Original Article
Down-regulation of LINC00341 predicts a poor prognosis and acts as a tumor suppressor in gastric cancer

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Abstract: Accumulating evidence has suggested that long noncoding RNAs (lncRNAs) play critical roles in tumor cell development and in the progression of human cancer. However, the significance and role of most lncRNAs, especially long intergenic ncRNAs (lincRNAs, the main type of lncRNAs), in gastric cancer is unclear. This study aimed to identify the clinical significance and potential biological function of LINC00341 in gastric cancer. Here, a qRT-PCR assay indicated that the relative expression level of LINC00341 were significantly down-regulated in gastric cancer tissues compared to matched adjacent normal tissues. Levels of LINC00341 in gastric cancer cell lines (MGC-803, BGC-823 and SGC-7901) were also significantly lower than in human normal gastric epithelial cell (GES-1). Patient with low LINC00341 expression were found to be negatively correlated with the TNM stage and lymph node metastasis. A Kaplan-Meier analysis showed that low expression of LINC00341 was significantly correlated with shorter overall survival (OS) of gastric cancer patients. Furthermore, an *in vitro* assay indicated that the over-expression of LINC00341 inhibited cell growth and migration and induced cell apoptosis in gastric cancer. In summary, this study provides the first evidence that the down-regulation of LINC00341 predicts a poor prognosis and acts as a tumor suppressor in the carcinogenesis of gastric cancer, indicating that LINC00341 may serve as a potential therapeutic target for gastric cancer.

Keywords: lncRNAs, LINC00341, gastric cancer, prognosis, suppressor

Introduction

Gastric cancer is the second leading cause of cancer-related death and the most common gastrointestinal malignancy worldwide [1]. Most gastric cancer patients, upon receiving a confirmed diagnosis, are already at an advanced stage accompanied by malignant proliferation, lymphatic metastasis, and extensive invasion, and over half of patients relapse after surgical treatment [2, 3]. Despite the significant achievements that have been made in the treatment of early gastric cancer, the long-term survival rate for advanced gastric cancer is still unsatisfactory [4]. The 5-year overall survival rate of patients with gastric cancer is lower than 40% in China [5]. Therefore, the identification of new effective biomarkers is essential for developing novel and effective therapeutic strategies against gastric cancer.

Recent improvements in whole-genome sequencing technology have revealed that the human genome contains only ~20,000 protein-coding genes, representing <2% of the total genome, and the remainder are non-coding RNAs (ncRNAs) [6, 7]. Among them are long non-coding RNAs (lncRNAs), which are more than 200 nucleotides in length and unable to be translated into proteins [8]. As of now, over 3,000 lncRNAs have been identified and understood to participate in a large number of cell biological processes, such as growth, invasion, cell cycle, migration, and apoptosis [9]. Mounting evidence has suggested that some lncRNAs can serve as promising biomarkers and targets for novel therapeutic approaches for cancer [10, 11]. For instance, silencing MALAT1 expression in hepatocellular carcinoma is a potential anticancer therapy to prevent tumor recurrence after liver transplantation

Table 1. Relationship between expression levels of LINC00341 and clinicopathological factors of 42 gastric cancer patients

Categories	Cases	LINC00341 expression		P value
		Low (n=23, %)	High (n=19, %)	
Age (years)				0.55
<60	20	10, 23.8%	10, 23.8%	
≥60	22	13, 31.0%	9, 21.4%	
Histological type				0.39
Adenocarcinoma	38	20, 47.6%	18, 42.9%	
Other types	4	3, 7.1%	1, 2.4%	
Gender				0.32
Male	19	12, 28.6%	7, 16.7%	
Female	23	11, 26.2%	12, 28.6%	
Tumor diameter (cm)				0.49
<4	35	20, 47.6%	15, 35.7%	
≥4	7	3, 7.1%	4, 9.5%	
Invasive depth				0.47
T1+T2	31	18, 42.9%	13, 31.0%	
T3+T4	11	5, 11.9%	6, 14.3%	
TNM stage				0.02*
I+II	33	15, 35.7%	18, 42.9%	
III+IV	9	8, 19.0%	1, 2.4%	
Differentiation stage				0.43
High	15	7, 16.7%	8, 19.0%	
Moderate+low	27	16, 38.1%	11, 26.2%	
Distal metastasis				0.26
No	36	21, 50.0%	15, 35.7%	
Yes	6	2, 4.8%	4, 9.5%	
Lymph node metastasis				<0.01*
No	13	12, 28.6%	1, 2.4%	
Yes	29	11, 26.2%	18, 42.9%	

TNM: Tumor Node Metastasis. *P<0.05.

In this study, we demonstrated that the down-regulation of LINC00341 expression was a characteristic molecular change in gastric cancer and investigated the function of altered LINC00341 levels on the phenotypes of gastric cancer cells *in vitro*. Our data suggest that LINC00341 may represent a new biomarker of prognosis and is a promising therapeutic target for gastric cancer intervention.

Material and methods

Tissue collection

Forty-two gastric cancer tissue samples and corresponding non-cancerous tissue samples (located 5 cm away from the edge of the gastric cancer) were obtained from patients who had undergone surgery at the Department of Gastrointestinal Surgery, The Second Hospital of Tianjin Medical University (Tianjin, China) between January 2010 and January 2014. Tumor samples and corresponding non-cancerous tissues were confirmed by two pathologists from our hospital. None of the patients had received radiotherapy or chemotherapy before their operations. Tumor stage evaluation is based on the tumor-node-metastasis (TNM) classification system of the International Union Against Cancer (5th ed. 1997), and histological grading assessments were based on the *NCCN Clinical Practice Guidelines in Oncology*. All specimens were immediately soaked in RNAfixer Reagent (Bioteke, Beijing, China) and frozen in liquid nitrogen until RNA extraction. The study was approved by the Research Ethics Committee of The Second Hospital of Tianjin Medical University. Informed consents were obtained from all patients. The patients' clinical pathology information is presented in **Table 1**.

Cell culture

The human gastric cancer cell lines (MGC-803, BGC-823 and SGC-7901) were purchased from the American Type Culture Collection (ATCC;

[12]. UCA1 promotes cell progression in prostate cancer, and the inhibition of UCA1 expression may be a promising strategy for prostate cancer therapy [13]. PVT1 acts as an oncogene in melanoma and can be a potential diagnostic biomarker and therapeutic target for melanoma [14].

Long intergenic ncRNAs (lincRNAs) are the main type of lncRNAs. LINC00341 is part of a complex regulating transcription of MEF2C [15, 16]. Previous studies indicated that LINC00341 was down-regulated and represented a potential biomarker in many human cancers such as breast cancer, colorectal cancer, cervical cancer, and pancreatic cancers [16]. However, very little is known about LINC00341 expression and its biological role in gastric cancer pathogenesis.
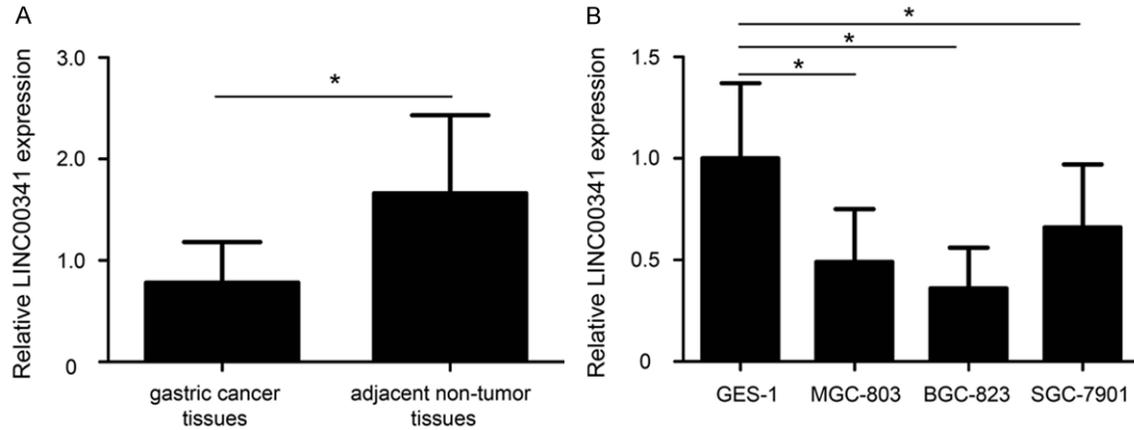
Figure 1. Expression of LINC00341 is down-regulated in gastric cancer tissues and cell lines. A. LINC00341 expression levels were lower in gastric cancer tissues than in corresponding adjacent non-tumor tissues. B. LINC00341 expression was decreased in gastric cancer cell lines (MGC-803, BGC-823 and SGC-7901) compared with normal gastric epithelial cell line (GES-1). *P<0.05.
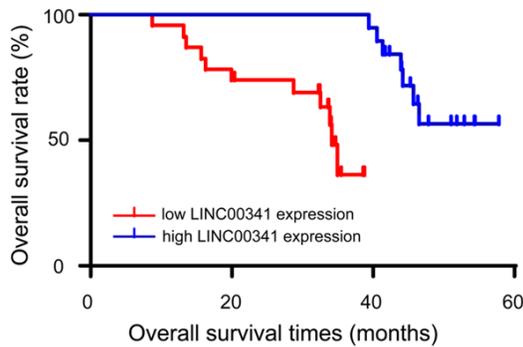

Figure 2. Kaplan-Meier survival analysis of association between LINC00341 expression and overall survival of gastric cancer patients. Patients with low LINC00341 expression showed poor overall survival times compared with those patients with high LINC00341 expression.

Manassas, VA, USA). The human normal gastric epithelial cell line, GES-1, was bought from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured with RPMI 1640 Medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin in a humidified incubator at 37°C with an atmosphere of 5% CO_2.

RNA extraction, reverse transcription and qRT-PCR assay

Total RNA was extracted from the frozen tissues and cultured cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufac-

turer's instructions. The concentrations of the total RNA samples were measured using a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA) at A_{260}/A_{280}. RNA was reversely transcribed into cDNA using a PrimeScript RT reagent Kit with a gDNA Eraser (Takara, China) according to the manufacturer's protocols. Quantitative real-time PCR (qRT-PCR) was performed using a SYBR Prime-Script RT-PCR kit (Takara, Japan) and on an ABI 7500 real-time PCR System (Applied Biosystems, Bedford, MA, USA).

PCR reactions were run using the following conditions: 95°C for 10 min, then 40 cycles at 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s. The primers were designed with Primer Premier 5.0 Software (Premier Biosoft International, Palo Alto, CA, USA) and purchased from GenePharma (Shanghai, China). Their sequences were as follows: LINC00341, 5'-AAAAGGGATTAGGACACCG-3' (forward) and 5'-GAGGCTGGCTGATGAGTG-3' (reverse); GAPDH (glyceraldehydes 3-phosphate dehydrogenase), 5'-GTCAACGGATTTGGTCT-3' (sense) and 5'-AG-TCTTCTGGGTGGCAGT-3' (antisense). The relative expression of LINC00341 was calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to GAPDH.

Cell transfection

The full-length cDNA of LINC00341 was amplified from the BGC-823 cell cDNA library and cloned into the pCMV-HA vector to generate a pCMV-HA-LINC00341 expression plasmid.
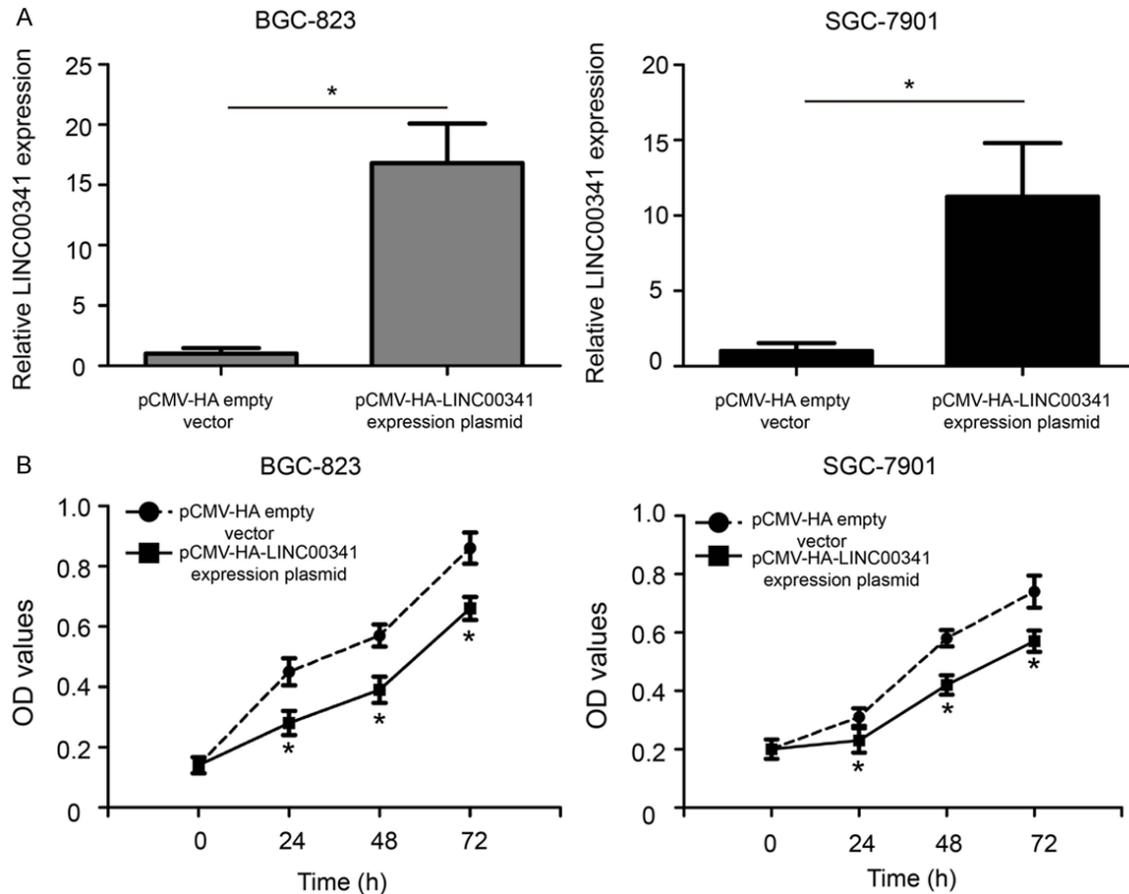
Figure 3. Over-expression of LINC00341 inhibited the growth of gastric cancer cells. A. Expression changes of LINC00341 after pCMV-HA-LINC00341 expression plasmid or pCMV-HA empty vector. The relative expression levels were evaluated by quantitative real-time PCR (qRT-PCR) assay. B. The proliferation of gastric cancer cells was detected by an MTT assay, and cell proliferation inhibition was observed in **BGC-823** and **SGC-7901** cells after treatment with pCMV-HA-LINC00341 expression plasmid. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. *$P<0.05$.

Accurate reading frame insertion was verified by Sanger DNA sequencing. The pCMV-HA empty vector was used as a negative control. A 5 μg pCMV-HA-LINC00341 expression plasmid or pCMV-HA empty vector was used to transfect the BGC-823 and SGC-7901 cells once they reached 70% confluency by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Transfection efficiency was assessed by qRT-PCR assay after 48 hours of transfection.

Cell proliferation, migration, and apoptosis assays

Cell proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay. Migration was determined using a

wound healing assay. Apoptosis was determined using flow cytometry. The procedures for cell proliferation, migration, and apoptosis were carried out as reported elsewhere [17, 18].

Statistical analysis

The statistical analysis was conducted using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The data was shown as the mean ± SD from at least three independent experiments. The statistical significance between groups was determined using the Student's t test or one-way ANOVA. The association between the expression of LINC00341 and the clinicopathologic parameters was evaluated using a Chi-square (χ^2) test. Patient survival was evaluated using the Kaplan-Meier method and compared using a log-rank test. A two-sided *P* value of
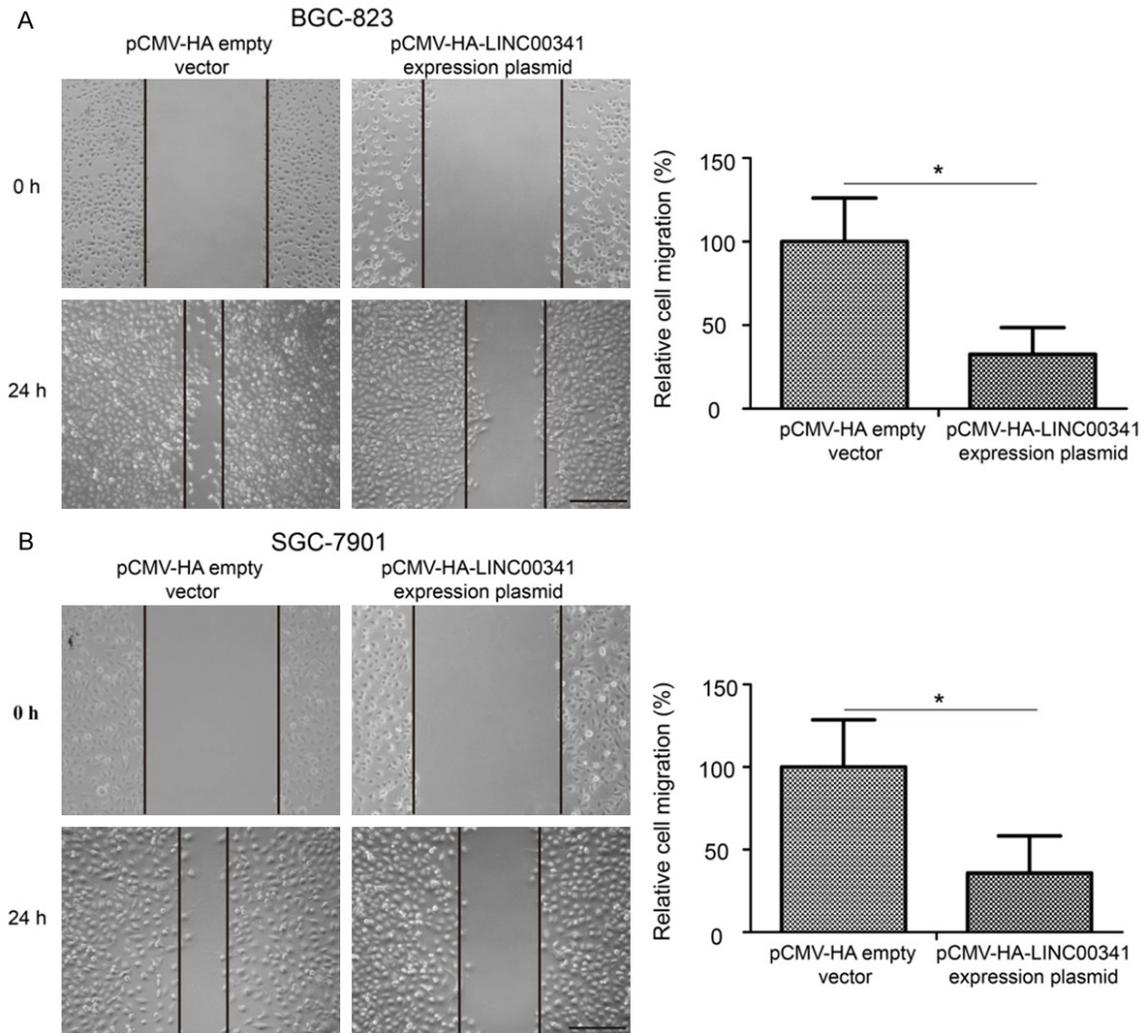
Figure 4. Enforced expression of LINC00341 suppressed the migration of gastric cancer cells. A. Wound healing assay was used to detect the migratory ability of **BGC-823 cells**. B. The migratory ability of **SGC-7901** cells was decreased after they were transfected with a pCMV-HA-LINC00341 expression plasmid. Bar =100 μm, *P<0.05.

less than 0.05 was considered statistically significant.

Results

The expression of LINC00341 is down-regulated in gastric cancer tissues and cell lines

We firstly examined LINC00341 expression levels in 42 paired gastric cancer tissues and corresponding non-cancerous tissues by qRT-PCR. As shown in **Figure 1A**, after normalization to GAPDH expression, the expression levels of LINC00341 in the gastric cancer tissues was significantly down-regulated in compared with the corresponding adjacent non-tumor tissues (P<0.05). In order to assess the role of

LINC00341 in the gastric cancer cells, we further performed qRT-PCR to detect LINC00341 expression in gastric cancer cell lines (MGC-803, BGC-823 and SGC-7901) and a normal gastric epithelial cell line (GES-1). Meanwhile, LINC00341 also had a lower expression in gastric cancer cells than it did in GES-1 cells (**Figure 1B**, P<0.05).

The correlation between LINC00341 expression and clinicopathological features in patients with gastric cancer

To assess the correlation of LINC00341 expression with the clinicopathological data, the expression levels of LINC00341 in gastric cancer tissues were categorized as low (n=23) or
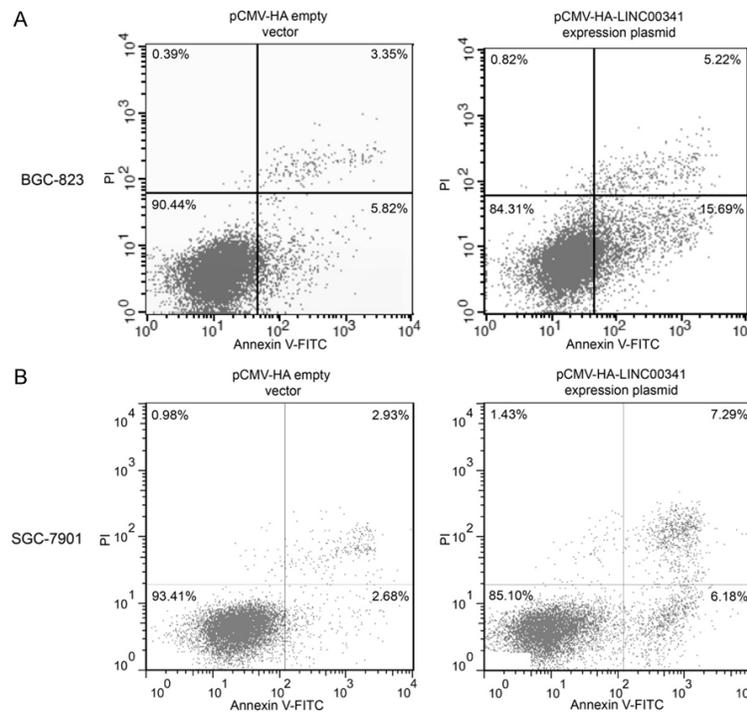
Figure 5. Restoration of LINC00341 induced the apoptosis of gastric cancer cells. A. The apoptosis of BGC-823 cells was detected by flow cytometry. B. More apoptotic cells were detected in the pCMV-HA-LINC00341 expression plasmid group compared with the pCMV-HA empty vector in SGC-7901 cells.

The over-expression of LINC00341 inhibited the growth of gastric cancer cells

BGC-823 and SGC-7901 cells were cultured and transfected with a pCMV-HA-LINC003-41 expression plasmid or a pCMV-HA empty vector. At 48 h after transfection, the efficiency of the transfection was detected by qRT-PCR. As shown in **Figure 3A**, we found that the relative levels of LINC00341 in BGC-823 and SGC-7901 cells were significantly up-regulated after they were transfected with the pCMV-HA-LINC00341 expression plasmid ($P<0.05$). Subsequently, we examined the effect of increased LINC00341 expression on the growth of gastric cancer cells *in vitro*. The growth curves determined by an MTT assay showed that cell growth arrest was observed in BGC-823 and SGC-7901 cells after they were treated with a pCMV-HA-LINC00341 expression plasmid (**Figure 3B**, $P<0.05$).

The enforced expression of LINC00341 suppressed the migration of gastric cancer cells

To further examine the effect of LINC00341 on the migration of BGC-823 and SGC-7901 cells at 24 hours after transfection, a wound healing assay was performed. Compared with the pCMV-HA empty vector group, the migration of gastric cancer cells was drastically suppressed by the pCMV-HA-LINC00341 expression plasmid. A Wound healing assay illustrated that the ratio of the relative migration in the pCMV-HA-LINC00341 expression plasmid group was reduced by 72.46% in BGC-823 (**Figure 4A**, $P<0.05$) and decreased by 61.62% in SGC-7901 (**Figure 4B**, $P<0.05$).

The restoration of LINC00341 induced the apoptosis of gastric cancer cells

Lastly, to investigate the effect of LINC00341 on cell apoptosis in gastric cancer cells, flow cytometry was applied. BGC-823 and SGC-

high (n=19) in relation to the mean value. As shown in **Table 1**, an examination of the correlation between the expression and the clinicopathological features showed that patients with a low LINC00341 expression were negative correlated with TNM stage and lymph node metastasis ($P<0.05$). However, we did not find any association between the expression levels of LINC00341 and other clinicopathological features including patients' gender, age, tumor diameter, histological type, invasive depth, differentiation stage, and distant metastasis.

The correlations of LINC00341 expression with the overall survival of gastric cancer patients

To further investigate the correlation of LINC-00341 expression with the overall survival (OS) of gastric cancer patients, a Kaplan-Meier analysis with the log-rank test was performed. As shown in **Figure 2**, we found that the levels of LINC00341 expression were significantly associated with the OS of gastric cancer patients, as patients with low LINC00341 expression had poorer OS times than those patients with high LINC00341 expression ($P<0.05$).

7901 cells were transfected with a pCMV-HA-LINC00341 expression plasmid or a pCMV-HA empty vector. Forty-eight hours after transfection, the rate of cell apoptosis was remarkably increased in the BGC-823 and SGC-7901 cells after they were transfected with the pCMV-HA-LINC00341 expression plasmid (**Figure 5**, P< 0.05).

Discussion

LncRNAs represent an emerging player in gastric cancer, contributing to tumor proliferation, metastasis, apoptosis, and survival [19]. For example, Sun, et al. [20] reports that down-regulated MEG3 is associated with poor prognosis and promotes cell proliferation in gastric cancer. Zhou, et al. [21] shows that the down-regulation of LET is associated with poor prognosis in gastric cancer. Chen, et al. [22] demonstrates that LINC00152 is highly expressed in gastric cancer and associates with the depth of invasion in patients with gastric cancer. Zhang, et al. [23] reveals that Sox2ot over-expression serves as a poor prognostic biomarker in gastric cancer.

As a newly identified lincRNA, LINC00341 is located in the chromosome 14q32.13 gene desert and represents a potential biomarker in many human cancers [16]. The expression of LINC00341 in gastric cancer is still unknown. Our study first reported that the expression of LINC00341 was obviously decreased in gastric cancer tissues and cell lines compared with paired non-cancerous tissues and the normal gastric epithelial cell line. Similarly, Liao, et al. demonstrated that the levels of LINC00341 are significantly lower in breast cancer tissues compared with healthy breast tissues [16].

We then used the mean expression level of LINC00341 as a cutoff to divide the 42 patients with gastric cancer into the low and high LINC00341 expression groups to further investigate the association between LINC00341 expression and clinicopathological features. Patients with a low expression of LINC00341 showed higher TNM stages and more lymph node metastasis than patients with high LINC-00341 expression. These results indicate that the down-regulation of LINC00341 is associated with the development and progression of gastric cancer. In order to investigate the prognostic role of LINC00341 on gastric cancer, we performed a Kaplan-Meier analysis of OS. The data showed that patients with low LINC00341 expression tend to have poor OS times in comparison to patients with high LINC00341 expression.

Finally, we attempted to explore the potential role of LINC00341 in gastric cancer cells by MTT, wound healing, and flow cytometry assays. Cell proliferation, migration and apoptosis were observed in BGC-823 and SGC-7901 cells after they were transfected with a pCMV-HA-LINC00341 expression plasmid or a pCMV-HA empty vector. The results showed that over-expression of LINC00341 inhibited cell growth and migration, and induced cell apoptosis in gastric cancer *in vitro*. These results suggest that LINC00341 acts as a tumor suppressor in the carcinogenesis of gastric cancer.

In conclusion, this study is the first to report that decreased LINC00341 is correlated with malignancy status and poor prognosis in gastric cancer. The restoration of LINC00341 expression suppresses cell growth and migration and induces apoptosis in gastric cancer. LINC00341 may serve as a new biomarker and a potential therapeutic target for gastric cancer. More work will be needed to determine the molecular mechanisms of LINC00341 in gastric cancer.

Acknowledgements

Disclosure of conflict of interest

None.

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