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ORIGINAL RESEARCH

RETRACTED ARTICLE: The opposite role of alternatively spliced isoforms of LINC00477 in gastric cancer

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Background: Aberrant transcript alternative splicing is an important regulatory process closely connected with oncogenesis.

Purpose: The objective of this study was to determine the phenotype and function of a novel long noncoding RNA (lncRNA) LINC00477 in gastric cancer.

Patients and methods: The gastric cancer samples of 140 from Oncomine database and 17 from our own hospital, as well as three gastric cancer cell lines MKN-45, AGS and KATO III were used in this study. The expression of the splicing isoforms of LINC00477 were tested. The tumor effects of LINC00477 on gastric cancer were investigated in vitro and in vivo. The mechanism of LINC00477 interacted with aconitase 1 (ACO1) was further examined by RIP and pull down assay.

Results: The overall expression of LINC00477 was reduced in gastric cancers compared to normal gastric tissues. The isoform 1 of LINC00477 was down-regulated while the isoform 2 was up-regulated in gastric cancer cells. The opposite role of isoforms 1 and 2 in the proliferation and migration of cancer cells in vitro and in vivo was observed. Furthermore, isoform 1 of LINC00477 was determined to interact with ACO1 and suppress the conversion ability from citrate to isocitrate by ACO1.

Conclusion: we presented the important roles of the spliced isoforms of long noncoding RNA LINC00477 in gastric carcinogenesis.

Keywords: LINC00477, gastric cancer, lncRNA, ACO1

Introduction

Gastric cancer (GC) is one of the most common gastrointestinal malignancy, ranking the second leading cause of cancer-related death worldwide.[1] The five-year survival rate is only 20%.[2] Most of the cases are diagnosed at a terminal stage, which is accompanied by malignant multiplication and extensive invasion in lymph node or distant metastasis.[3,4] Even though the improved chemotherapy protocols reduced the five-year mortality rate, it is still an urgent need to clarify the metastatic mechanisms and identify new prognostic biomarkers or potential therapeutic target for GC.

Long non-coding RNAs (lncRNAs) are a series of non-protein coding transcripts in excess of 200 nt, which have been determined to play crucial roles in multiple biological processes, such as transcriptional regulation, alternative splicing, chromatin remodeling, X-chromosome imprinting and cell differentiation, as well as metastasis and drug resistance in cancer.[5–7] Alternative splicing is a posttranscriptional regulation via generating multiple spliced isoforms with a tissue- and cell-specific manner to increase the diversity of the transcriptome. Alternative splicing of lncRNAs further

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expands their regulatory and functional complexity in cancerogenesis and cancer development. Therefore, illustration of the differential profiles of lncRNA variants in GC can be beneficial to identify gastric cancer-specific biomarkers, provide the potential therapeutic targets, and figure out the underlying mechanisms of lncRNAs in stomach tumorigenesis.

In this study, we investigated the lncRNA profiles in GC patients of oncomine database and found that LINC00477, a novel lncRNA with no detailed research currently, was downregulated in GC cancers compared to adjacent tissues. Interestingly, we further found that LINC00477 had two spliced isoforms, whose transcriptional levels cannot be reflected from oncomine data. Therefore, we focused on LINC00477 and explored the roles of different variants played in GC.

Materials and methods

Samples, cells, vectors, RNA oligos, and antibodies

Tissues including 7 gastric mucosal epithelium from ulcer patients, 5 squamous carcinoma, and 5 adenocarcinoma of stomach and their corresponding para-carcinoma tissu were harvested from Department of Gastroenterology the First Affiliated Hospital of Zhengzhou University. The clinical information of the patients are liste in Ta e 1. Signed informed consent and ethics comm tee doc ents of Ethics Committee of The First A iliate Hospital of Zhengzhou University were all pr ded to ap ve this study. Gastric cancer cell lines KN- AGS and ATO III and gastric epithelial cell GES-1 were urchased from American Type Culture ollection (ATCC). LINC00477 isoform 1 and 2 abbre ted as 1 and L2 were cloned the full length from GES cells d generated into pcDNA3.1. Th RNA oligos f L and L2 were designed from (Ther o, USA and inserted into pLKO.1-GFP vector. All the s ce of primers and oligos are listed in Table 2. The lnc NA stably expressing or blocking cell lines were prepared a vectors transfection and screening using 600 μg/mL G418 or 1.5 μg/mL puromycin. The second and third exons of LINC00477 were synthetic with biotin labeled (Thermo Fisher Scientific, USA) respectively, for RNA pull down assay. GAPDH and streptavidin primary antibodies were obtained from CST. IP grade Aconitase 1 (ACO1) primary antibody was obtained from Abcam.

Table 1 The clinical information of the patients used in this study

Serial number	Age (years)	Gender	Disease type	Stage
U_1	56	M	Ulcer	–
U_2	38	M	Ulcer	–
U_3	54	M	Ulcer	–
U_4	69	F	Ulcer	–
U_5	41	F	Ulcer	–
U_6	54	M	Ulcer	–
U_7	50	F	Ulcer	–
A_1	58	M	Adenocarcinoma	IV
A_2	62	F	Ad oma	III
A_3	49	F	denocarcin a	III
A_4	33	M	denocarcinom	II
A_5	57	M	nocarcinom	III
S_1	67	F	Squa us carcinom	II
S_2	49		Squa ous c inoma	IV
S_3		M	squamous carcinoma	II
S_4	63	M	Squamous carcinoma	II
S_	49	F	Squamous carcinoma	III

Abbrevia s: F, f le; M, male.

RNA extraction

Freshly prepared cells or tissues were washed by PBS and dded into 1 mL RNAiso plus (Takara, Japan). Tissues needed to be ground within liquid nitrogen. Samples were added 200 μL chloroform, vortex to mix and centrifuged in highest speed at 4°C. The supernatant was transferred to new tubes, added isopropanol with the same volume, and centrifuged in highest speed at 4°C, then washed by cold ethanol and 75% ethanol, and dissolved in DEPC water. A total of 100 ng RNA measured by Nanodrop was conducted reverse transcription using PrimeScript™ RT-PCR Kit (Takara, Japan). Real-time PCR was performed using FastStart Universal SYBR Green Master (Roche, USA). All primers used in this study are listed in Table 2.

CCK-8 assay

A total of 1×10^4 cells within 100 μL volume were transplanted into 96 well plates, changed the medium with 10% CCK8 (Solarbio, China) for culture 4 hrs after cell adherence, and examined the absorption within the wavelength of 450–490 nm using MK3 well reader (Thermo Fisher Scientific, USA) to evaluate the cell growth.

Table 2 All the primers and RNA oligos used in this study were listed

Primer/RNA oligo	Sequence	Tm (°C)
L1 for cloning	CGGGATCCAGTCTCTTCTTGCAAGGCCTTTCGC	52
	GAATTCCGACCTTAGCCTATTTTCATAAGGC	
L2 for cloning	CGGGATCCCTCTTCTTGCAAGGCCTTTCGCCC	55
	GAATTCCGAGATATATCTAATGCTAGATG	
L1 RNAi oligo	ACCTCGCCGTCACAGGATTTCATACTTCAAGAAGTATGAAATCCTGTGACGGCTT	–
L2 RNAi oligo	ACCTCGCACCCACTAACTCATCATCTTCAAGAGAGATGATGATGGAGTGGGTGCTT	–
L1 for detection	CACAAATTTTCTTCCACTTC	58
	GGCCTTAGCTGAGGTGGCAGG	
L2 for detection	CACAAATTTTCTTCCACTTC	58
	ATAAACAGTCTATTAACACAT	
L1&2 for detection	CACAAATTTTCTTCCACTTC	60
	CAATCATTAGATGGAAGTGGAT	
Ferritin for detection	TTCCAGG ACATCAAG	55
	AAGTCACAGAGATGGGGG	
ACO1	TCATAATGAC CATAAG	60
	ATTTACTCCCAATGGC	
GAPDH	GGAGCGAGATCCCTCCAAAAT	60
	GGCTGTTGTCATACTTCTCATGG	

Scratch wound healing assay

A total of 2×10^5 cells were resuspended and subcultured into 6 well plates overnight to spread out a monolayer. The cells were scratched by sterile tips every 0.5 cm on di[illegible]d washed away the floated cells by PBS twice, cultured w[illegible]thin non-FBS medium and captured images of the [illegible]race eve[illegible] hrs. The edge distance and the scratch ar[illegible] were [illegible]arvest[illegible] using ImageJ to access the cell migrati[illegible]ability [illegible]he migra[illegible]tion rate was calculated as ($Area_T$ [illegible]rea_T [illegible]$ea_{T0}\times100\%$.

Xenograft mouse m[illegible]del

This animals feeding an[illegible] experiments [illegible]re approved by the Institutional Animal [illegible]are and Use Committee of Zhengzhou University. Briefly, 6–8 [illegible]eek-old nude female mice (SLACCAS, China) w[illegible] prepar[illegible]. And 1×10^7 AGS cells with stabl[illegible]L1 ov[illegible]express[illegible]n[illegible]ere injected into the armpits of nude [illegible]ice. Tw[illegible]ve individual mice were used in each group for this stu[illegible]he tumors were isolated and accessed the tumor volume ($V=$ le[illegible]th $\times$ width$^2\times0.5$)[8] after one and two weeks.

RNA pull down assay

One microgram biotinylated ribonucleic exons of LINC00477 (L1: 4,567–4,695; L2: 15,390–15,993; L1&2: 16,800–17,202) (Figure 1B) were added with structure buffer (10 mM Tris pH 7.4, 0.1 M KCl, 10 mM $MgCl_2$) to form the RNA secondary structure and incubated at 95°C for 2 mins, on ice for 3 mins, and room temperature for 30 mins in order [illegible]stabilize RNA. The denatured RNA was suspended by 100 [illegible]L streptavi[illegible]n magnetic beads (Thermo Fisher Scientific, [illegible]A) and ro[illegible]ted overnight at 4°C. The next day, mixture wer[illegible]t[illegible]uged at 3,000 rpm for 1 min and washed by wash [illegible]ffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 100 U/mL RNAase inhibitor, 1× Protease inhibitors cocktail) three times, followed by the incubation and rotation with 1 mL AGS cell lysate at 37°C for 1 hr. Beads-RNA-proteins mixture was centrifuged at 3,000 rpm, washed by wash buffer additional three times, added 5×SDS for denaturation at 95°C for 10 mins and detected ACO1 and streptavidin level by SDS-PAGE.

RNA immunoprecipitation (RIP)

A total of 1×10^7 AGS cells were harvested, resuspended in nuclear isolation buffer (1.28 M sucrose, 40 mM Tris pH 7.5, 20 mM $MgCl_2$, 4% Triton X-100) and kept on ice for at least 30 mins with frequent mixing. The pellet nuclei were centrifuged by highest speed for 15 mins, resuspended by wash buffer (same with RNA pull down) and shear the chromatin through sonication by 30% power, 3 s on, 6 s off for 9 mins. After that, 90% nuclei were incubated with 1 µg ACO1 antibody overnight and 40 µl Protein A/G beads 2 hrs by gentle rotation at 4°C while the rest of 10% were harvested as input. The pellet beads were centrifuged by 3,000 rpm for 3 mins, washed three times. Both the input and pellet beads were purified by RNAiso

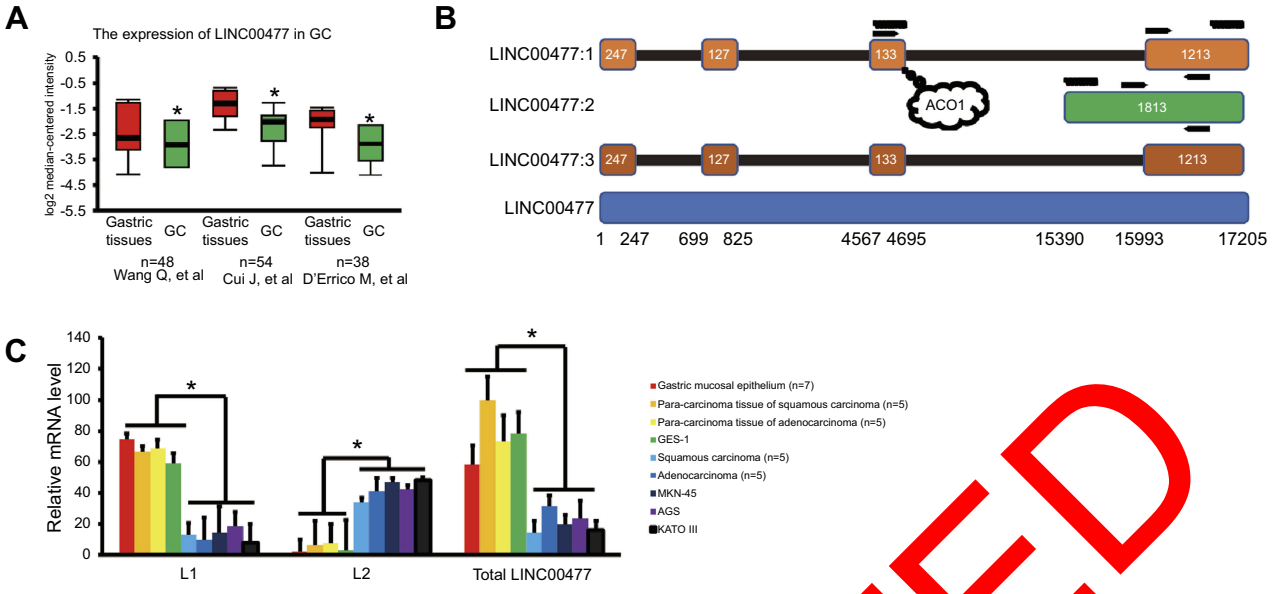

Figure 1 The transcripts of LINC00477 in GC. (**A**) The total expression of LINC00477 in the individual GC studies from Oncomine database. Data from these studies.[9–11] (**B**) The scheme of LINC00477. LINC00477:1–3 represent isoform 1–3. The bottom means the genomic location of this lncRNA. And we mean the location of primers used in this study. The curves mean the synthetic target RNAs labeled by biotin. The bubble means the binding site of ACO1. (**C**) The mRNA level of L1, L2 and total LINC00477 in GC tissues, cells, and stomach cells. Each experiment was performed at least three duplications. "*" and "**" represents *p*-value < 0.05 and 0.01, respectively.
Abbreviation: GC, gastric cancer.

plus and conducted reverse transcription which was described in RNA extraction.

Biochemical assay

The levels of intracellular citrate and isocitrate were measured by Citrate/Isocitrate Assay Kit (Bio-Vision, USA). The cellular ferritin protein level was measured by ELISA (Abcam, UK). The experimental procedure was followed by the instructions provided.

Statistical analysis

All statistical analysis was performed in SPSS 20 (IBM Corporation, Armonk, NY, USA). Delta-delta method was used to normalize the real-time PCR data. One-way ANOVA was used to analyze the difference among the groups. *p*-Value < 0.05 was considered as statistical significance.

Results

The aberrant transcriptional pattern of LINC00477 in GCs

The presence of LINC00477 transcript profiles in three individual datasets of GC patients (the cases of 48,[9] 54,[10] and 38[11] paired gastric cancers) from Oncomine database (https://www.oncomine.org/) was observed (Figure 1A). And we confirmed that LINC00477 happened to be obviously downregulated in GC tissues compared to stomach tissues or mucosa controls from three individual labs, although the actual expression of LINC00477 might be very low in cells. When we further tried to investigate the transcriptional regulation on LINC00477, we noticed that this lncRNA had three different spliced isoforms (LNCipedia database, https://lncipedia.org/)[12] generated by alternative exon splicing, whose expression could not be reflected in detail from the RNA-seq data of Oncomine. And we found that isoform 1 and isoform 3 are nearly the same sequence except the difference of three nucleotides in 3′ terminal (Figure 1B). Therefore, we designed a series of specific primers and focused on the level of LINC00477 isoform 1 and 2 (abbreviated as L1 and L2) in GC and stomach associated tissues and cells (Figure 1C). We observed that L1 was lowly while L2 was highly expressed in GC tissues and GC cells MKN-45, AGS, and KATO III compared to non-cancer tissues and cells. Moreover, the overall LINC00477 was reduced in GC, which is consistent with the results of GC patients in vivo. Taken together, we validated the reduction of LINC00477 in GC and the variant expression of its alternative spliced isoforms.

The opposite function of spliced isoforms of LINC00477 in GC cells

Next, we further investigated the role of LINC00477 played in GC. Because we have found the presence of downregulation of L1 and upregulation of L2 in GC, we determined to

change inversely but not intensify their transcriptional levels. Therefore, the overexpression of L1 and silencing of L2, respectively, in MKN-45, AGS, and KATO III cells were conducted and studied the impact on cancer proliferation and migration ability. We observed that the overexpression of L1 could robustly repress the cancer cell growth using CCK-8 assay (Figure 2A) and migration using wound healing assay (Figure 2B), while the silencing of L2 displayed more slight effect compared to L1. Furthermore, the stably L1 expression AGS cells and the xenograft mouse models were prepared to verify the tumor suppression of L1 in vitro. Consistently, we observed the inhibitory effect of AGS cells growth in vivo (Figure 2C). Collectively, our results demonstrated that the isoform 1 of LINC00477 acted as a tumor suppressor in GC cells.

The unique interaction between variant 1 of LINC00477 and ACO1 in GC cells

Next, we investigated the underlying mechanism of LINC00477 in GC. RBPDB database (http://rbpdb.ccbr.utoronto.ca/)[13] was utilized to seek the candidate protein between L1 and L2 of LINC00477 via RNA recognition motif alignment. Given the 21 and 23 candidate proteins of L1 and L2, except the enzymes for pre-mRNA processing, alternative splicing and RNA metabolism and transport (Figure 3A), we noticed aconitase 1 (ACO1), an essential enzyme for TCA cycle and intracellular iron controlling, potentially interacted with the specific CAGUGU motif of L1 (Figure 1B). RNA pull down assay was used to authenticate the actual interaction between isoform of LINC00477 and ACO1. And we determined that the specific exon of L1 but not L2 contributed to the interaction with ACO1 (Figure 3B). Moreover, RIP assay was further validated in gastric associated cells of GES-1, MKN-45, AGS, and KATO III. Consistent with RNA pull down assay, ACO1 protein was observed to bind with L1 not L2 and be able to capture more L1 in GSE-1 cells compared to the other three GC cells (Figure 3C). Furthermore, we questioned the role of L1 for the interaction with ACO1 in GC. Due to the extremely low expression of L1 in GC cells, we blocked L1 using ASO in gastric epithelial cells GSE-1 and overexpressed L1 in AGS cells. Although ferritin and ACO1 displayed an L1-independently transcription manner in GSE-1 and AGS cells, the ferritin protein was elevated upon L1 blocking (Figure 3D). Consistently, we also observed the presence of substantially declining proportion of citrate/isocitrate (Figure 3E) in cells with L1 low expression compared to high expression. Overall, the results above concluded that L1 could bind with ACO1 to suppress its function of ferritin translation translation for cell iron- and glycol-metabolism.

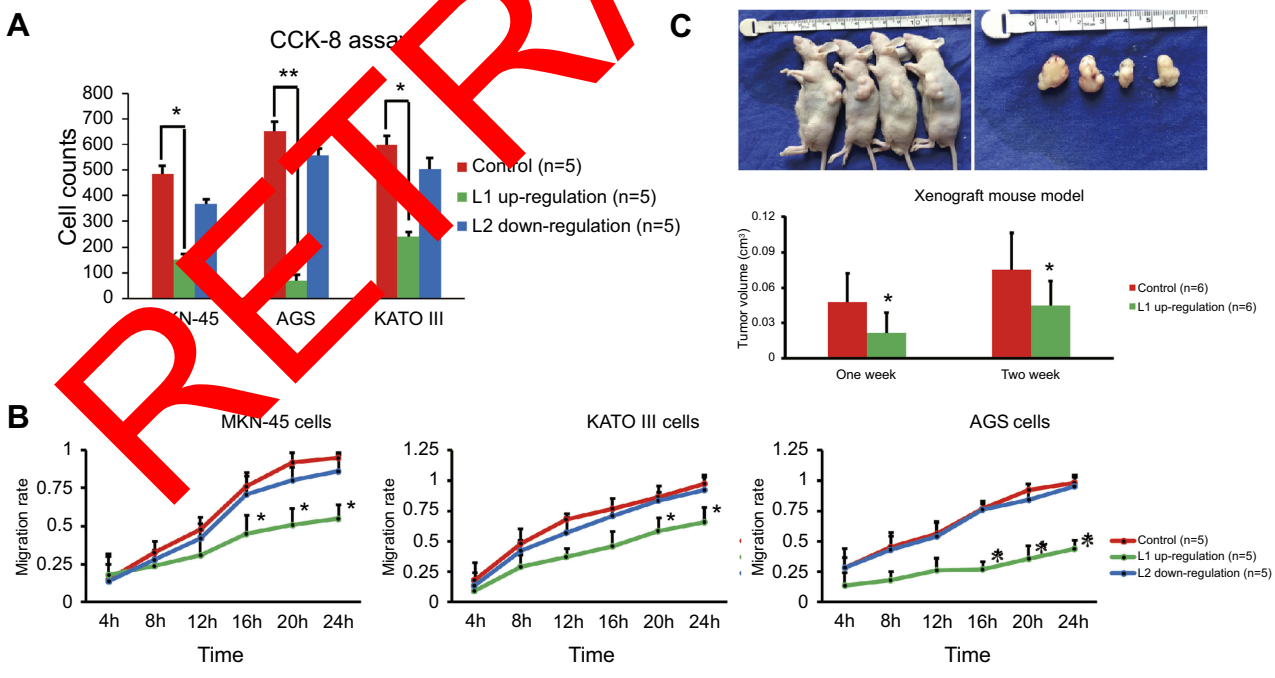

Figure 2 The function of LINC00477 in GC cells. (**A**) The cell growth of MKN-45, AGS, and KATO III with L1 overexpression or L2 silence assessed by CCK-8 assay. (**B**) The migration ability of MKN-45, AGS, and KATO III with L1 overexpression or L2 silence assessed by wound healing assay. (**C**) The cell growth of AGS with L1 overexpression assessed by xenograft mouse model. Each experiment was performed at least three duplications. "*" represents p-value < 0.05.
Abbreviation: GC, gastric cancer.

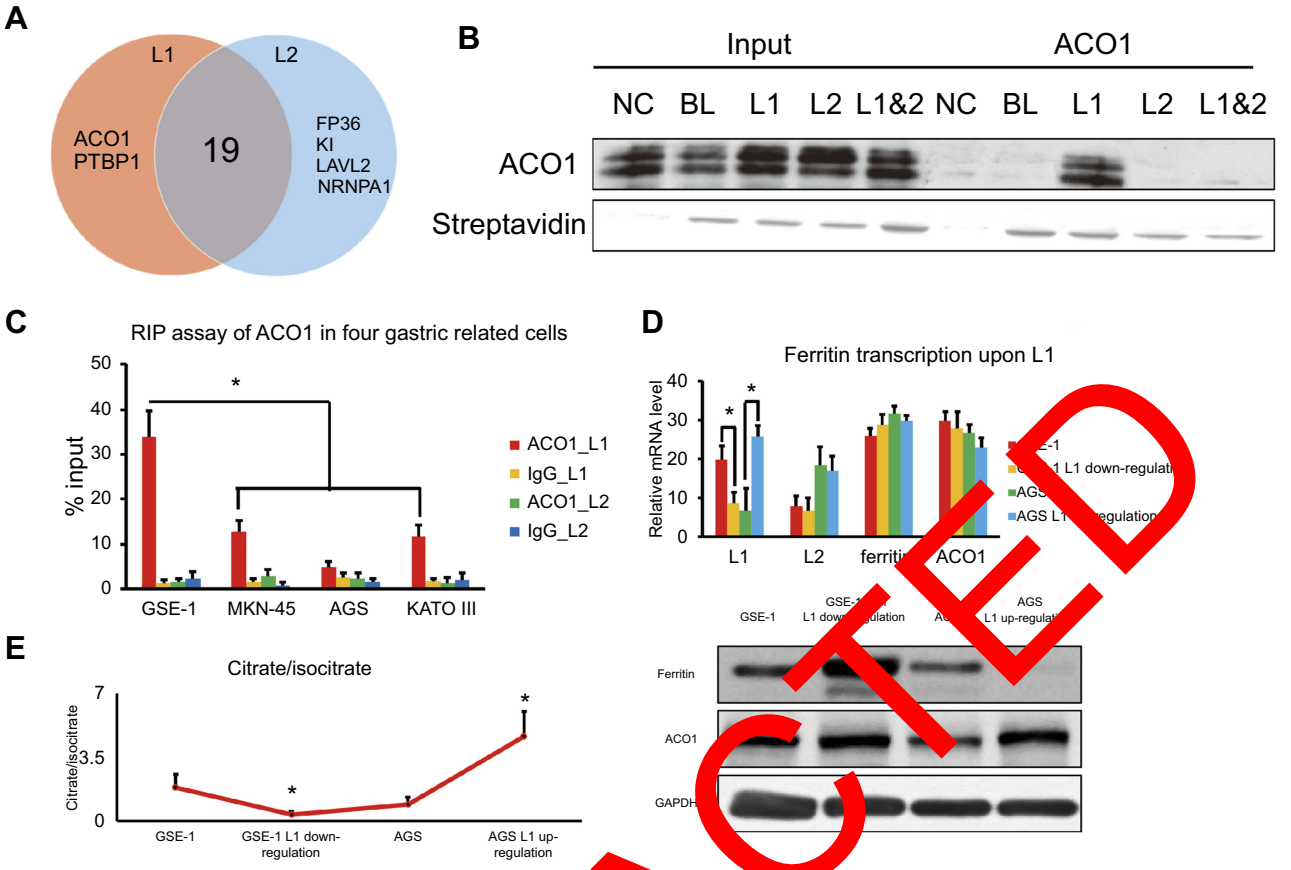

Figure 3 The tumor suppressive role of L1 in GC. (**A**) The Venn diagram of RNA binding p[illegible] of L1 and L2 predicted from RBPDB database. (**B**) RNA pull down assay for the interaction between L1 and ACO1 in AGS cells. NC, BL, L1, L2, and L1&2 represe[illegible] negative control, non-LINC00477 RNA oligos, 4,567–4,695 of L1; 15,390–15,993 of L2; 16,800–17,202 of both L1 and L2, respecti[illegible] (**C**) RIP [illegible] for the interaction between L1 and ACO1 in four cells. ACO1_L1, IgG_L1, ACO1_L2, and IgG_L2 mean the transcriptome of the four cel[illegible] pulled [illegible]wn by A[illegible]O1 or IgG antibody and detected the enrichment of L1 or L2. (**D**) The RNA and protein level of ferritin upon L1 up-/downregulation in GSE-1 [illegible] AGS cells. (**E**) The pr[illegible]portion of citrate and isocitrate upon L1 up/downregulation in GSE-1 and AGS cells. Each experiment was performed at least three duplications. "*" represe[illegible] value < 0.[illegible]5.
Abbreviation: GC, gastric cancer.

Discussion

Recently, with the develop[illegible]ent of next-ge[illegible]ation sequencing, an increasing nu[illegible]er of lncRNAs which were previously considered a[illegible] the junk DNA have been characterized and [illegible]ed w[illegible]spread [illegible]traction on the fields on epigenet[illegible]s of [illegible]histone [illegible]odification and DNA methylation,[14,15] tra[illegible]n regulation via enhancer activation or insulato[illegible]ocking[16,17] as well as post-transcriptional regulation inclu[illegible]g alternative splicing,[18,19] RNA stability,[20] and subcellular localization[21] in various biological events and diseases.

In our study, we found that LINC00477 was downregulated in GC patients compared to control stomach tissues or cells (Figure 1A). Unlike normal coding RNAs, a number of lncRNAs presented the low abundance transcripts because of an expression-dependent bias[22] and the enormous isoforms due to the alternative splicing in RNA-seq.[23] Therefore, RNA-seq might not reflect the true level of the multiple isoforms of LINC00477. We validated the expression pattern of two given LINC00477 isoforms in various stomach associated tissues or cells using real-time PCR, observed that the overall level of LINC00477 was downregulated in cancer although L2 was upregulated, and confirmed that LINC00477 was a potential biomarker that downregulated in GC compared to normal stomach control (Figure 1C) which was consistent with RNA-seq data. Here, we speculated that those multiple spliced isoforms might represent less biological significance, but the universally alternatively splicing specifically happened in noncoding exons.[24]

Moreover, we focused on L1 and L2 of LINC00477, and figured out that L1 played an important role of tumor suppressor in GC cells growth and migration (Figure 2). Compared to the robust tumor suppression

presence of L1, L2 displayed a relatively slight effect on GC development. We speculated that L2 might be an alternative splicing product with less significant function. We did not further investigate the RNA secondary structure of L1 and L2, but assumed that the different exons of L1 distinct from L2 might contribute to the tumor suppressor function of GC. Based on this assumption, we next predicted the potential RNA binding proteins using RBPDB database. Most of the proteins included in the pattern of L1 and L2 binding proteins were mainly involved in pre-mRNA processing, alternative splicing, and RNA metabolism and transport (Figure 3A). In this study, we were more interested in the biological function of L1 in GC but not the underlying mechanism of LINC00477 splicing isoforms generation. Thus, we focused on ACO1 which was a unique cytosolic enzyme for regulating transferrin translation to control the level of intracellular iron and citrate/isocitrate conversion in tricarboxylic acid cycle, as well as aberrantly upregulated in leukemia and rectal carcinoma in previous studies.[25,26] Interestingly, we determined that LINC00477 could repress the activation through the interaction with ACO1 by RNA pull down and RIP assay (Figure 3B and C). And an unknown [illegible] resulted in L1 of LINC00477 reduction in GC los[illegible] the compromising activation of ACO1 and further gave [illegible] to the disorder of glycometabolism (F[illegible]ure 3[illegible] and [illegible] for tumorigenesis.

Abbreviation list

GC, gastric cancer; L[illegible]RNA, long non-coding RNA; ACO1, aconitase 1; [illegible]IP, RNA immunoprecipitation.

Ethicals [illegible]pro[illegible]al an[illegible] informed consent

Guidelin[illegible] for [illegible]e ca[illegible]d use of animals of Institutional Animal Car[illegible]nd Use Committee of Zhengzhou University were followed.

Informed consent was obtained from all individual participants included in the study.

Acknowledgment

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Disclosure

The authors report no conflicts of interest in this work.

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