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Involvement of the MicroRNA-1-LITAF Axis in Gastric Cancer Cell Growth and Invasion

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Abstract. *Background/Aim: Lipopolysaccharide-induced tumor necrosis factor alpha factor (LITAF) has been identified as a tumor suppressor in human cancers. Present study, we assessed biological role of LITAF in human gastric cancer. Materials and Methods: The clinical impacts of LITAF expression were assessed in gastric cancer using public databases. The biological role of LITAF was assessed in gastric cancer cells using siLITAF transfection. Results: High LITAF expression was correlated well with worse prognosis, including pathological stage (p=0.034) and pathological T stage (p=0.047), as well as with shorter survival. Herein, we present a novel finding that miR-1-3p could inhibit LITAF expression by directly binding to the 3'-untranslated region of LITAF mRNA. Cell functional assays revealed that LITAF knockdown could significantly suppress gastric cancer growth and motility. Conclusion: High LITAF expression resulting from low miR-1-3p expression is a biomarker for poor prognosis or therapeutic targets in gastric cancer.*

Gastric cancer is a common disease ranked 6th in cancer incidence and 5th in cancer mortality worldwide (World Health Organization GLOBOCAN database, https://gco.iarc.fr/). Infection with *Helicobacter pylori* with resultant chronic inflammation has been identified as a primary cause of both intestinal-type and diffuse-type gastric adenocarcinoma (1, 2). *H. pylori* infection can induce inflammation, affect cell growth, differentiation and renewal, impair gastric mucosal integrity, and lead to gastric injury. *H. pylori* infection usually occurs from infancy or early childhood and develops into invasive intestinal-type gastric cancer through chronic non atrophic gastritis, multifocal atrophic gastritis, intestinal metaplasia, and atypical hyperplasia (3, 4).

The lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α factor gene (*LITAF*) was first identified by Polyak *et al.* (5) in 1997. Its encoded protein product, LITAF, translocates into the nucleus once activated by LPS, followed by the up-regulation of TNF-α transcription (6-8). LITAF may be an enhancer of inflammatory diseases, such as inflammatory bowel disease (9) and arthritis (10). Moreover, *LITAF* has been identified as a potential tumor suppressor gene because its expression can be induced by p53 (5). *LITAF* expression is significantly lower in tumor tissues, including those in breast cancer, pancreatic cancer, and acute leukemia (11-13) compared to the corresponding normal tissues. However, not all cancer cells exhibit low *LITAF* expression. For example, Matsumura *et al.* (14) have observed higher *LITAF* expression in extramammary Paget's disease tissue compared to the isogenic normal tissue in three of four individuals.

LITAF may serve as a switch in the balance between classical inflammation and alternative activation in cancer (15). In this study, we investigated the *LITAF* function and subsequent pathway in gastric cancer and found that miR-1-3p can inhibit LITAF expression by directly binding to the 3'-untranslated region (UTR) of LITAF mRNA. This novel finding may contribute to the development of a viable therapeutic strategy to improve outcomes in patients with gastric cancer.

Materials and Methods

Cell lines. Six human cell lines: i) AGS, ii) AZ-521, iii) HR, iv) N-87, v) SNU-1, and vi) TSGH, were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% inactivated FBS (Invitrogen).

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Key Words: Gastric cancer, miR-1, LITAF.

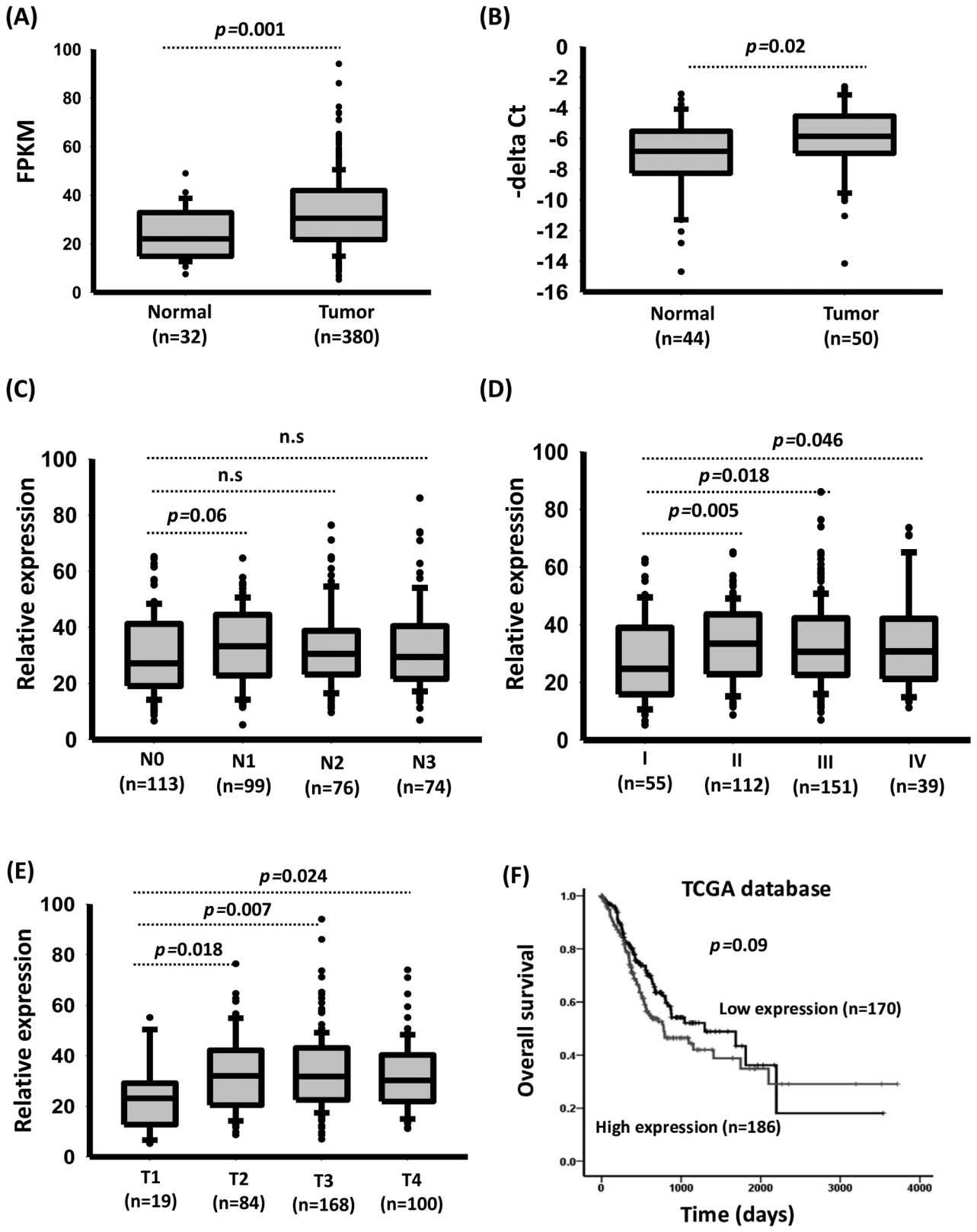

Figure 1. *Continued*

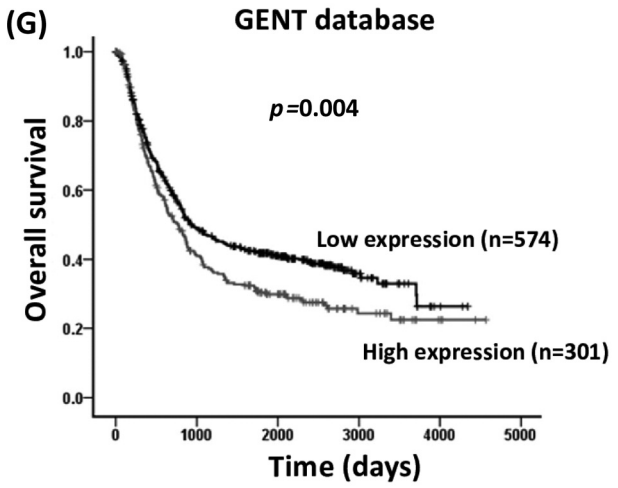

Figure 1. *Expression levels of LITAF are associated with poor prognosis in gastric cancer. (A) and (B) Expression levels of LITAF in gastric cancer tissues and corresponding adjacent normal tissues were examined by analyzing the TCGA database and real-time PCR. (C) High LITAF expression was not significantly associated with the lymph node metastasis status. (D) High LITAF expression was associated with the pathological stage and (E) with pathological T stage. Fragments per kilobase of transcript per million (FPKM) were used to quantify the gene expression. Effects of LITAF expression on overall survival of gastric cancer were examined by Kaplan–Meier survival curve following analysis from the TCGA database (F) and GENT (G).*

Table I. *Correlation of LITAF expression with clinicopathological characteristics of 360 gastric cancer patients.*

Variables	LITAF (n=360)			
	No. (%)	Mean±SD	Median	*p*-Value
Pathology stage				
I	54 (15.0)	27.12±14.24[c]	24.04	0.034[a]
II	116 (32.2)	33.61±12.59[c]	33.56	
III	151 (41.9)	32.55±14.42	30.55	
IV	39 (10.8)	33.64±16.08	30.51	
pT stage				
T1	19 (5.3)	23.87±14.08	23.15	0.047[a]
T2	77 (21.4)	33.30±15.05	33.19	
T3	165 (45.8)	33.06±14.10	31.58	
T4	99 (27.5)	31.48±13.07	30.66	
pN stage (n=357)				
N0	114 (31.9)	30.48±13.81	27.78	0.420[a]
N1	94 (26.3)	33.56±13.24	33.14	
N2	75 (21.0)	32.76±14.10	30.55	
N3	74 (20.7)	32.81±15.53	29.42	
pM stage				
M0	335 (93.1)	31.81±13.84	30.06	0.061[b]
M1	25 (6.9)	37.30±17.15	31.91	

[a]*p*-Values were estimated by one-way ANOVA; [b]p-Values were estimated by student's *t*-test; [c]*p*=0.050. M0 indicates patients without occurring distance metastasis. M1 indicates patients with occurring distance metastasis.

Clinical samples. Thirty clinical samples, including 44 of corresponding adjacent normal tissues, and 50 of gastric cancer tissues were obtained from the Biobank of Kaohsiung Veterans General Hospital and Taipei Tzu Chi Hospital for processing. This study was approved by the Institutional Review Board of Kaohsiung Veterans General Hospital Kaohsiung and Taipei Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Taiwan (VGHKS13-CT4-09 and No. 09-XD-038).

Expression data from The Cancer Genome Atlas and Gene Expression Database of Normal and Tumor Tissues. The transcriptome expression profiles of gastric cancer were downloaded from The Cancer Genome Atlas (TCGA) data portal (https://www.genome.gov/Funded-Programs-Projects/Cancer-Genome-Atlas). The RNA expression profiles of 380 samples of cancer tissues and 32 samples of adjacent normal tissues of patients with gastric cancer and the detailed clinical data of these patients were also downloaded from the TCGA database, including pathology stage, pT stage, pN stage, pM stage and survival time. The microarray expression data for gastric cancer were obtained from the Gene Expression Database of Normal and Tumor Tissues (GENT, http://genome.kobic.re.kr/GENT/). In total, expression data and survival data of 875 samples of gastric cancer tissues were collected. Then, the impacts of LITAF expression on overall survival were analyzed in patients with gastric cancer using the Kaplan–Meier method.

RNA extraction. Total RNA was extracted using TRIzol reagent (Invitrogen) as described previously (16).

Real-time reverse transcription polymerase chain reaction (PCR). We completed the procedure according to the manufacturer's instructions (Invitrogen) and as described previously (17). A 2 μg of total RNA was reverse transcribed using oligo $(dT)_{15}$ primers and SuperScript III Reverse Transcriptase. The sequences of primers are as follows:
LITAF-F: 5'-CCATCCGCACCTCCATCCTA-3'
LITAF-R: 5'-TGGATAGGGCGGTCCAAAAA-3'
GAPDH-F: 5'-TGCACCACCAACTGCTTAGC-3'
GAPDH-R: 5'-GGCATGGACTGTGGTCATGAG-3'

Stem–loop reverse transcription PCR. We performed this procedure according to the manufacturer's instructions (Invitrogen) and as we described previously (18), 1 μg of total RNA was reverse-transcribed through a stem–loop reverse transcription reaction using microRNA reverse transcription primers and SuperScript III Reverse Transcriptase. Gene expression was detected using an SYBR Green I assay (Applied Biosystems, Foster City, CA, USA) and the expression level of miR-1-3p was normalized to that of U6 (ΔCt=miR-1-3p Ct-U6 Ct). To simplify data presentation, relative expression values were multiplied by 10^5. The sequences of primers are as follows:
miR-1-3p-RT:
5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATACATAC-3'
miR-1-3p-GSF: 5'-CGGCGGTGGAATGTAAAGAAGT-3'
Universal reverse: 5'-CTGGTGTCGTGGAGTCGGCAATTC-3'
U6-F: 5'-CTCGCTTCGGCAGCACA-3'
U6-R: 5'-AACGCTTCACGAATTTGCGT-3

Table II. *Univariate and multivariate Cox's regression analysis of gene expression for overall survival of 356 patients with gastric cancer.*

Characteristic	No. (%)	OS			
		CHR (95% CI)	*p*-Value	AHR (95% CI)	*p*-Value
LITAF	(n=356)				
Low	170 (47.8)	1.00	0.090	1.00	0.192
High	186 (52.2)	1.34 (0.96-1.87)		1.26 (0.89-1.77)	

OS: Overall survival; CHR: crude hazard ratio; AHR: adjusted hazard ratio. AHR were adjusted for AJCC pathological stage (II, III and IV *vs*. I).

Small interfering RNA transfection. Small interfering RNA (siRNA) targeting LITAF (siLITAF, sense: 5'-AGAUGAUCGUGAGU CAGCUTT-3'; antisense: 5'-AGCUGACUCACGAUCAU CUTG-3') and a scrambled oligonucleotide used as a negative control were designed and synthesized by GenDiscovery Biotechnology (Taipei, Taiwan). The cells were transfected with a final concentration (10 mM) of individual siRNA or control using Lipofectamine RNAiMAX (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA), according to the manufacturer's instructions.

miR-1-3p overexpression. The gastric cancer cells were transfected with 10 nM of miRNA-1-3p mimics (sense: 5'-UGGAAUG UAAAGAAGUAUGUAU-3'; antisense: 5'-ACAUACUUCUUU ACAUUCCAUU-3') or an appropriate miRNA mimic control (sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense: 5'-ACGUGACACGUUCGGAGAATT-3') using Lipofectamine RNAiMAX reagent (13778150, Thermo Fisher Scientific Inc.), according to the manufacturer's instructions.

miRNA target candidates and luciferase reporter assay. The prediction of the miRNA candidates for binding to 3'-UTR of LITAF mRNA was done using the prediction tool on microRNA.org (19). The 3'-UTR sequences and seed region mutant of LITAF were cloned into a *pMIR-REPROT* vector (AM5795, Thermo Fisher Scientific). Subsequently, the *pMIR-REPROT-LITAF* or *pMIR-REPROT-LITAF*$_{(mutant)}$ vector was co-transfected with or without the miR-1-3p expression vector into a 293T cell line using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific). After 24 h of transfection, cell lysates were used to measure luciferase activity using the Dual-Glo Luciferase Assay System (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions.

Western blotting. Western blotting was performed as described previously (18). The cells were washed with phosphate-buffered saline (PBS) and then lysed with a lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.02% sodium azide, 1 μg/ml of aproteinin, and 1 mM PMSF) at 4˚C for 30 min. The lysate was then collected and centrifuged to remove cell debris. Protein assays were performed using a Bio-Rad Protein Assay kit (Bio-Rad Inc., Hercules, CA, USA) based on the Bradford dye-binding procedure. Protein samples (60 μg) were separated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 10% resolving gel by using a Mini-PROTEAN 3 cell apparatus. The proteins were then electrotransferred onto nitrocellulose membranes (NEF1002001PK, PerkinElmer, Inc., Waltham, MA, USA). After blocking at 4˚C overnight by using PBS-Tween containing 5% skim milk, the membranes were incubated with anti-LITAF antibody (1:1000, 611614, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and an anti–beta-actin antibody (1:2000, MAB1501, Merck Millipore, Billerica, MA, USA).

Cell proliferation assay or colony formation assay. For cell proliferation analysis, 5000 living cells were plated onto 96-well plates. After transfecting with siLITAF or control, the number of viable gastric cancer cells was determined at 0, 1, 2, 3, and 4 days using the CellTiter-Glo One Solution Assay (Promega Corporation, Madison, WI, USA). For the colony formation assay, 4000 living cells were seeded onto 6-well plates. Transfection with siLITAF was implemented. The cells were then cultured for 2 weeks.

Cell invasion assay, and cell migration assay. Cell invasion and migration ability were evaluated using the transwell migration/invasion assay (Costar, Lowell, USA) as described previously (17). The cells were tested for migration and invasion abilities *in vitro* in transwell chambers (Costar, Lowell, MA, USA). The lower side or the upper side of the polycarbonate membranes (containing 8-μm pores) of the transwell was coated with 50 μg/ml of type I collagen or 80 μg per well of Matrigel and then used for migration or invasion assays, respectively. AGS or AZ-521 cells were transfected with siLITAF, or a control. Subsequently, the cells were added to the upper chamber of a transwell. After incubation for 24 h at 37˚C, the cells at the lower side were prepared for Giemsa staining. The level of migration or invasion was determined using a microscope at 200× magnification.

Statistical analysis. LITAF and miR-1-3p expression levels between adjacent normal and gastric cancer were analyzed using Student *t*-tests. The expression levels of LITAF in differ pathology stage, pT stage, pN stage, pM stage of patients with gastric cancer were assessed using Student *t*-tests. Cumulative survival curves were estimated using the Kaplan–Meier method and comparison between survival curves was performed using the log-rank test. Differences were considered to be significant when $p<0.05$. The cell proliferation assay, colony formation assay, and the invasion/migration experiments were performed in triplicate. The histograms present the mean values and the error bars indicate the standard deviation. These data were analyzed using Student *t*-test.

Results

High LITAF expression associated with poor prognosis in gastric cancer. We examined the expression levels of LITAF in gastric cancer by analyzing the TCGA database. As shown

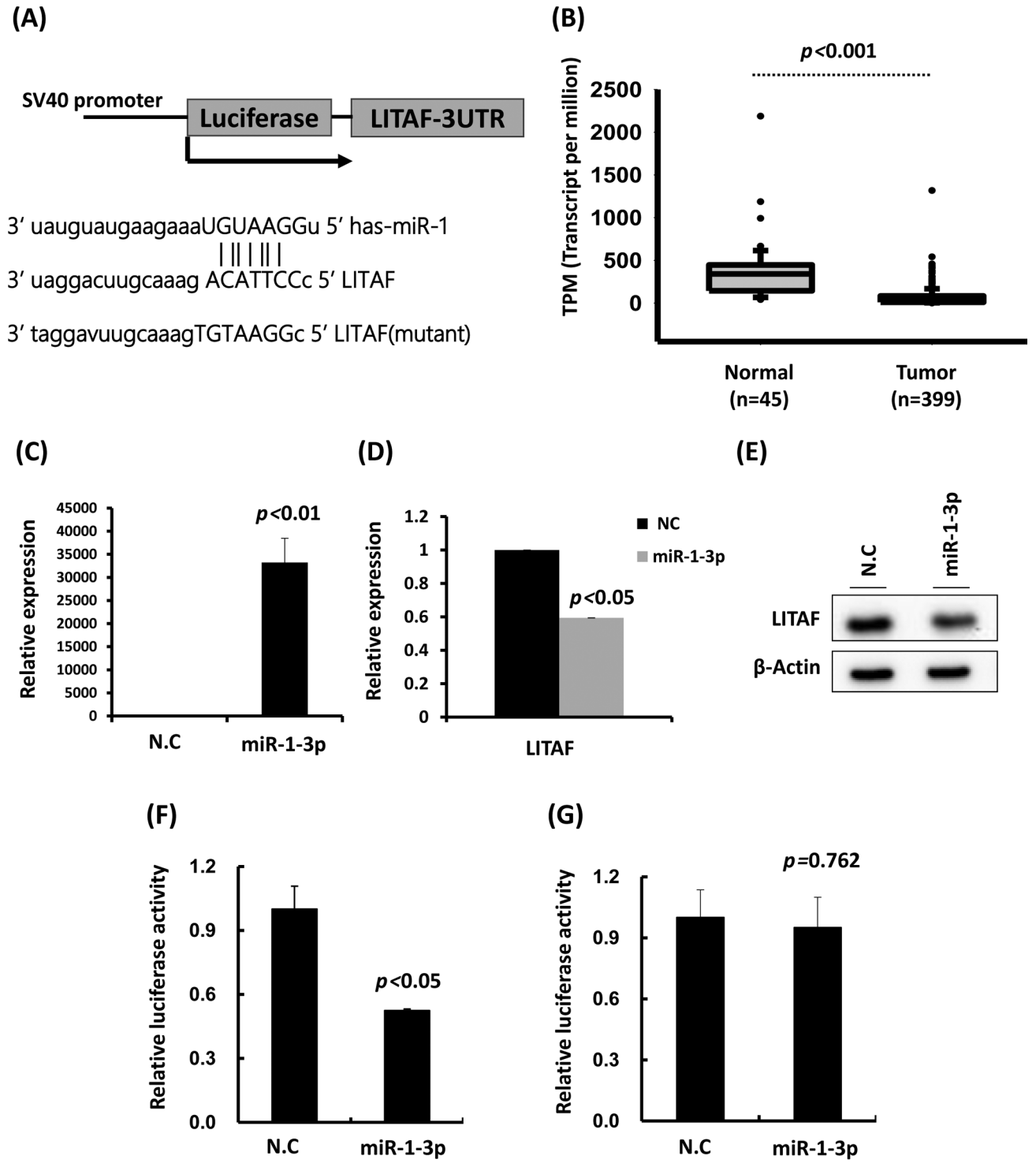

Figure 2. *MicroRNA-1-3p suppresses LITAF expression by directly targeting 3'-UTR of LITAF mRNA in gastric cancer. (A) Schematic diagram of the binding sites of miR-1-3p with 3'-UTR of LITAF mRNA. (B) The expression levels of miR-1-3p were assessed by analyzing the TCGA database. (C) miR-1-3p expression was examined in AGS cells transfected with miR-1-3p mimics. (D) and (E) the mRNA and protein of LITAF were assessed in AGS cells transfected with miR-1-3p and a negative control. (F) and (G) Reporter constructs with LITAF's wild-type 3'-UTR or s mutated binding site co-transfected with miR-1-3p mimics into 293T cells. Luciferase activity was then assessed. Transcript per million (TPM) was used to quantify miR-1-3p expression.*

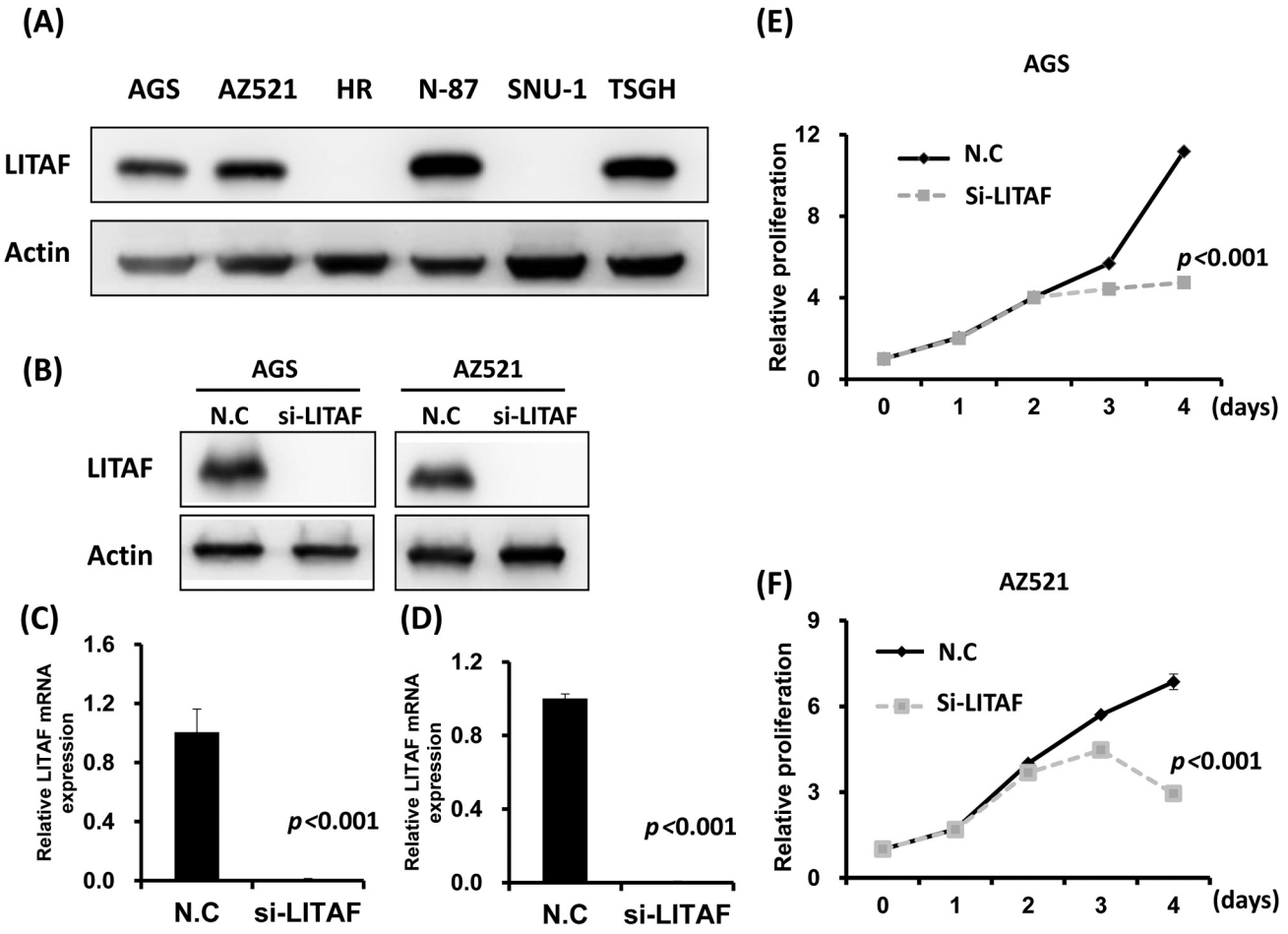

Figure 3. *LITAF knockdown suppresses gastric cancer cell proliferation. (A) LITAF protein expression was examined in human gastric cancer cells using Western blotting. (B) and (C) LITAF expression was examined in AGS and AZ-521 cells with siLITAF transfection using Western blotting and real-time PCR, respectively. (D) and (E) Cell proliferation was examined in AGS cells and AZ-521 cells with LITAF knockdown, respectively.*

in Figure 1A, significant LITAF up-regulation was observed in gastric cancer tissues compared to corresponding adjacent normal tissues. To confirm this finding, we examined *LITAF* expression levels in corresponding adjacent normal tissues, and gastric cancer tissues through real-time PCR. The mRNA levels of LITAF were significantly higher in the gastric cancer tissues compared to the corresponding adjacent normal tissues (Figure 1B). The association between LITAF expression and clinicopathological features of gastric cancer were also examined by analyzing the TCGA database. High LITAF expression was significantly associated with pathological stage (p=0.034) and pathological T stage (p=0.047), but not with lymph node metastasis status (p=0.42) (Figures 1C-E and Table I). In addition, it was borderline-associated with poor survival in patients with gastric cancer (p=0.09) (Table II and Figure 1F). To confirm the effects of LITAF expression on the overall survival of patients with gastric cancer, we performed further assessment using a cohort from the GENT. In total, expression data and survival data of 875 samples of gastric cancer tissues were collected. Then, the impact of LITAF expression on overall survival was analyzed in patients with gastric cancer by Kaplan–Meier method. As shown in Figure 1G, high LITAF expression was significantly associated with worse survival in patients with gastric cancer (p=0.004).

LITAF overexpression in gastric cancer due to low miR-1-3p levels. To explore the mechanisms of the abnormal LITAF expression in gastric cancer, we further investigated whether LITAF miRNA was up-regulated in gastric cancer. Using the microRNA.org prediction tool, we determined that miR-1-3p potentially targets 3'-UTR of LITAF mRNA (Figure 2A) and that miR-1-3p expression is significantly reduced in gastric cancer (Figure 2B). Ectopic miR-1-3p expression revealed that it could significantly suppress LITAF mRNA and protein levels (Figure 2C–E). The luciferase reporter assay revealed that miR-1-3p significantly inhibited luciferase activity by directly

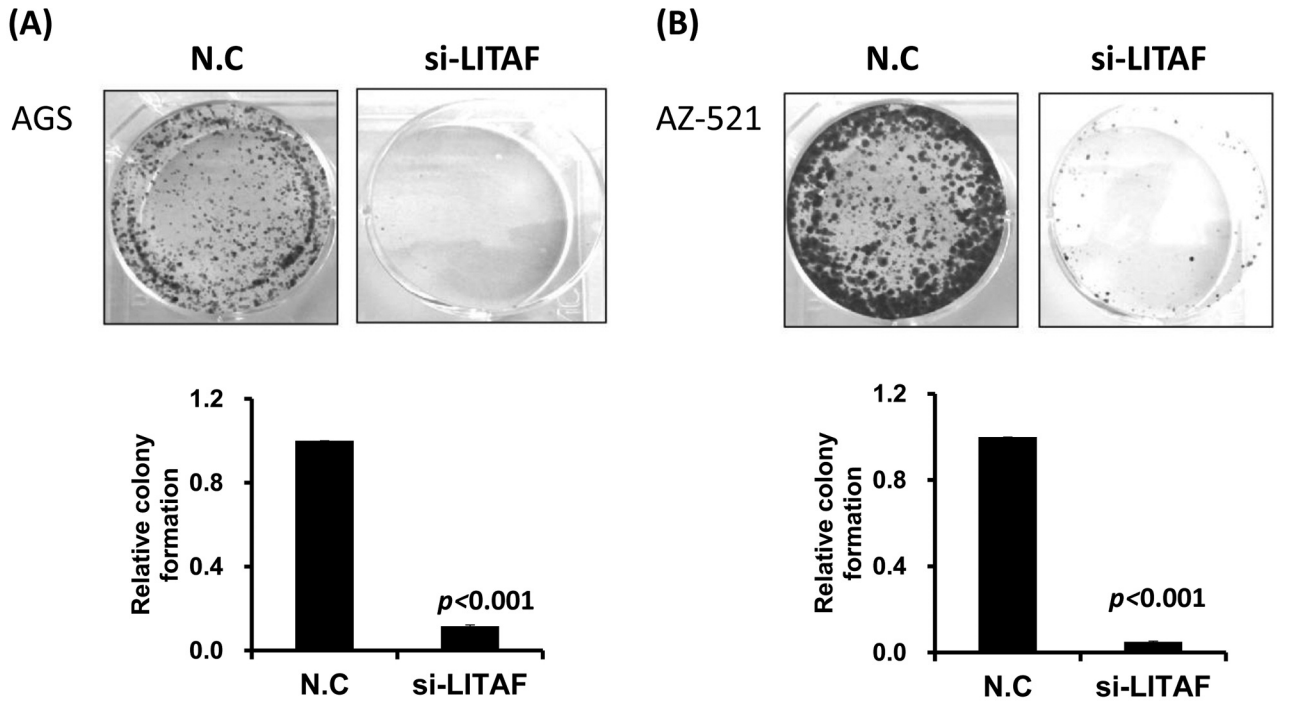

Figure 4. *LITAF knockdown could inhibit colony formation in gastric cancer cells. (A) and (B) Colony formation ability was examined and quantified in AGS cells and AZ-521 cells with LITAF knockdown.*

targeting the 3'-UTR of LITAF mRNA (Figure 2F). After the binding sites of miR-1-3p were mutated, luciferase activity was restored (Figure 2G). In summary, our data showed that miR-1-3p could suppress LITAF expression in gastric cancer through direct binding to 3'-UTR of LITAF mRNA.

LITAF knockdown suppresses gastric cancer growth and motility. Database analysis revealed that LITAF expression is higher in advanced rather than at early pathological and pathological T stages, suggesting that LITAF expression is involved in gastric cancer cell growth. To explore the biological role of LITAF in gastric cancer, we designed siRNA to silence LITAF expression in human gastric cancer cells. As shown in Figure 3A, LITAF expression was high in AGS, AZ-521, N-87, and TSGH cells. We selected AGS and AZ-521 cells, which showed the high LITAF expression, for subsequent analysis. After siLITAF transfection for 48 h, the expression levels of LITAF protein and mRNA were significantly reduced in both AGS and AZ-521 cells (Figures 3B and 3C). LITAF knockdown significantly suppressed AGS cell proliferation, colony formation, and cell motility (Figures 3D, 4A, 5A, and 5B). Similar effects were observed for the AZ-521 cells, indicating that LITAF knockdown can significantly reduce cell growth and motility (Figures 3E, 4B, 5C and 5D). Our results suggest that LITAF might play an oncogenic role, regulating gastric cancer cell growth and metastasis.

Discussion

LITAF is considered a tumor suppressor gene because its expression is regulated by p53 (5). Recent studies have shown that LITAF is frequently down-regulated in different cancers, including breast cancer, acute leukemia, lymphoma, prostate cancer, and pancreatic cancer (11-13, 20-23). The possible mechanisms of the tumor-suppressive activity of LITAF (15) include promoting p53- and/or p72-mediated cell apoptosis following tumor growth restriction (24-26), promoting the ubiquitin–proteasome system in mediating the degradation of pro cancerous proteins (27), and acting as a downstream target of the tumor suppressor factor AMPK to stimulate *TNFSF15* expression and then restrain angiogenesis, thereby inhibiting tumor growth (23). However, LITAF has not always been regarded as a tumor suppressor gene and has higher expression levels in some types of cancer tissues. Matsumura *et al*. in 2004 (14) observed that extramammary Paget's disease tissue showed higher *LITAF* expression levels compared to the isogenic normal tissue. The researchers suggested that the overexpression could be caused by *LITAF* mutations, which would lead to functional inactivation of normal LITAF, followed by accumulation of nonfunctional LITAF.

To the best of our knowledge, few studies have reported that LITAF contributes to gastric tumorigenesis (28, 29). Xu *et al*. have identified three new gene fusions (i) BMX-

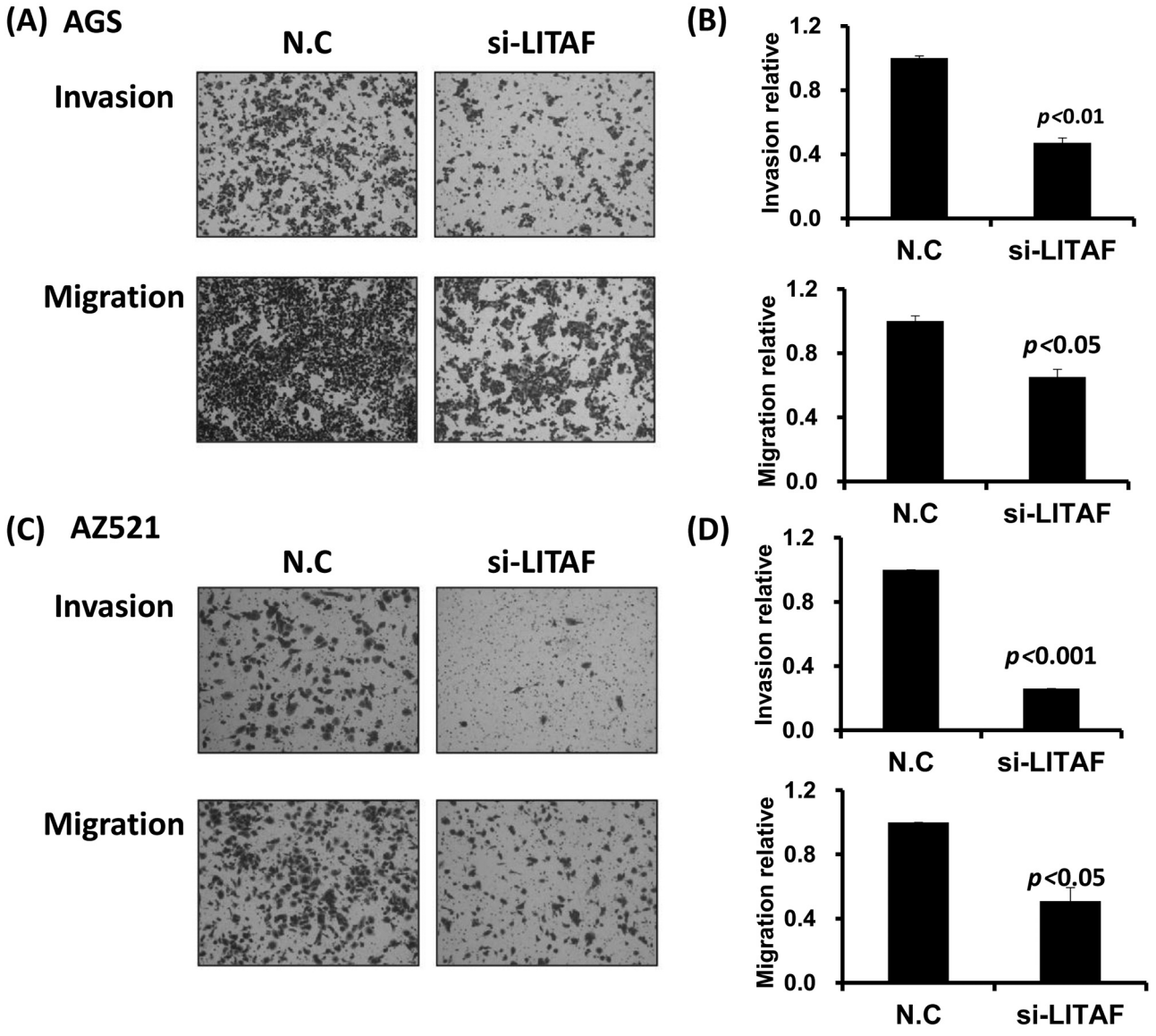

Figure 5. *LITAF knockdown suppressed gastric cancer cell motility (A) and (B) Cell migration and invasion were examined in AGS cells with LITAF knockdown. The invading and migrating cells were quantified. (C) and (D) Cell migration and invasion were examined in AZ-521 cells with LITAF knockdown. Relative invading and migrating abilities were quantified.*

ARHGAO12, ii) LRP5-LITAF, and iii) C15orf75-CBX3) in gastric cardia adenocarcinoma by analyzing next-generation sequencing data (28). However, the biological role and clinical importance of LRP5-LITAF fusion in gastric cancer remain unclear. Metformin treatment has been found to increase LITAF expression and reduce Bmi-1 expression in AGS cells (29). Knockdown of LITAF could induce miR-15a, miR-194, miR-128, and miR-192 expression (29). Huang *et al*. have concluded that metformin regulation of miR-15a, miR-194, miR-128, and miR-192 is abolished by knockdown of LITAF (29). In addition, the researchers identified miR-15a, miR-194, miR-128, and miR-192 as downstream effectors of LITAF in tumor cells with metformin treatment. Additional studies have found that miR-15a, miR-194, and miR-128 play a tumor-suppressive role on regulating the growth, migration, and invasion of gastric cancer cells (30-32). These results may suggest that LITAF plays an oncogenic role in gastric cancer possibly through the regulation of miR-15a, miR-194, and miR-128 expression. The former is confirmed by our findings that LITAF knockdown can significantly suppress the proliferation, colony formation, migration, and invasion of gastric cancer cells.

The chronic inflammation of stomach cells that can be induced by *H. pylori* infection plays a critical role in gastric adenocarcinoma progression (1, 2). It has also been reported that reported that *H. pylori* infection can induce chronic inflammation, hyperplasia, and gastric motor dysfunction by modeling the miR-1-HDAC4/SRF axis (33). A separate study examining the Gene Expression Omnibus database has revealed that *H. pylori* infection can significantly induce LITAF expression (34). *H. pylori* is known to stimulate TNF-α production in gastric epithelial cells (35, 36). Therefore, the up-regulation of LITAF expression may be due to increased TNF-α in gastric cells infected by *H. pylori*. The present study reports a novel finding that miR-1-3p can suppress LITAF expression by directly targeting 3'-UTR. Saito *et al*. have reported that *H. pylori* infection significantly suppresses miR-1 and miR-133a/b expression using a mouse model (33). MicroRNA-1 is tumor-suppressor regulating growth, angiogenesis, and metastasis in gastric cancer cells (37, 38). MicroRNA-106a has been also reported to confer aggressive radioresistance to prostate cancer cells by suppressing LITAF expression (39). Recently, the miR-10b-3p-LITAF axis was identified to contribute to hypoxia-induced inhibition of cellular viability, migration, and invasion in preeclampsia (40).

In conclusion, our findings suggest that the miR-1-LITAF axis may be involved in gastric carcinogenesis, suggesting this insight has potential as a therapeutic target in gastric cancer

Conflicts of Interest

The Authors declare that they have no competing interest.

Authors' Contributions

CYC executed this study and drafted the manuscript. CYC, WCC and CYR assisted in collecting clinical samples and assessed the expression levels of LITAF on gastric cancer. TYT and LMC performed the *in vitro* assays. TKW supervised the study and edited the manuscript.

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