LINC00115 promotes gastric cancer partly by the miR-212-5p/ATPAF1 axis

QINGXI ZHU, JIE TAN, TING ZHAN, MENG LIU, YANLI ZOU & WEIJIE LIU

Abstract: LncRNAs are known to be key regulators in the initiation and development of diverse cancers. Whether LINC00115 is involved in the regulation of gastric cancer (GC) progression remains unclear. Here, we aimed to show the function of LINC00115 in GC. RT-qPCR was used to measure gene expression in GC tissues and cells. Colony formation, EdU, TUNEL, and wound healing assays were used to analyze cellular processes in GC. The in vivo GC xenograft model was established. We observed that LINC00115 was highly expressed in GC. Functionally, silencing LINC00115 inhibited GC cell proliferation, and migration but facilitated GC apoptosis. Mechanistically, LINC00115 sponged miR-212-5p, while miR-212-5p targeted ATPAF1 in GC cells. Rescue assays showed ATPAF1 overexpression counterbalanced the inhibitory role of LINC00115 depletion in GC progression in vitro and in vivo. Overall, LINC00115 promoted GC progression by upregulating ATPAF1 via miR-212-5p.

Key words: LINC00115, gastric cancer, miR-212-5p, ATPAF1.

INTRODUCTION

Gastric cancer (GC) is a common malignancy worldwide, and it is associated with high mortality (Siegel et al. 2023). In 2020, there were over one million newly diagnosed patients with GC, with an estimated 769,000 deaths globally (Sung et al. 2021). Owing to population aging, it is expected that by 2040, the global cancer burden will be 50% higher than that in 2020 (Dinmohamed et al. 2020, Sung et al. 2021). It is reported that a variety of non-genetic factors, such as obesity, smoking, and chronic infections, contribute to GC development (Compare et al. 2010). While many efforts have been made to improve GC diagnosis and treatment, patients in most countries still exhibit a 5-year survival rate of under 30% (Bonnot et al. 2019, Newhook et al. 2019). Hence, deeper understanding of the regulatory mechanisms of GC tumorigenesis is required to improve the prevention and treatment for GC.

Long noncoding RNAs (lncRNAs) are noncoding transcripts with over 200 nucleotides, and many of them have been confirmed to exert key effects on the regulation of cancer development through various mechanisms, including regulating miRNA expression (DiStefano 2018, Hombach & Kretz 2016). Importantly, it has been demonstrated that a variety of lncRNAs display crucial roles in GC development. LncRNA LINC00978 was shown to promote GC proliferation and epithelial-mesenchymal transition (EMT) (Fu et al. 2018). LncRNA LINC00675 restrained GC development by facilitating phosphorylation of vimentin (Zeng et al. 2018). It was reported that DGCR5 sponged miR-23b to inhibit GC cell growth and boost apoptosis through the regulation of PTEN and BTG1 (Xu et al. 2019). LncRNA LINC00115, also known as NCRNA00115, was found to exert an important effect on some cancer types. For example, LINC00115 was upregulated and enhanced glioma stem-like cell...
self-renewal (Tang et al. 2019). LINC00115 was identified to be associated with bladder cancer patients' outcome and it was considered as an underlying prognostic or diagnostic marker for this cancer (Jiang et al. 2018). Also, LINC00115 might be a potential therapeutic target for lung adenocarcinoma by acting as ceRNA to mediate miRNA-mRNA axis (Li et al. 2016). However, whether LINC00115 participates in the regulation of GC development remains unknown and needs an exploration.

In this study, the main purpose was to investigate the role of LINC00115 in GC. First, we measured the LINC00115 level in GC tissues and cells, followed by evaluation of the biological functions of LINC00115 in GC cells and tumor growth. We further probed into the regulatory mechanism of LINC00115.

MATERIALS AND METHODS

Tissue samples
GC tissues (n=30) and adjacent normal tissues (n=30) were obtained from GC patients at Tongren Hospital of Wuhan University (Wuhan Third Hospital). The collected tissue samples were maintained at -80°C. No patients had received anti-cancer treatment before the operation. Informed consents of the research were provided by all participants. The research was approved by the Ethics Committee of Tongren Hospital of Wuhan University (Wuhan Third Hospital).

Cell lines
GC cell lines (HGC-27, AGS, SNU-1, and ALF) and a human gastric mucosal cell line (GES-1) were obtained from the ATCC (VA; USA). All cells were cultivated in RPMI 1640 medium with 10% FBS (Gibco, USA) in a 37°C incubator with 5% CO₂.

Transfection
Sh-LINC00115#1 (or sh-LINC00115#2), pcDNA3.1/ATPAF1 (ATPAF1), miR-212-5p mimics, and their negative controls were all transfected into GC cells. Transfection was conducted utilizing Lipofectamine 2000 (Invitrogen, USA). All plasmids were from GenePharma (Shanghai, China). Transfected cells were collected for analysis at 48 h post-transfection.

RT-qPCR
TRizol (Invitrogen, USA) was used to isolate total RNA from transfected GC cells. Then, first-strand cDNA was generated by ImProm-II Reverse Transcription System (Promega, USA). Later, RT-qPCR was conducted by using SYBR Green qPCR assay (Takara, Japan) on an ABI 7500 Real-Time PCR System. GAPDH or U6 was used as internal control, and gene expression was calculated using the 2⁻ΔΔCt method.

Western blot
RIPA lysis buffer (Life Technologies, USA) was utilized to isolate the total protein. After that, protein concentration was quantified by BCA Assay Kit (Beyotime, China). Before transfer into PVDF membranes, the protein samples were separated by 10% SDS-PAGE gel electrophoresis. The membranes were blocked with 5% skim milk and were incubated with primary antibody against MMP9 (ab76003, Abcam), MMP2 (ab92536, Abcam), ATPAF1 (ab101518, Abcam), and β-actin (ab8226, Abcam), followed by incubation with secondary antibody (Abcam) for 1 h at room temperature. Band exposure was achieved using ECL and quantification was performed by ImageJ software.

Colony formation assay
Cell proliferation was evaluated by conducting this assay. Briefly, cells were inoculated in 6-well plates at 1×10⁵ cells per well and incubated for 14 days. The medium was replaced every three
days. Finally, cells were fixed with methanol, stained with crystal violet (Sigma, USA), and counted under a light microscope.

**EdU staining**

A kFluor488-EdU kit (KeyGEN, Jiangsu, China) was used for this assay. At 48 h after transfection, cells were digested and added into each well of 96-well plates. Afterwards, 25 μl EdU reagent was added to each well. The plates were maintained for 2 h. Then, the cells were fixed with 4% paraformaldehyde, treated with 0.5% Triton X-100, and stained with DAPI. Finally, the results were recorded by a fluorescence microscope (Leica DMI3000B, Germany).

**TUNEL assay**

TUNEL assay was performed to evaluate GC cell apoptosis. Apoptotic cells were identified with a TUNEL detection kit (Roche, Germany). Nuclei were stained with DAPI. An inverted laser scanning confocal microscope was used to capture the images. Finally, ImageJ software was used to analyze TUNEL-positive cells.

**Wound healing assay**

HGC-27 and AGS cells were seeded in 96-well plates for 24 h. The cells attached to the bottom were scratched using a 10 μl pipette tip. Then, the cells were washed 3 times with PBS to remove detached cells, and the cells were incubated in serum-free medium in an incubator at 37°C. The wounds were recorded at 0 h and 24 h at the same position using an inverted microscope.

**RNA pulldown assay**

Biotin-coupled LINC00115 probe and control probe were designed by RiboBio (Guangzhou, China). Briefly, GC cells were fixed with 1% formaldehyde and then lysed. Biotin-coupled RNA complex was added to the cell lysates with streptavidin-coated magnetic beads (Invitrogen) and cultured overnight at 4°C. Afterwards, the RNA attached to the mixture was extracted with TRizol and measured using RT-qPCR.

**Luciferase reporter assay**

LINC00115 or ATPAF1 3’-UTR fragments sharing the miR-212-5p binding site (wild-type or mutant) were separately subcloned into the pmirGLO vectors (Promega, Madison, MI), and co-transfected with NC mimics or miR-212-5p mimics into GC cells using Lipofectamine 2000. After incubation for 48 h, luciferase reporter assay system (Promega) was utilized to evaluate the luciferase activity.

**Subcellular fractionation assay**

Nuclear and cytoplasmic fractions were separated using a PARIS Kit (Invitrogen). Briefly, the cells were added and incubated in 500 μl fractionation buffer for 20 min at 4°C. The mixtures were centrifuged at 500 ×g/min for 5 min. The pellets were used for RNA extraction. Then, the expression level of LINC00115 was tested using RT-qPCR. U6 or GAPDH was as nuclear and cytoplasmic control, respectively.

**Xenograft tumor model experiments**

The Animal Care and Use Ethics Committee of Tongren Hospital of Wuhan University approved all animal studies. Nine BALB/c nude mice (4-weeks-old) from the Institute of Medical Laboratory Animals, Chinese Academy of Medical Sciences were housed in a specific-pathogen-free condition with free food and water access for a 7-day period. Mice were then randomized into sh-NC, sh-LINC00115 and sh-LINC00115+ATPAF1 groups (n=3 each). These animals then received a subcutaneous 0.15 mL injection of 2×10⁶ HGC-27 cells infected with stable expression of the corresponding plasmids in the femoral region. Tumor growth was measured every 5 days using calipers, with tumor volume being calculated...
as follows: \( V = \frac{1}{2}(\text{length} \times \text{width}^2) \). After 30 days, mice were then euthanized and tumors were collected, weighed, and utilized for other analyses.

### Statistical analysis

Statistical analysis was conducted with Graphpad Prism 5 software (GraphPad Software, La Jolla, CA, USA), and data are presented as the mean ± SD. Each assay was repeated three times. Comparisons between or more than two groups was performed using student’s t test or one-way ANOVA. Pearson correlation analysis was performed to evaluate the expression association among genes. \( p < 0.05 \) was considered statistically significant.

### RESULTS

#### LINC00115 knockdown suppressed GC cell growth

To figure out the role of LINC00115 in GC, we first determined the expression of LINC00115 in GC tissues and cells. The LINC00115 level in GC tissues was markedly higher than in nontumor tissues (Figure 1a). Besides, LINC00115 expression was higher in GC cells than in normal human gastric mucosal cell line (GES-1) (Figure 1b). Subsequently, the biological function of LINC00115 in GC was explored. HGC-27 and AGS cells were transfected with sh-LINC00115#1 or sh-LINC00115#2, and the results of RT-qPCR showed that LINC00115 expression in the sh-LINC00115#1 group was lower than in the sh-NC group (Figure 1c). We thus used sh-LINC00115#1 (sh-LINC00115) in the subsequent assays. Then, through colony formation and EdU assays, we observed that LINC00115 silencing significantly hampered GC cell proliferation (Figure 1d and e). Next, the results from TUNEL assay suggested that LINC00115 depletion boosted GC cell apoptosis (Figure 1f). As shown in Figure 1g, LINC00115 suppression impaired the migration of cells. Furthermore, the expression levels of matrix metalloproteinases (MMP2 and MMP9) were reduced by loss of LINC00115 (Figure 1h). Taken together, LINC00115 knockdown reduced cell proliferation and migration of GC.

#### LINC00115 bound to miR-212-5p

Subsequently, we aimed to investigate the mechanisms of LINC00115 in GC, and subcellular fractionation assay showed that LINC00115 mostly existed in the cytoplasm of GC cells (Figure 2a). We thus predicted that LINC00115 acted as a ceRNA in GC. According to the ceRNA network, we tried to search for the specific miRNA sharing binding site with LINC00115 using StarBase website. StarBase database predicted that 3 miRNAs sharing binding sites with LINC00115 (search category: Pan-Cancer: 2 cancer types). The results of RNA pulldown assay showed that miR-212-5p had the most significant enrichment in HGC-27 and AGS cells among these miRNAs (Figure 2b). Moreover, downregulation of miR-212-5p in GC tissues and cell lines was found (Figure 2c and d). The potential binding site between LINC00115 and miR-212-5p is presented in Figure 2e. We then overexpressed miR-212-5p in GC cells (Figure 2f). Results from luciferase reporter assay exhibited a reduction in the luciferase activity of LINC00115-Wt in GC cells overexpressing miR-212-5p (Figure 2g), confirming that LINC00115 could bind to miR-212-5p. Furthermore, the expression of miR-212-5p was negatively correlated with that of LINC00115 in GC tissues (Figure 2h).

#### ATPAF1 was a target of miR-212-5p

Similarly, StarBase website was searched to predict the potential downstream target for miR-212-5p. Two candidates were found (search category: CLIP Data: strict stringency (>=5), Pan-Cancer: 10 cancer types): RPLP0 and ATPAF1. We
observed that the expression levels of ATPAF1 were significantly decreased by overexpression of miR-212-5p and RPLP0 had no detectable change (Figure 3a). Additionally, overexpression of miR-212-5p reduced the ATPAF1 protein expression in GC cells (Figure 3b). Therefore, ATPAF1 was selected for the subsequent study. Then, we found that the luciferase activity of ATPAF1-Wt was attenuated by miR-212-5p overexpression, while the luciferase activity of ATPAF1-Mut exerted no change after miR-212-5p overexpression (Figure 3c and d), suggesting that miR-212-5p targeted ATPAF1. RT-qPCR indicated that miR-212-5p was knocked down in GC cells after transfection with miR-212-5p inhibitor (Figure 3e). As shown, the expression of ATPAF1 mRNA and protein was decreased by LINC00115 silencing in HGC-27 and AGS cells.
while the expression was restored after miR-212-5p knockdown (Figure 3f). We further discovered that ATPAF1 was downregulated in GC tissues and cells (Figure 3g and h). Furthermore, a high ATPAF1 level was closely related to a poor overall survival of GC patients (Figure 3i).

**LINC00115 silencing hampered GC cell growth by regulating ATPAF1**

To further explore whether LINC00115 regulates GC cell processes by regulating ATPAF1, a series of rescue assays were carried out. For this purpose, HGC-27 cell was transfected with ATPAF1 (Figure 4a). Overexpression of ATPAF1 offset the inhibitory effect of LINC00115 depletion on the proliferation of HGC-27 cells (Figure 4b and c). Also, promoted cell apoptosis of HGC-27 arising from LINC00115 knockdown was reversed by enhancement of ATPAF1 (Figure 4d). Moreover, LINC00115 depletion-induced decrease in HGC-27 cell migration was restored by overexpression of ATPAF1 (Figure 4e). Furthermore, ATPAF1 upregulation counteracted the suppression of LINC00115 silencing in the expression of MMP2 and MMP9 (Figure 4f).

**LINC00115 silencing suppressed tumorigenesis in vivo by downregulating ATPAF1**

Animal experiments were performed to explore the roles of LINC00115 and ATPAF1 in tumor growth in vivo. LINC00115 knockdown led to less tumor formation and reduced tumor size compared to the sh-NC group, and the effect of LINC00115 knockdown on tumor growth was significantly reversed by ATPAF1 overexpression (Figure 5a, b and c). These results highlighted the inhibition of LINC00115 suppressed tumorigenesis and tumor growth of GC in vivo by decreasing ATPAF1 expression.
DISCUSSION

GC is a common digestive system tumor, and it poses a huge threat to human health for its high incidence and mortality (Torre et al. 2016). The aberrant expression levels of tremendous lncRNAs have been shown to be closely related to the processes of various cancers, such as cervical cancer (Zhang et al. 2019), colorectal cancer (Huang et al. 2019), gallbladder cancer (Yang et al. 2018), and GC (Wang et al. 2020). Previous studies elucidated the oncogenic role of LINC00115 in several cancers (Jiang et al. 2018, Li et al. 2016, Tang et al. 2019). In this study, we focused on the role of LINC00115 in GC. Our data exhibited that LINC00115 was upregulated in GC tissues and cells. Additionally, LINC00115 depletion repressed the proliferation and migration of GC cells, and it facilitated GC cell apoptosis, as well as inhibited tumor growth in vivo. Therefore, these results were consistent with that of previous studies indicating LINC00115 exerted an oncogenic effect on cancer progression.

It is known that lncRNAs could modulate cancer development via various mechanisms, including acting as ceRNAs by competitively
binding to miRNAs to mediate the expression of mRNAs (Karreth & Pandolfi 2013, Tay et al. 2014). MiRNAs are key mediators in the development of multiple cancers, including GC (Petrocca et al. 2008, Ueda et al. 2010). MiR-212-5p was found to be correlated with the regulation of quite a lot of tumors (Chen et al. 2020, Fang et al. 2020, Lv et al. 2017). Importantly, miR-212-5p was identified as a tumor inhibitor in GC (Li et al. 2018). Consistently, our findings indicated miR-212-5p was underexpressed in GC tissues and cells. Besides, we found that miR-212-5p was sponged by LINC00115. Generally, miRNA activity can be antagonized with antisense oligonucleotides which sequester or degrade mature miRNAs, and expressed miRNA sponges which compete with target transcripts for miRNA binding (Roberts & Wood 2013). Here, we also found that miR-212-5p expression was negatively related to LINC00115 expression in GC clinical samples. It is worth examining whether fine tuning of LINC00115 expression is part of an autoregulatory loop in which it’s inhibitory to expression and activity of

Figure 4. LINC00115 knockdown restrained GC cellular processes through the regulation of ATPAF1. (a) The transfection efficiency of ATPAF1 overexpression in HGC-27 cells. HGC-27 cell proliferation after transfection with sh-NC, sh-LINC00115, or sh-LINC00115+ATPAF1 was detected by (b) colony formation and (c) EdU assays. (d) The apoptosis of HGC-27 cells in the groups of sh-NC, sh-LINC00115, or sh-LINC00115+ATPAF1 was assessed with TUNEL assay. (e) Wound healing assay was adopted to evaluate the migration of HGC-27 cells in the three groups. (f) Western blot analysis detected the protein levels of matrix metalloproteinases (MMP2 and MMP9) in HGC-27 cells transfected with the indicated plasmids. ***p<0.001.
miR-212-5p, which allows the establishment of the lncRNA up-regulated expression in GC.

ATPAF1 (ATP synthase mitochondrial F1 complex assembly factor 1) was reported to influence some diseases, such as clear cell renal cell carcinoma (Brüggemann et al. 2017), androgen-independent prostate cancer (Schinke et al. 2014), and asthma (Schauberger et al. 2011). Nevertheless, research on the role of ATPAF1 in cancer development is still limited, and whether ATPAF1 is involved in GC development remains obscure. Here, we identified that ATPAF1 was a target of miR-212-5p in GC cells. ATPAF1 was positively related to LINC00115 but negatively related to miR-212-5p in GC cells. In addition, we observed that ATPAF1 was overexpressed in GC tissues and cells, and abnormal expression of ATPAF1 was closely associated with the poor survival rate of GC patients. Furthermore, increased ATPAF1 counteracted the suppressive role of LINC00115 depletion in GC progression in vitro and in vivo, suggesting that ATPAF1 expression was responsible for the regulatory action of LINC00115 in GC.

In conclusion, the present study elucidates the biological role of LINC00115 in GC, and our study provides new evidence exhibiting the ceRNA network. We demonstrated that LINC00115 facilitates cell proliferation and migration of GC via sponging miR-212-5p to target ATPAF1. This finding may shed some light on GC diagnosis and treatment.

Acknowledgments

The work was supported by Special projects for central and local guidance of Hubei Province (Approval number: 2019ZYDYD067 (XH)).

REFERENCES


How to cite

Manuscript received on May 4, 2023; accepted for publication on August 9, 2023

QINGXI ZHU
https://orcid.org/0000-0001-7154-9939

JIE TAN
https://orcid.org/0000-0002-8861-1134

PING ZHAN
https://orcid.org/0000-0003-0622-7890

MENG LIU
https://orcid.org/0000-0002-3332-1316

YANLI ZOU
https://orcid.org/0000-0001-8818-9001

WEIJIE LIU
https://orcid.org/0000-0002-5763-7286

Tongren Hospital of Wuhan University (Wuhan Third Hospital), Department of Gastroenterology, No.241, Pengliuyang Road, Wuhan 430060, Hubei, China

Correspondence to: Yanli Zou, Weijie Liu
E-mail: haisizou@163.com, 172077205@qq.com

*These authors contributed equally to the work.

Author contributions
QZ and JT conceived and designed the experiments. TZ, ML, YZ, WL, QZ and JT carried out the experiments. WL, YZ, QZ and JT analyzed the data. WL, YZ, QZ and JT drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.