European Review for Medical and Pharmacological Sciences 2019; 23: 10769-10775

Knockdown of LINC00461 inhibits cell proliferation and induces apoptosis in gastric cancer by targeting LSD1

X. SHI, X. YOU, W.-C. ZENG, Y.-J. DENG, H.-L. HONG, O.-X. HUANG, M.-F. WANG

Department of Medical Oncology, The First Affiliated Hospital, Fujian Medical University, Fuzhou, China

Abstract. – OBJECTIVE: To uncover the function of LINC00461 in regulating cellular behaviors of gastric cancer (GC) *via* targeting LSD1.

PATIENTS AND METHODS: LINC00461 level in GC tissues with different tumor node metastasis (TNM) staging and lymphatic metastasis statues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). In vitro influences of LINC00461 on proliferative and apoptotic rates were evaluated in AGS and SGC-7901 cells. The interaction between LINC00461 and LSD1 was explored by RNA immunoprecipitation (RIP) assay and qRT-PCR. Finally, the potential role of LSD1 in the proliferative ability of GC cells mediated by LINC00461 was assessed.

RESULTS: LINC00461 level was higher in GC tissues relative to matched control ones. It was positively correlated to TNM staging and lymphatic metastasis of GC. Knockdown of LINC00461 markedly attenuated viability and the proliferative ability of AGS and SGC-7901 cells, but induced apoptosis. RIP assay demonstrated the interaction between LINC00461 and LSD1. Moreover, LSD1 could reverse the regulatory effect of LINC00461 on the proliferative ability of GC cells.

CONCLUSIONS: LINC00461 is upregulated in GC, which is positively related to TNM staging and lymphatic metastasis. LINC00461 mediates proliferation and apoptosis of GC cells, thereafter aggravating the progression of GC.

Key Words:
Gastric cancer (GC), LINC00461, Proliferation, Apoptosis.

Introduction

Gastric cancer (GC) is a severe malignancy that threatens human health. It ranks first in the incidence of digestive system tumors[1]. Environmental, genetic and other predisposing factors are considered to be risk factors for tumorigenesis of GC. Currently, *Helicobacter pylori* infection is believed to be the leading risk factor for GC[2,3]. Owing to the unsatisfactory diagnostic rate of early-stage GC in our country, the prognosis of GC is poor, especially for advanced GC patients[2]. It is urgent to develop specific and sensitive hallmarks for early-stage screening and diagnosis of GC. Hence, explorations on the tumorigenesis and tumor progression of GC are extremely important.

Long non-coding RNA (lncRNA) is a class of RNAs with over 200 nt transcript length. It has a specific spatial secondary structure and highly conserved sequence elements[4]. LncRNA is widely present in tissues and cells. Initially, lncRNA was considered to be a non-functional by-product of RNA polymerase II transcript as a transcriptional noise for the genome[5]. They are unable to encode proteins, but could regulate gene expressions and thus participate in biological processes[6,7]. LncRNAs have been identified as crucial regulators in biology. Serving as carcinogenic or tumor-inhibiting roles, lncRNAs widely influence tumor cell behaviors and tumor-related pathways[7-9]. Therefore, in-depth researches on differentially expressed lncRNAs in tumor diseases contribute to developing novel strategies for tumor treatment.

LINC00461 is reported to be a potential molecular target in multiple types of tumors[10-13]. Its role in GC, however, has not been fully explored. This study mainly investigated the regulatory effects of LINC00461 on influencing proliferation and apoptosis of GC cells. We aim to provide a promising target for the diagnosis and treatment of GC.

Corresponding Author: Xi Shi, MM; e-mail: sxi91@163.com

Patients and Methods

Subjects and Clinical Samples

Tumor tissues and matched adjacent tissues were harvested from 25 GC patients undergoing surgery in The First Affiliated Hospital, Fujian Medical University from September 2016 to September 2018. Resected samples were placed into liquid nitrogen until analyses. Enrolled GC patients were pathologically diagnosed and denied the medical history of other malignancies. This investigation was approved by the Medical Ethics Committee of The First Affiliated Hospital, Fujian Medical University and informed consent was obtained from each subject.

Cell Culture

Epithelial cells of gastric mucosa (GES-1) and GC cell lines (MG803, AGS, and SGC-7901) provided by the American Type Culture Collection (ATCC; Manassas, VA, USA) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C.

Transfection

Cells were pre-seeded in the 6-well plates. 4 μg transfection vectors and 10 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were respectively diluted in 250 μL of serum-free Opti-MEM and mixed together. 20 min later, the transfection mixture was applied in each well. Complete medium was replaced 4 h later. Sequences of transfection vectors were as follows: sh-LINC00461 1#: 5'-CTGCAAAGAAGCATAAAATGA-3'; sh-LINC00461 2#: 5'-CAGCATCAAAATCGAATAATA-3'; sh-LINC00461 3#: 5'-AATTCTCCGAACGTGTCACGT-3'; sh-LSD1: 5'-GCCACCCAGAGAUAUUACU-3'.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNA extraction was performed using the TRIzol method (Invitrogen, Carlsbad, CA, USA). The extracted RNA was quantified and reversely transcribed into complementary deoxyribose nucleic acid (cDNA). The mRNA level of LINC00461 was detected using the SYBR PrimeScript™ RT-PCR kit (TaKaRa, Otsu, Shiga, Japan) on the Roche LightCycler480 fluorescence quantitative PCR system, and 3 repeated wells were set for each specimen. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, for a total of 40 cycles. The relative level was calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study were as follows: LINC00461, F: 5'-CGGAGGTCATCCTCGGTACTC-3', R: 5'-GCTAGCCATGTGTAGAAGCCA-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in the 96-well plate with 2×10^3 cells per well. Absorbance (A) at 450 nm was recorded at the established time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

Cells were seeded in the 24-well plate with 2×10^4 cells per well and labeled with 50 μmol/L EdU at 37°C for 2 h. After 30-min fixation in 4% paraformaldehyde, cells were incubated with phosphate-buffered saline (PBS) containing 0.5% Triton-100 for 20 min. After washing with PBS containing 3% bovine serum albumin (BSA), 100 μL of the dying solution was applied per well for 1-h incubation in the dark and cells were counterstained with Hoechst 33342 for 30 min. The ratio of EdU-positive cells was calculated.

Colony Formation Assay

Cells were seeded in a 6-well plate with 1×10^3 cells per well. After 2 weeks of culture, cell colonies were fixed for 15 min, dyed with 0.05% crystal violet, and captured for calculation.

Flow Cytometry

Cells were prepared for single-cell suspension. Cells were double-stained with 5 μL of Annexin V and 5 μL of fluorescein isothiocyanate (FITC) in the dark for 10 min. The apoptotic rate was finally determined using a flow cytometry (Partec AG, Arlesheim, Switzerland).

RNA Immunoprecipitation (RIP)

Cells were treated according to the procedures of Millipore Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). The cell lysate was incubated with anti-LSD1 or IgG antibody at 4°C for 6 h. A protein-RNA complex was captured and digested with 0.5 mg/mL proteinase K containing 0.1%

sodium dodecyl sulphate (SDS) to extract RNA. The magnetic beads were repeatedly washed with RIP washing buffer to remove non-specific adsorption as much as possible. Finally, the extracted RNA was subjected to mRNA level determination using qRT-PCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

LINC00461 Level Was Correlated to Malignancy of GC

QRT-PCR revealed a higher abundance of LINC00461 in GC tissues relative to matched normal tissues (Figure 1A). To uncover the potential correlation between LINC00461 level with malignancy of GC, its level in GC tissues with different TNM staging and metastatic statues was determined. Compared with GC patients in stage I-II, those in stage III-IV presented a higher level of LINC00461 (Figure 1B). GC patients accompanied with lymphatic metastasis showed a higher abundance of LINC00461 relative to those non-metastatic patients (Figure 1C). It is suggested that LINC00461 may be related to malignant progression of GC.

LINC00461 Promoted Proliferative Ability and Suppressed Apoptosis of GC Cells

LINC00461 was identically upregulated in GC cells relative to epithelial cells of the gastric mucosa (Figure 2A). AGS and SGC-7901 cells were selected owing to their high abundances of LINC00461. Three shRNAs targeting LINC00461 were established, namely sh-LINC00461 1#, sh-LINC00461 2#, and sh-LINC00461 3#. The former two shRNAs presented a pronounced transfection efficacy in AGS and SGC-7901 cells (Figure 2B). Cell viability was greatly inhibited after transfection of sh-LINC00461 1# or sh-LINC00461 2# in GC cells (Figure 2C, D). Similar trends were obtained through the colony formation assay that knockdown of LINC00461 suppressed clonality in GC cells (Figure 2E). On the contrary, the apoptotic rate was markedly enhanced after transfection of sh-LINC00461 1# or sh-LINC00461 2# (Figure 2F).

Furthermore, EdU assay was conducted to assess the proliferative change affected by LINC00461. Transfection of sh-LINC00461 1# or sh-LINC00461 2# in GC cells greatly decreased the number of EdU-positive cells, indicating the attenuated proliferative ability (Figure 3).

LINC00461 Accelerated Proliferative Ability of GC Cells Via Interacting with LSD1

RIP assay showed a higher enrichment of LINC00461 in anti-LSD1 relative to anti-IgG (Figure 4A). Hence, it is proved that LINC00461 was interacted with LSD1. In GC cells, transfection

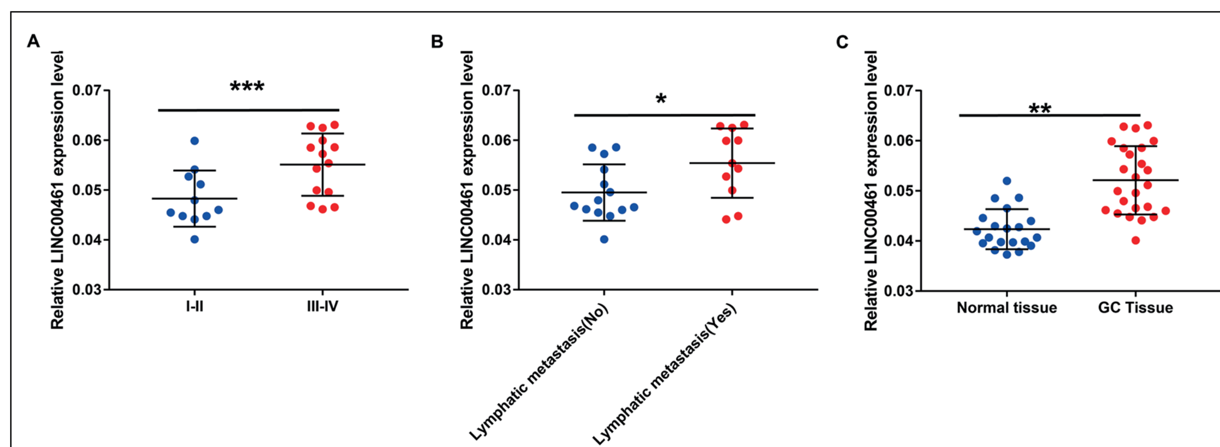

Figure 1. LINC00461 level was correlated to malignancy of GC. **A,** Relative level of LINC00461 in GC tissues and matched normal tissues. **B,** Relative level of LINC00461 in GC patients with stage III-IV and stage I-II. **C,** Relative level of LINC00461 in GC patients either with lymphatic metastasis or not.

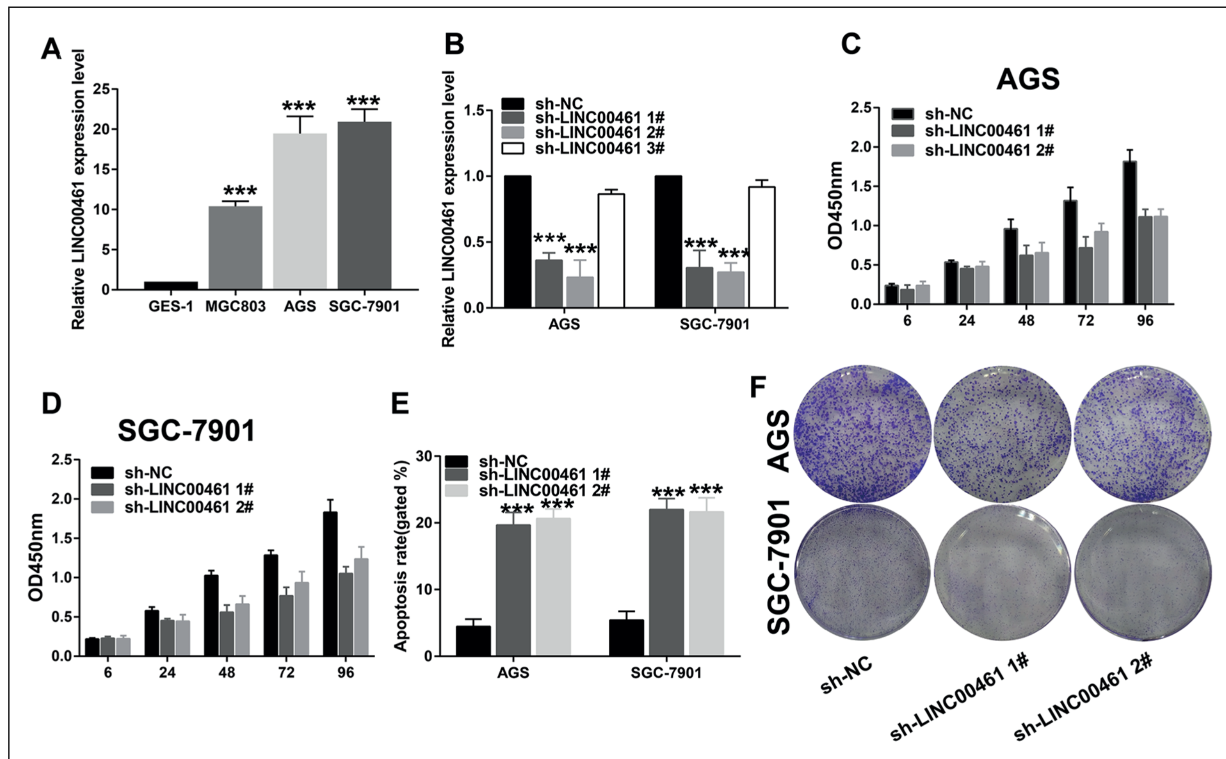

Figure 2. LINC00461 promoted viability and suppressed apoptosis of GC cells. **A,** Relative level of LINC00461 in epithelial cells of gastric mucosa and GC cells. **B,** Transfection efficacy of sh-LINC00461 1#, sh-LINC00461 2# and sh-LINC00461 3# in AGS and SGC-7901 cells. **C,** CCK-8 showed the viability in AGS cells transfected with sh-NC, sh-LINC00461 1# or sh-LINC00461 2#. **D,** CCK-8 showed the viability in SGC-7901 cells transfected with sh-NC, sh-LINC00461 1# or sh-LINC00461 2#. **E,** Colony formation assay showed the colonies in AGS and SGC-7901 cells transfected with sh-NC, sh-LINC00461 1# or sh-LINC00461 2#. **F,** Apoptotic rate in AGS and SGC-7901 cells transfected with sh-NC, sh-LINC00461 1# or sh-LINC00461 2# (magnification × 40).

of sh-LSD1 markedly downregulated LSD1 level, verifying its sufficient efficacy (Figure 4B). We, therefore, speculated whether LSD1 was involved in LINC00461-mediated cellular behaviors of GC. Transfection of sh-LINC00461 1# remarkably decreased the number of EdU-positive cells, but it was further reversed after co-transfection of sh-LSD1 in GC cells (Figure 4C). Collectively, LINC00461 was able to mediate proliferative ability and apoptotic rate of GC cells *via* interacting with LSD1.

Discussion

The mortality of GC is high in our country due to the lack of effective early-stage diagnostic and therapeutic methods[14]. LncRNAs have been reported to participate in the occurrence and progression of human tumors[15]. Several lncRNAs are identified to affect the occurrence, metastasis, drug-resistance, and prognosis of GC, such as H19 and SNHG1[16,17]. Ji et al[12] illustrated that LINC00461 could accelerate the proliferative, metastatic, and invasive abilities of hepatocellular carcinoma through the miR-149-5p/LRIG2 axis. The biological function of LINC00461 in GC remains to be further explored. Clarification of LINC00461 function in the progression of GC helps to uncover effective diagnostic hallmarks.

The tumor is a complex disease involving genetic variations and epigenetic mutations, and further influences tumor cell behaviors[18,19]. Tumor cells are manifested with excessive proliferation and enhanced anti-apoptotic ability[9,20]. This study examined the expression pattern and biological functions of LINC00461 in GC. LINC00461 was upregulated in GC tissues relative to matched normal ones, and it was positively correlated to TNM staging and lymphatic metastasis. It is suggested that LINC00461 may exert a carcinogenic role in the progression of GC. Subsequently, *in vitro* studies demonstrated that knockdown of LINC00461 attenuated proliferative ability and induced apoptosis of GC cells.

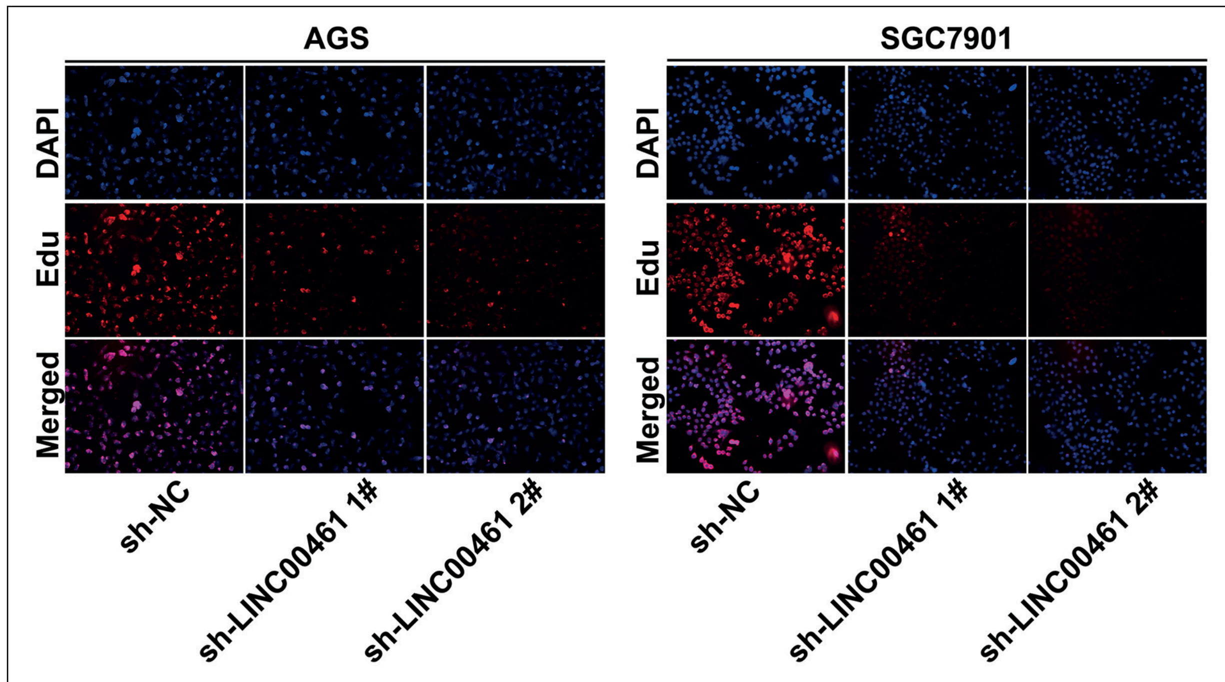

Figure 3. LINC00461 promoted proliferation of GC cells. EdU assay showed DAPI-labeled nucleus (blue), EdU-labeled cells (red) and merged images in AGS (left) and SGC-7901 cells (right) transfected with sh-NC, sh-LINC00461 1# or sh-LINC00461 2# (magnification × 40).

In this paper, the RIP assay showed the interaction between LINC00461 and LSD1, indicating the potential involvement of LSD1 in LINC00461-mediated cellular behaviors of GC cells. LSD1 is upregulated in many types of malignant tumors[21]. It has become an important epigenetic target for tumor treatment[22,23]. Huang et al[24] pointed out that LINC00673 inhibits ex-

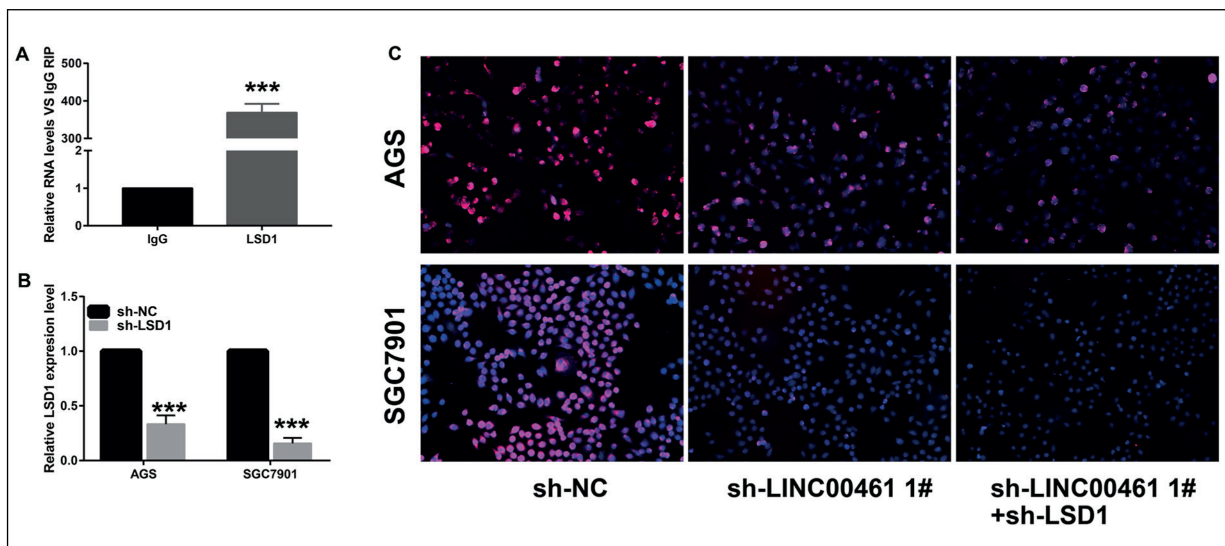

Figure 4. LINC00461 accelerated proliferative ability of GC cells *via* interacting with LSD1. **A,** RIP assay showed enrichment of LINC00461 in anti-IgG or anti-LSD1. **B,** Transfection efficacy of sh-LSD1 in AGS and SGC-7901 cells. **C,** EdU assay showed EdU-positive cells in AGS and SGC-7901 cells transfected with sh-NC, sh-LINC00461 1# or sh-LINC00461#+sh-LSD1 (magnification × 40).

pression levels of KLF2 and LATS2 through interacting with LSD1, thus aggravating the progression of GC. Liu et al[25] showed that lncRNA FEZF1-AS1 suppresses p21 level through LSD1-induced H3K4me2 demethylation, and further accelerates GC cell proliferation. Our results consistently verified that LSD1 is responsible for LINC00461-mediated proliferation of GC. This report provides a new theory for underlying the progression mechanism of GC and a promising target for GC treatment.

Conclusions

In summary, that LINC00461 is upregulated in GC, which is positively related to TNM stage and lymphatic metastasis. LINC00461 mediates proliferation and apoptosis of GC cells, thereafter aggravating the progression of GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

Preliminary study on the inhibitory effect of MFSD2A in gastric cancer and its mechanism, Natural Science Foundation of Fujian (2018J01159).

References

1) Karimi P, Islami F, Anandasabapathy S, Freedman ND, Kamangar F. Gastric cancer: descriptive epidemiology, risk factors, screening, and prevention. Cancer Epidemiol Biomarkers Prev 2014; 23: 700-713.
2) Correa P. Gastric cancer: overview. Gastroenterol Clin North Am 2013; 42: 211-217.
3) Zhang XY, Zhang PY. Gastric cancer: somatic genetics as a guide to therapy. J Med Genet 2017; 54: 305-312.
4) Sun M, Kraus WL. From discovery to function: the expanding roles of long noncoding RNAs in physiology and disease. Endocr Rev 2015; 36: 25-64.
5) Struhl K. Transcriptional noise and the fidelity of initiation by RNA polymerase II. Nat Struct Mol Biol 2007; 14: 103-105.
6) Esteller M. Non-coding RNAs in human disease. Nat Rev Genet 2011; 12: 861-874.
7) Chandra Gupta S, Nandan Tripathi Y. Potential of long non-coding RNAs in cancer patients: from biomarkers to therapeutic targets. Int J Cancer 2017; 140: 1955-1967.
8) Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.
9) Zhu CH, Xiao DH, Dai LG, Xu HG, Jiang YH, Zheng ZJ. Highly expressed lncRNA FAL1 promotes the progression of gastric cancer by inhibiting PTEN. Eur Rev Med Pharmacol Sci 2018; 22: 8257-8264.
10) Deng M, Yuan H, Liu S, Hu Z, Xiao H. Exosome-transmitted LINC00461 promotes multiple myeloma cell proliferation and suppresses apoptosis by modulating microRNA/BCL-2 expression. Cytotherapy 2019; 21: 96-106.
11) Dong L, Qian J, Chen F, Fan Y, Long J. LINC00461 promotes cell migration and invasion in breast cancer through miR-30a-5p/integrin beta3 axis. J Cell Biochem 2019; 120: 4851-4862.
12) Ji D, Wang Y, Li H, Sun B, Luo X. Long non-coding RNA LINC00461/miR-149-5p/LRIG2 axis regulates hepatocellular carcinoma progression. Biochem Biophys Res Commun 2019; 512: 176-181.
13) Yang Y, Ren M, Song C, Li D, Soomro SH, Xiong Y, Zhang H, Fu H. LINC00461, a long non-coding RNA, is important for the proliferation and migration of glioma cells. Oncotarget 2017; 8: 84123-84139.
14) Gullo I, Carneiro F, Oliveira C, Almeida GM. Heterogeneity in gastric cancer: from pure morphology to molecular classifications. Pathobiology 2018; 85: 50-63.
15) Huarte M. The emerging role of lncRNAs in cancer. Nat Med 2015; 21: 1253-1261.
16) Hu Y, Ma Z, He Y, Liu W, Su Y, Tang Z. LncRNA-SNHG1 contributes to gastric cancer cell proliferation by regulating DNMT1. Biochem Biophys Res Commun 2017; 491: 926-931.
17) Liu G, Xiang T, Wu QF, Wang WX. Long noncoding RNA H19-derived miR-675 enhances proliferation and invasion via RUNX1 in gastric cancer cells. Oncol Res 2016; 23: 99-107.
18) Brown JC, Winters-Stone K, Lee A, Schmitz KH. Cancer, physical activity, and exercise. Compr Physiol 2012; 2: 2775-2809.
19) Rajagopalan D, Jha S. An epi(c)genetic war: pathogens, cancer and human genome. Biochim Biophys Acta Rev Cancer 2018; 1869: 333-345.
20) Li L, Feng T, Lian Y, Zhang G, Garen A, Song X. Role of human noncoding RNAs in the control of tumorigenesis. Proc Natl Acad Sci U S A 2009; 106: 12956-12961.
21) Lynch JT, Harris WJ, Somervaille TC. LSD1 inhibition: a therapeutic strategy in cancer? Expert Opin Ther Targets 2012; 16: 1239-1249.
22) Fu X, Zhang P, Yu B. Advances toward LSD1 inhibitors for cancer therapy. Future Med Chem 2017; 9: 1227-1242.

23) Maiques-Diaz A, Somervaille TC. LSD1: biologic roles and therapeutic targeting. Epigenomics 2016; 8: 1103-1116.
24) Huang M, Hou J, Wang Y, Xie M, Wei C, Nie F, Wang Z, Sun M. Long noncoding RNA LINC00673 is activated by SP1 and exerts oncogenic properties by interacting with LSD1 and EZH2 in gastric cancer. Mol Ther 2017; 25: 1014-1026.
25) Liu YW, Xia R, Lu K, Xie M, Yang F, Sun M, De W, Wang C, Ji G. LincRNAFEZF1-AS1 represses p21 expression to promote gastric cancer proliferation through LSD1-mediated H3K4me2 demethylation. Mol Cancer 2017; 16: 39.