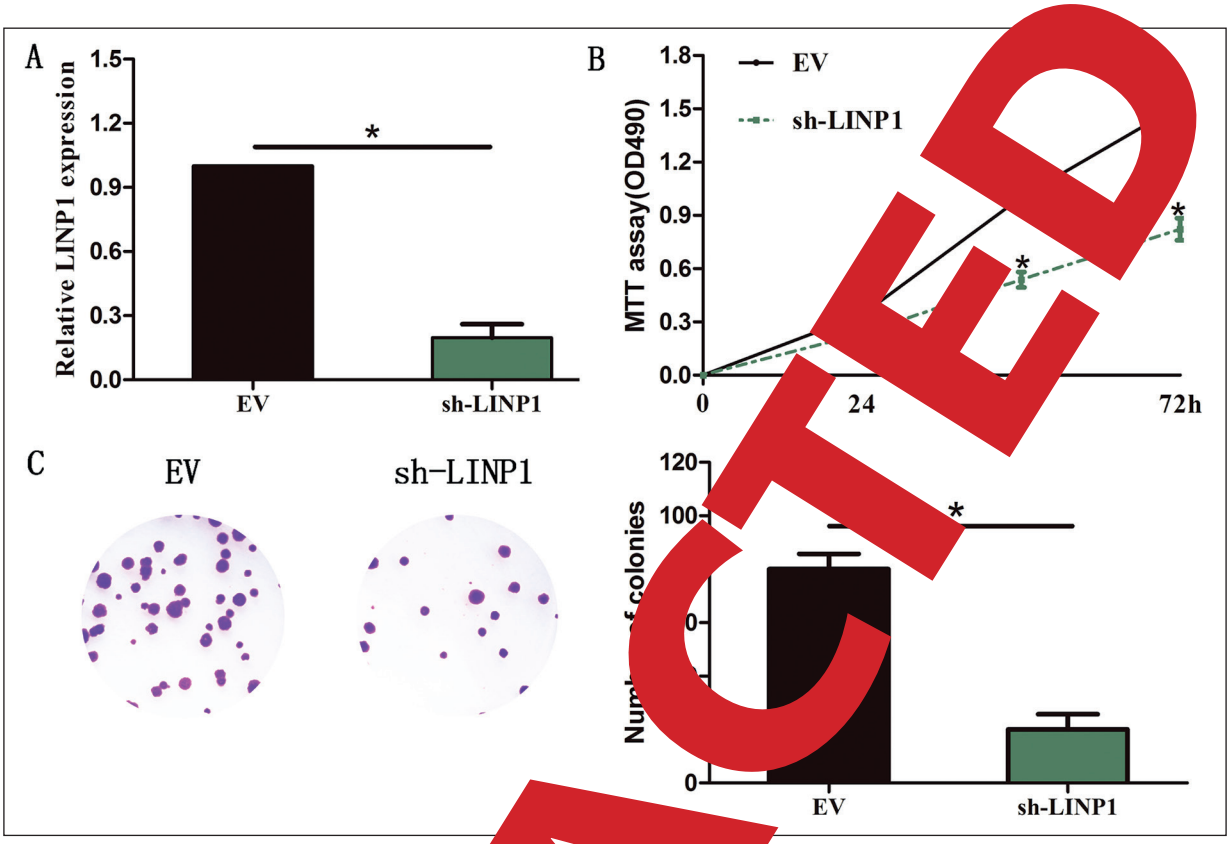

Figure 2. Knockdown of LINP1 inhibited GC cel feration expression in GC cells transduced with empty vector (EV) or LINP1 shRNA (sh-LINP1) was dete CR. GAPDH was used as an internal control. **B,** Cell proliferation assay showed that the viability of BGC-82 in LINP1 shRNA group was significantly inhibited when compared with the empty vector group. **C,** Colony forma say showed that knockdown of LINP1 markedly decreased the number of colonies in BGC-823 GC ce gnificatio The results represented the average of three independent experiments (mean ± standard error of t $p<0.05$.

(Figure 4A). Western blot its ful ther verified that the prot in ex level of RBM5 was markedly u gulated lls transfected with LINP RNA as wel ure

4B). To explore the interaction between LINP1 and RBM5, the expression level of RBM5 in GC tissues and cells was detected. As a result, RBM5 expression in GC tissues was remarkably

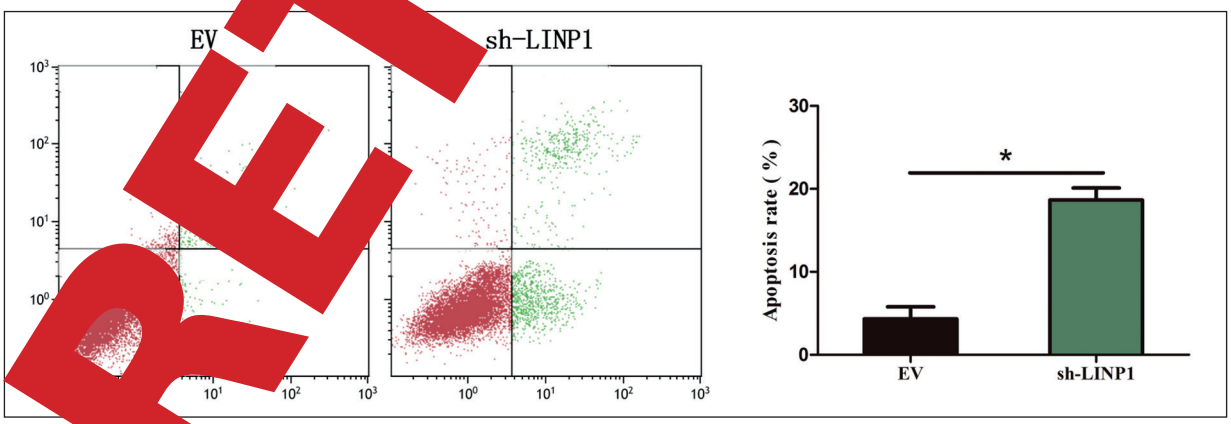

Figure 3. n of LINP1 promoted GC cell apoptosis. Apoptosis assay showed that the apoptosis rate of GC cells was remarkably pr *via* knockdown of LINP1. The results represented the average of three independent experiments (mean ± standard error o e mean). *$p<0.05$ compared with control cells.

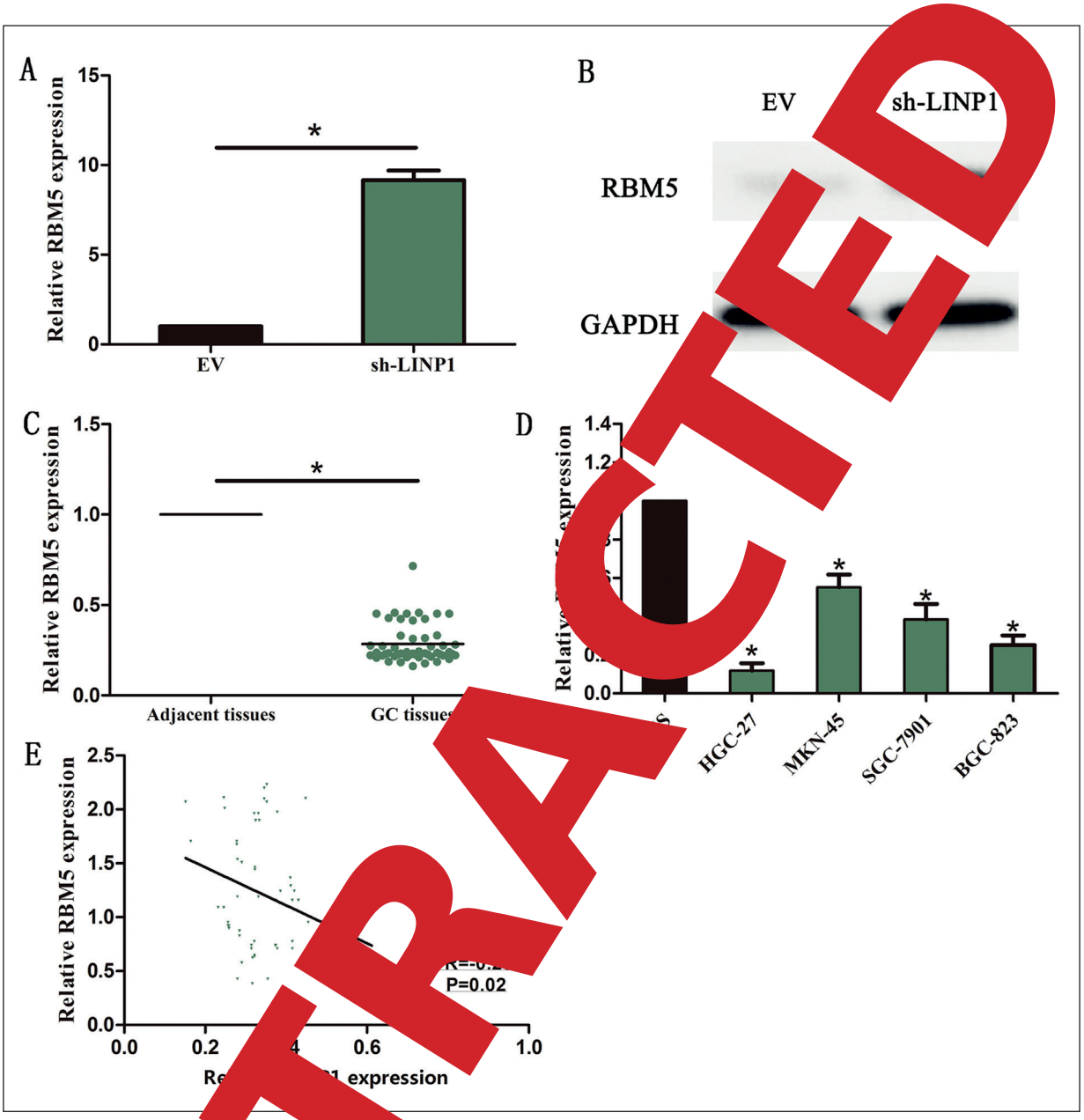

Figure 4. Interactio[n] between RBM5 a[nd] in GC. **A,** The mRNA expression level of RBM5 in BGC-823 cells of LINP1 shRNA (sh-LINP1) [gro]up was significantly [down]-regulated when compared with the empty vector (EV) group. **B,** The protein expression of RB[M5] [in]creas[ed] markedly after knockdown of LINP1 in BGC-823 cells. **C,** RBM5 was remarkably down-regulated in GC [tis]sues wh[en] [co]mpared with the adjacent tissues. **D,** RBM5 was significantly down-regulated in human GC cell lines whe[n] [co]mpared [with] GES [cells]. **E,** Correlation analysis showed that RBM5 expression in GC tissues was negatively associated wi[th] [LI]NP1 expression. [The] results represented the average of three independent experiments. Data were presented as mean ± standar[d] [fr]om the m[ean]. *$p<0.05$.

low[er] than [in] adjacent normal tissues ([P] < 4C[-2]). [The] expression in GC cells was sig[nifi]cantly lower than that of GES cells as well (Figure [4D]). Furthermore, RBM5 expression in GC tissues [was] negatively associated with LINP1 expression (Figure 4E).

LINP1 Knockdown Inhibited Tumor Formation In Vivo

Tumorigenicity assay was then performed to figure out the function of LINP1 in tumor formation *in vivo*. Tumor size in LINP1 shRNA group was significantly smaller when compared with

that of the empty vector group (Figure 5A). Moreover, the expression level of LINP1 in generated tumor tissues was detected by RT-qPCR. The results showed that LINP1 was lowly expressed in the LINP1 shRNA group when compared with the empty vector group (Figure 5B). The above results suggested that LINP1 could induce tumor formation *in vivo*.

Discussion

Accumulating evidence has indicated that a substantial portion of transcribed sequences may be non-protein-coding. They represent a higher percentage of transcribed sequences than protein coding transcripts. LncRNAs are a multifarious class of transcripts with longer than 200 base pairs in length. Meanwhile, lncRNAs have been reported to play regulatory roles in potential activity and splicing event *via* small RNA regulatory pathways. Recent studies have shown that many lncRNAs are aberrantly expressed in GC patients. Their abnormal expression is associated with pa prognosis as well. LncRNA SNHG5 se an important anti-oncogene in the progr of GC by trapping MTA2 in cytosol[8]. By cr talk with miR-21, lncRNA LINC-PINT serv as an anti-oncogene in GC, which s indicates poor survival of GC patients[9]. ession of lncRNA CTD-2510F5.4 is soci with the malignant phenotype of whic poor prognosis of GC pati studie have demonstrated that he p on and metastasis of GC can be regu RNAs. LncRNA LOC554202 enha he p tion and migration of GC cel rough the la-tion of E-cadherin and In addition, ng as a sponge of miR-49 RNA S G20 promotes the prolife ation a GC cells by up-regulat ZFX expre

Recently, lncR n NH pathway (LINP1) is up-regulated any rs. It has also been proved to par HEJ- iated DNA repair. In the pre ly, L 1 was found significantl -regula GC tissue and cells. Th e of LINP1 mor progression has att ch attention. LINP1 increases the io g resistance of cervical cancer[13,14]. LINP1 p he progression of prostat cer by regula p53[15]. LINP1 functions n oncogene in breast cancer and promotes moresistance[16]. Our results showed that after NP1 was kno d down, the growth of GC was signi tly inhibited, while cell apop- d. Besides, tumorigenesis assay *in* d that knockdown of LINP1 could markedly inhibit tumor formation *in vivo*. These data indicated that LINP1 functioned as an onco- .

binding motif 5 (RBM5) is located on e cancer inhibitor region 3p21.3[17]. In our work, we found that RBM5 was significantly down-regulated in GC tissues and cells. Previous studies ve shown that RBM5 acts as a regulator in the progression of several carcinomas. RBM5 depresses tumorigenesis of gliomas through the Wnt/β-catenin signaling[18]. RBM5 is related to poor clinicopathological characteristics of pan-

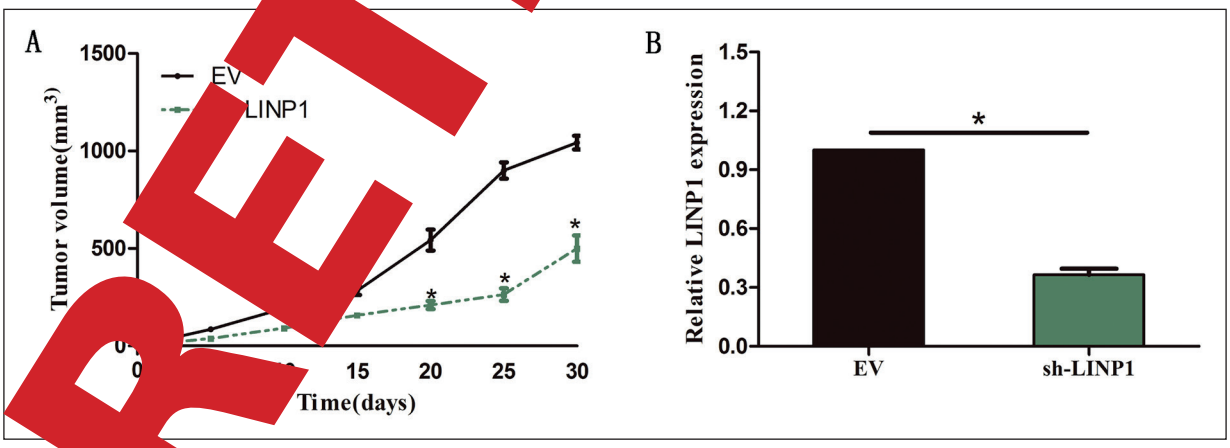

Figure 5. n of LINP1 inhibited tumor formation *in vivo*. **A,** After tumor extraction, tumor volume was calculated in empty vecto or LINP1 shRNA (sh-LINP1) group, respectively. **B,** The relative expression of LINP1 in tumors was examined by RT-q R. Data were presented as mean ± SD of three independent experiments. *$p<0.05$.

creatic ductal adenocarcinoma patients[19]. RBM5 is down-regulated in lung adenocarcinoma, which can serve as a diagnostic marker for patients[20,21]. In addition, RBM5 inhibits the progression of GC by enhancing the activity of p53 transcription[22]. In our study, the results indicated that RBM5 was remarkably up-regulated after LINP1 was knocked down *in vitro*. To further uncover the association between RBM5 and LINP1 in GC patients, we detected RBM5 and LINP1 expression in GC tissues. The correlation analysis showed that RBM5 expression in GC tissues was negatively associated with LINP1 expression. Our findings suggested that LINP1 functioned in human GC tissues by repressing RBM5.

Conclusions

LINP1 serves as a new biomarker in the development of GC, which can also be used as a promising mark.

Conflict of Interest

The Authors declare that they have no conflict of i s.

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