Long non-coding RNA LINC00857 promotes gastric cancer cell proliferation and predicts poor patient survival

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Abstract. Gastric cancer (GC) is a common malignancy worldwide and its pathogenesis remains unclear. Long non-coding RNAs (lncRNAs) serve an important function in cancer development, therefore identification of functional lncRNAs in GC is required. The results of the present study demonstrate that an lncRNA, LINC00857, was increased in GC tissues compared with adjacent non-tumor tissues. Overexpression of LINC00857 was positively associated with poor survival rate, as well as with the tumor size of patients with GC. LINC00857 knockdown induced by specific small interfering RNAs significantly inhibited GC cell proliferation in vitro. Genome-wide analysis revealed that LINC00857 knockdown deregulated the cell cycle. Western blot analysis confirmed that LINC00857 knockdown decreased protein expression of cyclin D1 and cyclin E1 in GC cells. Taken together, the results indicated that LINC00857 knockdown suppressed GC cell proliferation through deregulating the cell cycle, resulting in the downregulation of cyclin D1 and cyclin E1. Therefore, LINC00857 expression may be an independent biomarker for the diagnosis and prognosis of GC.

Introduction

Gastric cancer (GC) leads to a major health burden worldwide (1). Approximately 679,100 novel cases and 498,000 mortalities were estimated in China in 2015 (2). Although the incidence and mortality rates have declined in the West, the overall 5-year survival rate remains at ~20% (3). The need for an effective biomarker for the diagnosis of early asymptomatic GC remains challenging (4). In current clinical practices, several serum tumor markers, including CA (carbohydrate antigen) 19-9, carcinoembryonic antigen and CA125, have been widely used, but their specificity is ambiguous (5).

Furthermore, the current treatment options for advanced GC lack specificity, resulting in a large number of side effects and drug resistance. Thus, there is an urgent requirement to investigate novel biomarkers and therapeutic targets for GC. Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs of length >200 nucleotides that do not encode proteins (6). Previous studies have indicated the important function of lncRNAs in cancer development (7,8). Sun et al (9) reported that the lncRNA GClncl acted as a scaffold to recruit the WDR5 and KAT2A complex and regulated the transcription of target genes. Additionally, the lncRNA HOXA11-AS acts as a scaffold to link chromatin modification factors (PRC2, LSD1 and DNMT1), and promoted cell proliferation and invasion in GC (10), suggesting that these lncRNAs may serve as therapeutic targets in GC. Genome-wide analysis has revealed that lncRNAs exhibit tissue-specific and cancer-specific expression patterns (11,12). A recent study has identified several novel circulating lncRNAs for the diagnosis of GC, using genome-wide lncRNA microarrays (13). One of those lncRNAs was LINC00857, whose expression was increased in the tumor tissue and in the serum of patients with GC. In addition, LINC00857 was upregulated in patients with lung cancer and was able to promote cell proliferation, colony formation and invasion (14). However, the underlying molecular mechanism of LINC00857 in GC development remains unclear.

In the present study, it was demonstrated that LINC00857 expression was increased in GC tissues and associated with poor patient survival rates. Overexpression of LINC00857 in GC cells promoted cell proliferation and migration, suggesting that LINC00857 may be used as a novel biomarker and therapeutic target for GC.

Materials and methods

Patients and clinical samples. A total of 60 patients with GC were enrolled in the present study, which complied with the Ethics Review Board at the Yongchuan Hospital of Chongqing Medical University (Chongqing, China). Written informed consent was obtained from all patients. In total, 60 pairs of fresh GC tissues and adjacent non-tumor tissues were collected from patients who had not undergone any treatment at the Yongchuan Hospital of Chongqing Medical University between April 2012 and December 2015. The tissue samples were collected in the operating room and processed

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within 15 min, and the non-tumorous samples were taken at a distance of >5 cm from the tumor. Clinical features of patients with GC included in the present study are presented in Table I.

Cell culture. Four GC cell lines (AGS, BGC-823, MKN-45 and SGC-7901) and a gastric epithelial cell line (GES-1) were purchased from BeNa Culture Collection (Beijing China). AGS cells were cultured in Ham's F12 medium (HyClone, GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the remaining cell lines were cultured in Dulbecco's modified Eagle's medium/high glucose medium (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS at 37°C in 5% CO2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from tissue and cell samples using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was resuspended in 50 µl pre-heated (65°C) nuclease-free water, subpackaged into two nuclease-free tubes and stored at -80°C. A PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) and a Permix Ex Taq kit (Takara Biotechnology Co., Ltd.) were used to perform RT-qPCR assays according to the manufacturer's protocol. Briefly, cDNA was synthesized in a 20 µl reaction volume containing 4 µl 5X RT buffer, 4 µl nuclease-free water, 1 µl Prime reverse transcriptase, 1 µl RT primer mixture and 10 µl total RNA following genomic DNA removal at 37°C for 15 min. qPCR experiments were performed in a 20 µl reaction volume on a Bio-Rad IQ5 thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the parameters: 95°C for 2 min, 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The primer sequences for LINC00857 were as follows: 5’-CCCCTGCTTCTTGTGTTCC-3’ (forward) and 5’-AGCTTGTCTTCTTGGTACT-3’ (reverse). The primer sequences for GAPDH were as follows: 5’-GGTGGTGTCCTCTGACTACCA-3’ (forward) and 5’-TCTCTTCTCTTTTGCTTCCTG-3’ (reverse). LINC00857 expression was normalized using the 2^ΔΔCq method (15) relative to the mRNA expression of GAPDH.

Western blotting. SGC-7901 cells (5x10^4) were seeded in 6-well plates and transfected with small interfering RNAs (siRNAs). After 24 h, the cells were harvested and lysed in radioimmunoprecipitation assay buffer (Roche Diagnostics, Basel, Switzerland). Total proteins were measured using a BCA kit (Thermo Fisher Scientific, Inc.), and 20 µg was loaded for 12% SDS-PAGE and separated, followed by transferrence onto polyvinylidene difluoride (PVDF) membranes and blocked in PBS containing 3% bovine serum albumin at room temperature for 1 h. Anti-F cyclin D1 rabbit (dilution, 1:500; cat. no. 2978), anti-cyclin E1 mouse (dilution, 1:500; cat. no. 4129) and anti-GAPDH rabbit (dilution, 1:1,000; cat. no. 5174) antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) were incubated at 4°C overnight. The goat-anti-rabbit (cat. no. ZB-2301) or goat-anti-mouse (cat. no. ZB-2305) HRP-conjugated secondary antibodies (1:10,000; ZSGB-BIO, Beijing, China) were incubated at room temperature for 1 h. Finally, the PVDF membranes were visualized using SuperSignal West Dura Extended Duration Substrate kit (Thermo Fisher Scientific, Inc.).

Oligonucleotide transfection. SGC-7901 cells (5x10^4) and MKN 45 cells (5x10^4) were seeded in 6-well plates, incubated for 24 h, and then 200 ng/ml specific siRNAs for LINC00857 or negative control (NC) were transfected into the cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and the cells were harvested after 24 h. The sequences of siRNAs were as follows: siRNA1, 5'-GGUAAGGGAGUGUGGAAGUU-3'; siRNA2, 5'-GGCUAUGUCUGUAGAUCUU-3'.

Cell proliferation. SGC-7901 and MKN-45 cells transfected with LINC00857 siRNAs or NC were harvested and diluted at a density of 10^4 cells/ml, prior to seeding in 96-well plates. Cell proliferation was analyzed using a Cell Counting Kit-8 kit (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Absorbance at 450 nm was measured when the reagent reacted for 24, 48, 72 or 96 h at room temperature.

Statistical analysis. Data were analyzed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as the mean ± standard deviation. The differences between two groups were analyzed using the unpaired Student's t-test. One-way analysis of variance with post hoc comparison using Sidak's multiple comparisons test was used for multiple groups. Patient survival was calculated using the Kaplan-Meier estimator method. P<0.05 was considered to indicate a statistically significant difference.

Table I. Clinical characterization of patients with GC.

| Variable                  | Gastric cancer, n (%) |
|---------------------------|-----------------------|
| Sex                       |                       |
| Male                      | 36 (60)               |
| Female                    | 24 (40)               |
| Age, years                |                       |
| ≥57                       | 31 (52)               |
| ≤57                       | 29 (48)               |
| Histology                 |                       |
| Adenocarcinoma            | 52 (87)               |
| Mucinous adenocarcinoma   | 8 (13)                |
| TNM stage                 |                       |
| I/II                      | 18 (30)               |
| III/IV                    | 42 (70)               |
| Lymph node metastasis     |                       |
| Yes                       | 47 (78)               |
| No                        | 13 (22)               |

TNM, TNM classification of malignant tumors.
Results

Increased expression of LINC00857 is associated with poor survival rate in patients with GC. To investigate LINC00857 expression in GC, 60 pairs of GC tissues and adjacent non-tumor tissues were collected. RT-qPCR experiments demonstrated that LINC00857 expression was significantly increased in GC tissues compared with non-tumor tissues (P<0.0001; Fig. 1A). Additionally, receiver operating characteristic (ROC) curve analysis indicated that LINC00857 expression signature exhibited an increased area under the curve (AUC) value of 0.8631 with a sensitivity of 91.67% and a specificity of 78.33% in GC tissues (Fig. 1B). Further analysis revealed that LINC00857 expression was positively associated with tumor size (Fig. 1C). Furthermore, survival rate analysis indicated that patients with GC with increased expression of LINC00857 exhibited a poorer survival rate compared with those with low expression of LINC00857 (Fig. 1D). These results suggest that LINC00857 expression may serve as a novel biomarker for the prognosis of GC.

Downregulation of LINC00857 suppresses GC cell proliferation. To determine the function of LINC00857 in the development of GC, certain GC cell lines, including GES-1, AGS, BGC-823, MKN-45 and SGC-7901, were used. RT-qPCR experiments revealed that LINC00857 expression was significantly increased in MKN-45 and SGC-7901 cells compared with that in the remaining cell lines. In particular, the lowest expression of LINC00857 was observed in GES-1 cells (Fig. 2A). Since the highest expression of LINC00857 was observed in MKN-45 and SGC-7901 cells, those cell lines were selected for the subsequent gene transfection experiments as described previously (14). RT-qPCR experiments indicated that the two sets of siRNAs significantly knocked down the expression of LINC00857 in MKN-45 and SGC-7901 cells (Fig. 2B). Cell proliferation was analyzed using a CCK-8 assay and the results indicated that LINC00857 down-regulation significantly inhibited the proliferative capacity of MKN-45 and SGC-7901 cells (Fig. 2C and D), suggesting that LINC00857 may promote GC development.

LINC00857 activates multiple signaling pathways involved in the cell cycle. To investigate the molecular mechanisms by which LINC00857 knockdown suppresses cell proliferation, a whole transcriptome analysis using Affymetrix ST2.1 exon arrays was performed in LINC00857-knockdown SGC-7901 cells. SGC-7901 cells transfected with a negative control were used additionally. A hierarchical clustering analysis demonstrated the aberrant expression of multiple transcripts between the two groups (Fig. 3A). Pathway analysis revealed that LINC00857 led to a significant inactivation of the cell cycle signaling pathway (Fig. 3B). RT-qPCR and western blot analysis confirmed that the mRNA and protein expression
of cyclin D1 and cyclin E1 were significantly decreased in LINC00857-knockdown SGC-7901 cells compared with control cells (Fig. 3C and D). Furthermore, flow cytometric analysis indicated that LINC00857-knockdown SGC-7901
cells were arrested in G₁ phase (Fig. 3E). These data suggest that LINC00857 is able to regulate G₁/S transition in GC cells.

Discussion

In the present study, it was demonstrated that increased expression of LINC00857 was associated with the poor survival rate and tumor size of patients with GC. ROC analysis revealed that LINC00857 expression may serve as an independent biomarker for the diagnosis of GC. A cell proliferation assay revealed that knockdown of LINC00857 significantly inhibited the proliferative capacity of GC cells in vitro. Furthermore, genome-wide analysis demonstrated that LINC00857 knockdown resulted in the inactivation of the cell cycle signaling pathway. RT-qPCR and western blot analysis also confirmed that the mRNA and protein expression of cyclin D1 and cyclin E1 were decreased in LINC00857-knockdown SGC-7901 cells. Furthermore, flow cytometric analysis indicated that knockdown of LINC00857 inhibited G₁/S transition in SGC-7901 cells.

Recent evidence suggests that a number of IncRNAs were able to serve as potential biomarkers for GC (16-19). In a previous study by Zhang et al (13), IncRNA microarray profiling was applied to identify several novel IncRNAs, including LINC00857, for the diagnosis of GC. These data are consistent with the results of the present study in that LINC00857 exhibited an increased AUC value in GC tissues compared with that of non-tumor tissues. The association between LINC00857 expression and the survival rate of patients with GC was further assessed, and it was demonstrated that patients with GC with increased expression of LINC00857 exhibited a poor survival rate. These results suggest that LINC00857 expression may serve as a biomarker for the prognosis of GC.

Although a number of IncRNAs have been annotated, functional interpretation remains limited. The biological effect of LINC00857 on GC development remains largely unclear. The results of the present study demonstrated that LINC00857 knockdown significantly inhibited GC cell proliferation through regulating cell cycle signaling pathways, confirming the results of previous studies (14). Furthermore, LINC00857 expression might be regulated by cyclin D1 and cyclin E1, which induces G₁ arrest in GC cells. Previous studies have also demonstrated that the down-regulation of cyclin D1 and cyclin E1 significantly inhibited GC cell proliferation (20,21). Nevertheless, the molecular mechanisms by which LINC00857 regulates GC cell proliferation require investigation in future studies. Future in vivo experiments are also required to confirm the results of the present study.

In conclusion, the results of the present study demonstrated that LINC00857 expression is associated with the poor survival rate of patients with GC and that downregulating LINC00857 expression suppresses GC cell proliferation in vitro through cell cycle arrest.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FH designed the present study and wrote the manuscript. FHI and KP performed the experiments and analyzed the data. MJR, FWZ and TWY collected the samples of the patients and analyzed the data.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Review Board at the Yongchuan Hospital of Chongqing Medical University (Chongqing, China).

Consent for publication

The authors declare that the patients have provided written informed consent for the publication.

Competing interests

The authors declare that they have no competing interests.

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