

Original Article



LETM1 Promotes Gastric Cancer Cell Proliferation, Migration, and Invasion via the PI3K/Akt Signaling Pathway

Yunfeng Zhang , Lele Chen , Yifan Cao , Si Chen , Chao Xu , Jun Xing ,
Kaiguang Zhang

OPEN ACCESS

Department of Gastroenterology, The Affiliated Provincial Hospital of Anhui Medical University, Hefei, China

Received: Nov 27, 2019
Revised: Mar 4, 2020
Accepted: Mar 6, 2020

Correspondence to Kaiguang Zhang

Department of Gastroenterology, The Affiliated
Provincial Hospital of Anhui Medical University,
17 Lu Jiang Road, Hefei 230001, China.
E-mail: zhangkaiguang0097@163.com

Copyright © 2020. Korean Gastric Cancer
Association

This is an Open Access article distributed
under the terms of the Creative Commons
Attribution Non-Commercial License ([https://
creativecommons.org/licenses/by-nc/4.0](https://creativecommons.org/licenses/by-nc/4.0))
which permits unrestricted noncommercial
use, distribution, and reproduction in any
medium, provided the original work is properly
cited.

ORCID iDs

Yunfeng Zhang
<https://orcid.org/0000-0003-1078-0752>
Lele Chen
<https://orcid.org/0000-0002-6064-1569>
Yifan Cao
<https://orcid.org/0000-0002-2255-9994>
Si Chen
<https://orcid.org/0000-0001-9623-6787>
Chao Xu
<https://orcid.org/0000-0002-5711-088X>
Jun Xing
<https://orcid.org/0000-0002-2771-6725>
Kaiguang Zhang
<https://orcid.org/0000-0002-0740-3625>

Funding

This work was supported by the Fundamental
Research Funds for the Central Universities
(WK9110000004).

ABSTRACT

Purpose: Globally, there is a high incidence of gastric cancer (GC). Leucine zipper-EF-hand containing transmembrane protein 1 (LETM1) is reported to play a vital role in several human malignancies. However, there is limited understanding of the role of LETM1 in GC. This study aims to investigate the effects of LETM1 on proliferation, migration, and invasion of GC cells.

Materials and Methods: The expression levels of LETM1 in the normal gastric mucosal epithelial cells (GES-1) and GC cells were analyzed by quantitative real-time polymerase chain reaction and western blotting. CCK-8, wound healing, and Transwell invasion assays were performed to evaluate the effect of LETM1 knockdown or overexpression on the proliferation, migration, and invasion of the GC cells, respectively. Additionally, the effect of LETM1 knockdown or overexpression on GC cell apoptosis was determined by flow cytometry. Furthermore, the effect of LETM1 knockdown or overexpression on the expression levels of PI3K/Akt signaling pathway-related proteins was evaluated by western blotting.

Results: The GC cells exhibited markedly higher mRNA and protein expression levels of LETM1 than the GES-1 cells. Additionally, the knockdown of LETM1 remarkably suppressed the GC cell proliferation, migration, and invasion, and promoted the apoptosis of GC cells, which were reversed upon LETM1 overexpression. Furthermore, the western blotting analysis indicated that LETM1 facilitates GC progression via the PI3K/Akt signaling pathway.

Conclusions: LETM1 acts as an oncogenic gene to promote GC cell proliferation, migration, and invasion via the PI3K/Akt signaling pathway. Therefore, LETM1 may be a potential target for GC diagnosis and treatment.

Keywords: LETM1 protein, human; Phosphatidylinositol 3 kinase; Protein kinase B; Stomach neoplasms

INTRODUCTION

Globally, gastric cancer (GC) is the fifth most common cancer and the third leading cause of cancer-related mortality [1]. Each year more than 950,000 new cases of GC are diagnosed [2]. The risk factors that contribute to the occurrence and development of GC are *Helicobacter pylori* infection, chronic gastritis, and genetic mutations [3-5]. The accurate diagnosis of GC at the early stage is difficult as the patients are asymptomatic [6,7]. The 5-year survival rate for patients with advanced GC is approximately 25% after initial diagnosis [8]. There have

Author Contributions

Conceptualization: Z.Y., C.L., Z.K.; Funding acquisition: Z.K.; Methodology: Z.Y., C.L., C.Y., X.C.; Software: Z.Y., C.Y., X.C.; Supervision: Z.K., C.S.; Validation: Z.Y., C.L., C.Y.; Writing - original draft: Z.Y., C.L.; Writing - review & editing: Z.Y., X.J.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

been several advances in diagnostic modalities and therapeutic strategies for GC in the last few decades. However, the prognosis for patients with advanced GC is poor [9]. The median survival time of the metastatic GC cases is approximately one year [2]. Therefore, early diagnosis and therapy are vital for improving the long-term survival of patients with GC.

Leucine zipper-EF-hand containing transmembrane protein 1 (LETM1), which is localized to the inner mitochondrial membrane, is involved in the maintenance of mitochondrial morphology. LETM1 was first discovered in human Wolf-Hirschhorn syndrome, which is a complex malformation syndrome caused by the deletion of parts of the distal short arm of chromosome 4 [10,11]. Several studies have reported that LETM1 plays a pivotal role in mitochondrial ATP production and biogenesis, regulation of the mitochondrion ion channel, and mitochondrial respiration [11,12]. The dysregulation of LETM1 is reported to be an important factor that contributes to the initiation and progression of malignant tumors through cancerous metabolic alterations [12-14]. Chen et al. [14] reported that LETM1 is closely associated with the progression of carcinoma and that LETM1 is an independent poor prognostic factor in patients with head and neck squamous cell carcinoma. Yang et al. [15] reported that enhanced expression of LETM1 indicates poor prognosis and that LETM1 may be a potential cancer stem-like cell marker in patients with esophageal squamous cell carcinoma. However, the role of LETM1 in human GC has not been elucidated.

The phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) signaling pathway is one of the most frequently activated pathogenic signaling cascades in human malignancies, including GC [16-19]. The activity of Akt, which is the immediate downstream effector of PI3K, is regulated by phosphorylation. The phosphorylation stabilizes Akt and protects it against proteasome-mediated degradation [20]. Phosphorylated Akt (p-Akt), which is the active form of Akt, influences various cellular functions, including cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking [21]. Some studies have reported that the expression of LETM1 may be related to p-Akt protein. For example, Hwang et al. [22] reported that LETM1 altered the Akt signaling, suppressed the cell cycle, and promoted apoptosis in the lung cancer cells. Using immunohistochemical analysis, Piao et al. [23] revealed that LETM1 was strongly related to the expression of p-Akt in colorectal cancer. These studies only analyzed the expression level of LETM1 by immunohistochemical staining and did not verify the expression level by western blotting.

Previously, we had analyzed the immunohistochemical sections of 114 pairs of GC and adjacent normal tissues to investigate the expression level of LETM1. Additionally, we determined the correlation between LETM1 and clinicopathological characteristics of patients with GC, as well as the overall survival of patients with GC. The cancerous tissues exhibited significantly higher expression levels of LETM1 than the adjacent non-tumor tissues ($P < 0.01$). The expression level of LETM1 was closely associated with differentiation ($P = 0.030$), infiltration ($P = 0.003$), and lymph node metastasis ($P = 0.033$) of GC. Additionally, LETM1 was a negative prognostic factor for patients with GC ($P = 0.014$) [24]. These data indicated that LETM1 may play a crucial role in the carcinogenesis of GC.

In this study, we designed several functional experiments to analyze the role of LETM1 in the GC cells at the molecular level to investigate the role of LETM1 in human GC. The expression of LETM1 was markedly upregulated in the GC tissues and cell lines. Additionally, the knockdown of LETM1 effectively attenuated the proliferation, migration, and invasion of GC cells, which were reversed upon LETM1 overexpression. Furthermore, the western blotting analysis revealed that LETM1 exerts its function in GC via the PI3K/Akt signaling pathway.

MATERIALS AND METHODS

Cell culture

The GC cell lines (MGC-803, AGS, and MKN-45) and normal gastric mucosa epithelial cell line (GES-1) were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) and medium supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (HyClone). The cells were cultured in a humidified atmosphere at 5% CO₂ and 37°C.

Cell transfection

The small interfering RNA (siRNA) and overexpression plasmids targeting human LETM1 (si-LETM1 and oe-LETM1, respectively), as well as the corresponding negative controls (si-NC and oe-NC, respectively), were purchased from GenePharma (Shanghai, China). Each vector contained a green fluorescent protein reporter to determine transfection efficiency. The si-RNAs and overexpression plasmids were transfected into the cells using the Lipofectamine 2000 reagent (Invitrogen), following the manufacturer's instructions. The cells were harvested at 48-hour post-transfection and were used for subsequent experiments. The target sequences of the siRNAs are shown in **Table 1**.

Data mining

The immunohistochemistry staining data of LETM1 were obtained from the Human Protein Atlas database (<https://www.proteinatlas.org/>) [25]. The overall survival of patients with GC based on LETM1 expression was determined using Kaplan-Meier Plotter (<http://kmplot.com/analysis>) [26]. We selected the “exclude outlier arrays” option as the “array quality control,” while the other parameters were used as default settings.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the MKN-45 or MGC-803 cells using TRIzol reagent (Invitrogen), following the manufacturer's instructions. The isolated RNA was subjected to reverse transcription using the PrimeScript™ RT Master Mix (TaKaRa, Shiga, Japan). The complementary DNA was subjected to qRT-PCR in an ABI7500 PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix kit (TaKaRa). DNA was amplified under the following thermal cycling conditions: 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 30 seconds. The samples were analyzed in triplicates. The relative expression levels of LETM1 were calculated by the 2^{-ΔΔCt} method. The expression levels of LETM1 were normalized to those of the β-actin gene. The primer sequences used for qRT-PCR are listed in **Table 2**.

Table 1. The sequences used for siRNA duplexes

Gene name	Sense	Antisense
si-LETM1	5'-CCAUCGGCACCAACAACUUTT-3'	5'-AAGUUGUUGGUGCCGAUGGTT-3'
si-NC	5'-UUCUCCGAACGUGUCACGUTT-3'	5'-ACGUGACACGUUCGGAGAATT-3'

siRNA = small interfering RNA; NC, negative control; LETM1 = leucine zipper-EF-hand containing transmembrane protein 1.

Table 2. The primer sequences used for qRT-PCR

Gene name	Forward	Reverse
LETM1	5'-ATCAACGCCATGAAGCAAGTCAA-3'	5'-GTCCACCAGCTCAATCACCTT-3'
β-actin	5'-CTCCCTGGAGAAGAGCTACGAGC-3'	5'-CCAGGAAGGAAGGCTGGAAGAG-3'

qRT-PCR = quantitative real-time polymerase chain reaction; LETM1 = leucine zipper-EF-hand containing transmembrane protein 1.

Cell proliferation assay

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan), following the manufacturer's instructions. After 48 hours of transfection, the MKN-45 or MGC-803 cells were seeded in a 96-well plate. The analysis was performed with 3 replicates for each group. To each well, 10 μ L of CCK-8 solution was added and the cells were incubated at 37°C for 1 hour. The absorbance of the mixture was measured at 450 nm using a Multiskan FC plate reader (51119100, Thermo Scientific, Waltham, MA, USA). The proliferation of cells was determined every 24 hours.

Colony formation assay

The colony formation assay was performed following a previously described method [27]. Briefly, equal numbers of transfected cells were seeded into 6-well plates and cultured at 37°C for 14 days. Next, the cells were fixed in 4% paraformaldehyde at 25°C and stained with 0.1% crystal violet. The cells were photographed (Nikon, Japan) and counted.

Cell apoptosis

The MKN-45 or MGC-803 cells were transfected for 48 hours in a 6-well plate. Next, the cells were resuspended in a binding buffer containing Annexin V-fluorescein isothiocyanate and propidium iodide and incubated for 15 minutes in the dark at 25°C. The cells were then washed with phosphate-buffered saline (PBS, pH 7.2-7.4). The cells were subjected to fluorescence-activated cell sorting in a flow cytometer (BD Biosciences, San Jose, CA, USA).

Wound healing assay

The cells were cultured in a 6-well plate. A sterilized 10- μ L pipette tip was used to carefully scratch the monolayer (100% confluency). The exfoliated cells were washed with PBS and the medium was replaced with a serum-free medium. The photomicrographs were captured at 5 randomly chosen areas after scrapping for 24 hours. The cell migration areas were measured and the migration rates were calculated.

Transwell invasion assay

The cell invasion ability was measured using the Transwell insert chambers (Corning, New York, NY, USA) precoated with Matrigel (1:8 dilution; BD Biosciences). The cells were resuspended in a serum-free medium. Next, the cell suspension was added to the upper chamber. To the lower chamber, DMEM supplemented with 20% FBS was added. After incubation for 48 hours, the non-invading cells in the upper chamber were wiped. Finally, the membranes were fixed with 4% paraformaldehyde and stained with 1% crystal violet for 10 minutes. The number of invasive cells was counted in at least 3 randomly selected microscopic fields (200 \times magnification).

Western blotting

The cells were washed twice with pre-chilled PBS. The total cellular protein was extracted on ice using RIPA lysis buffer containing protease inhibitor. Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were electroblotted onto a 0.45 μ m polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA, USA). The PVDF membrane was blocked with 5% fat-free milk for 1 hour. Next, the membrane was incubated with specific primary antibodies at 4°C overnight. The membrane was then incubated with The membrane was incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1: 20,000; ab205718; Abcam, Cambridge, MA, USA) or goat anti-mouse (1: 5,000; ab6789; Abcam) dilution for 2 hours. The protein bands were visualized by enhanced chemiluminescence. The blot images were analyzed using the image analysis software Image

J (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The following primary antibodies were used in this analysis: anti-LETM1 (1:500; DF12417; Affinity, Cincinnati, OH, USA), anti-PI3K (1:1,000; 20584-1-AP; Proteintech, Chicago, IL, USA), anti-total Akt (t-Akt) (1:1,000; 10176-2-AP; Proteintech), anti-p-Akt (1:1,000; 66444-1-Ig; Proteintech), anti-p53 (1:1,000; sc-126; Santa Cruz Biotechnology, Dallas, TX), and anti-glyceraldehyde-3-phosphate dehydrogenase (1:1,000; sc-166545; Santa Cruz Biotechnology) antibodies.

Statistical analysis

The data are presented as mean±standard deviation from at least 3 independent experiments. The data were analyzed using the independent student's t-test in the GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The difference was considered statistically significant when the P-value was less 0.05. P-values of statistical significance are represented as *P<0.05, †P<0.01, and ‡P<0.001.

RESULTS

LETM1 is upregulated in the human GC cell lines and tissues

The immunohistochemical sections of GC exhibiting low and high expression levels of LETM1 are shown in **Fig. 1A**, which were obtained from the Human Protein Atlas database. The Kaplan-Meier Plotter analysis was performed by selecting the “exclude outlier arrays” option as the “array quality control.” The analysis revealed that high LETM1 expression level was significantly associated with poor overall survival in patients with GC (P=0.039) (**Fig. 1B**). The mRNA and protein expression levels of LETM1 in the GC cells and GES-1 cells were analyzed by qRT-PCR and western blotting, respectively. The mRNA and protein expression levels of LETM1 in the GC cell lines (MGC-803, AGS, MKN-45) were markedly higher than those in the GES-1 cells (**Fig. 1C and D**).

LETM1 knockdown inhibits proliferation and decreases the viability of the GC cells

To investigate the biological function of LETM1 in the GC cells, the MKN-45 cells were transfected with si-LETM1 or si-NC. The MKN-45 cells, which exhibit enhanced expression levels of LETM1, were chosen for this analysis. As shown in **Fig. 2A**, the transfection efficiency was greater than 70% after 48 hours. The mRNA and protein expression levels of LETM1 were analyzed in the LETM1 knockout cells. The si-LETM1-transfected MKN-45 cells exhibited downregulated mRNA and protein expression levels of LETM1 when compared to the si-NC-transfected MKN-45 cells (**Fig. 2B and C**). The results of CCK-8 assay and colony formation assays revealed that the cell viability and proliferative capacity of the si-LETM1-transfected cells were lower than those of the si-NC-transfected cells (**Fig. 2D and E**).

LETM1 knockdown facilitates apoptosis and inhibits migration and invasion of the GC cells

To further explore the underlying mechanisms that influence cell proliferation, flow cytometry was used to analyze cell apoptosis. The proportion of si-LETM1-transfected MKN-45 cells exhibiting apoptosis was higher than that of the si-NC-transfected MKN-45 cells (**Fig. 3A**). Furthermore, wound healing assay was employed to assess the effect of LETM1 knockdown on MKN-45 cell migration. The si-LETM1-transfected MKN-45 cells exhibited observably attenuated migration when compared to the si-NC-transfected MKN-45 cells (**Fig. 3B**). Next, the effect of LETM1 knockdown on the invasion of GC cells was evaluated using the Transwell chambers coated with Matrigel. The si-LETM1-transfected GC cells exhibited notably attenuated invasion when compared to the si-NC-transfected GC cells (**Fig. 3C**).

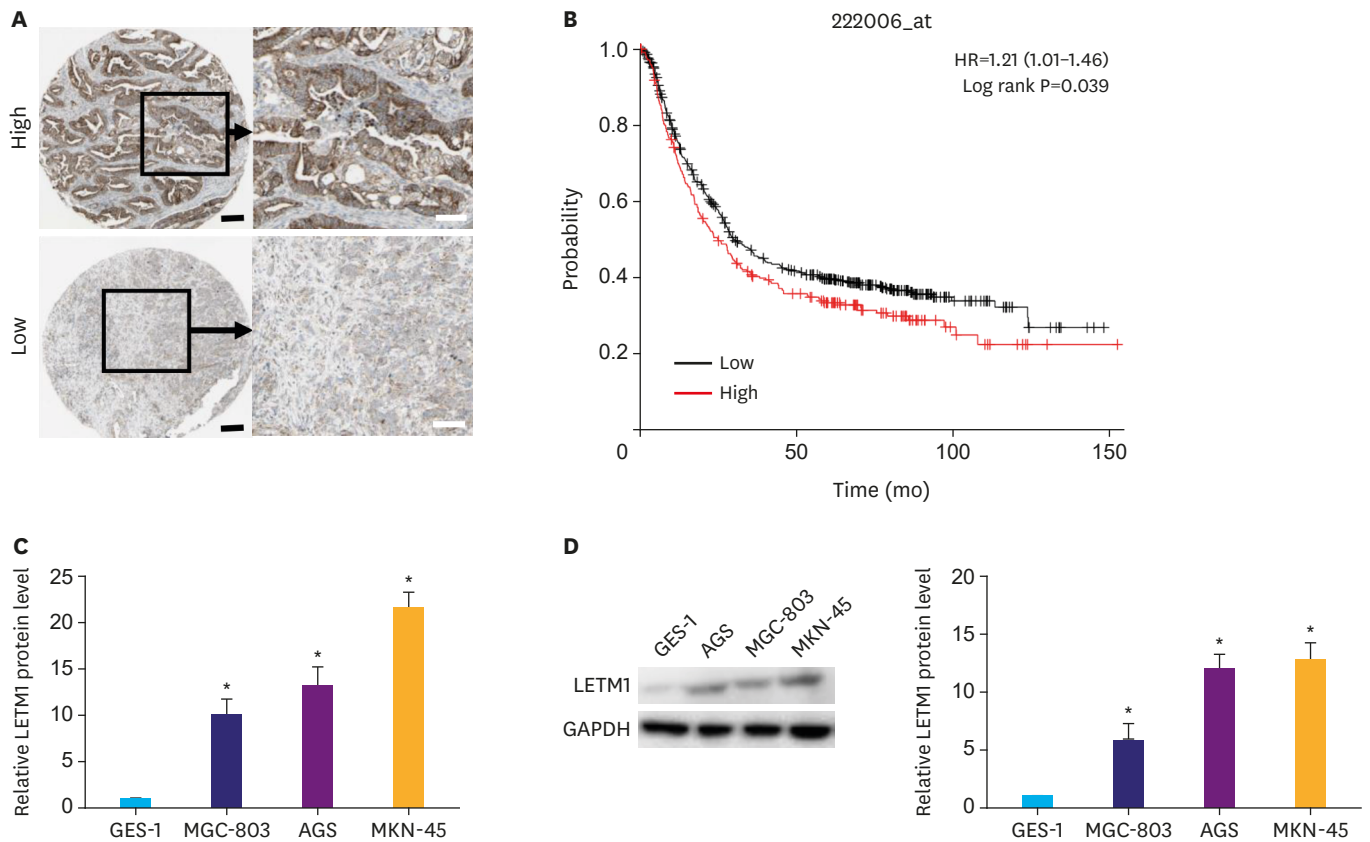


Fig. 1. LETM1 is overexpressed in the human GC cell lines and tissues. (A) The immunohistochemical images of GC cells exhibiting low and high expression levels of LETM1. Black bar, 200 μ m; White bar, 100 μ m. (B) The overall survival curves of patients with GC based on LETM1 expression obtained from Kaplan-Meier Plotter. The “exclude outlier arrays” option was selected as the “array quality control.” The low and high expression groups include 592 and 248 patients with GC, respectively. (n=840, P=0.039). (C) The mRNA expression levels of LETM1 in the GC cell lines (MGC-803, AGS, and MKN-45) and normal human gastric mucosal epithelial cell line (GES-1). (D) The protein expression levels of LETM1 in the GC cell lines (MGC-803, AGS, and MKN-45) and GES-1 cells. Data are presented as mean \pm standard deviation. LETM1 = leucine zipper-EF-hand containing transmembrane protein 1; GC = gastric cancer; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HR = hazard ratio. *P<0.01.

Overexpression of LETM1 promotes GC cell proliferation and viability

We also investigated the effects of LETM1 overexpression on GC cell proliferation, migration, and invasion. The MGC-803 cells, which exhibit downregulated expression levels of LETM1, were transfected with oe-LETM1 or oe-NC. As shown in **Fig. 4A**, the transfection efficiency was more than 80% after 48 hours of transfection. The transfection efficiency of the oe-LETM1 plasmid in the MGC-803 cells was confirmed by qRT-PCR and western blotting (**Fig. 4B and C**). The results of CCK-8 and colony formation assays demonstrated that the oe-LETM1-transfected cells exhibited increased cell viability and proliferation when compared to the oe-NC-transfected cells (**Fig. 4D and E**).

Overexpression of LETM1 suppresses GC cell apoptosis and enhances GC cell migration and invasion

The flow cytometry analysis revealed that the proportion of oe-LETM1-transfected MGC-803 cells exhibiting apoptosis was lesser than that of the oe-NC-transfected MGC-803 cells (**Fig. 5A**). Additionally, the oe-LETM1-transfected MGC-803 cells exhibited enhanced migration when compared to the oe-NC-transfected MGC-803 cells (**Fig. 5B**). The results of the Transwell invasion assays revealed that the oe-LETM1-transfected cells exhibited higher invasion than the oe-NC-transfected cells (**Fig. 5C**).

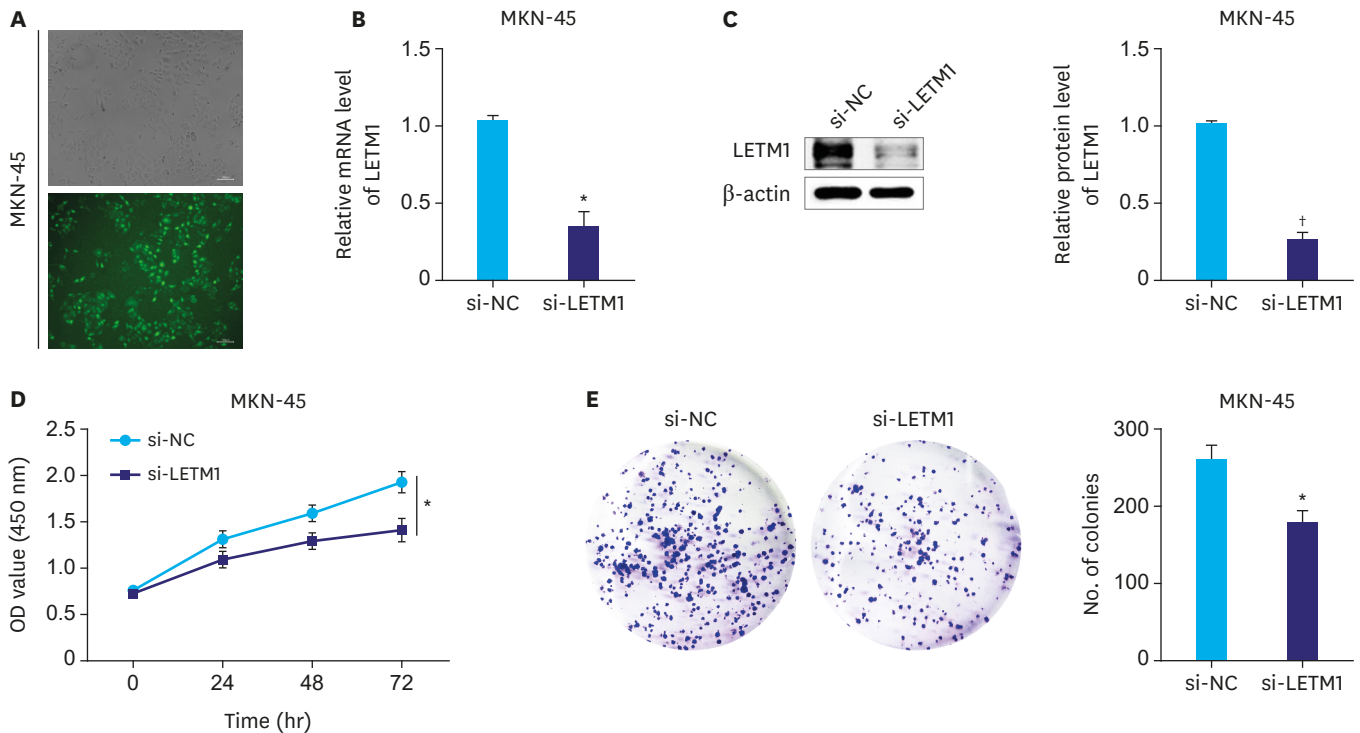


Fig. 2. LETM1 knockdown inhibits proliferation and decreases the viability of GC cells. (A) The transfection efficiency after 48 hours of transfection (magnification: 100×). (B) The mRNA expression level of LETM1 in the si-LETM1-transfected and si-NC-transfected MKN-45 cells was determined by qRT-PCR. (C) The protein expression level of LETM1 in the si-LETM1-transfected and si-NC-transfected cells was analyzed by western blotting. (D, E) CCK-8 assays and colony formation assays were performed to evaluate the proliferation capacity and the viability of the si-LETM1-transfected and si-NC-transfected GC cells, respectively. Data are presented as mean±standard deviation.

LETM1 = leucine zipper-EF-hand containing transmembrane protein 1; si-LETM1 = siRNA targeting LETM1; si-NC = negative control for the si-LETM1 group; qRT-PCR = quantitative real-time polymerase chain reaction; CCK-8 = Cell Counting Kit-8; GC = gastric cancer; OD = optical density.

*P<0.01; †P<0.001.

LETM1 exerts its function via the PI3K/Akt signaling pathway

To further investigate the underlying mechanism of LETM1 in proliferation, invasion, and migration of GC cells, we analyzed the protein expression levels of PI3K, t-Akt, p-Akt, and p53 in the si-LETM1-transfected and si-NC-transfected MGC-803 cells. The western blotting analysis revealed that the si-LETM1-transfected MGC-803 cells exhibited obviously lower protein expression levels of PI3K and p-Akt and markedly higher protein expression levels of p53 than the si-NC-transfected MGC-803 cells. The knockdown of LETM1 did not significantly affect the expression levels of t-Akt ($P>0.05$, Fig. 6A and B). In contrast, the oe-LETM1-transfected cells exhibited higher protein expression levels of PI3K and p-Akt and lower protein expression levels of p53 than the si-NC-transfected MGC-803 cells (Fig. 6C and D). These results suggested that LETM1 accelerates the malignant phenotype of GC via the activation of the PI3K/Akt signaling pathway.

DISCUSSION

In this study, we explored the role of LETM1 in GC. The human GC cell lines and tissues exhibit enhanced expression levels of LETM1. Previous studies have indicated that LETM1 is a negative prognostic factor for patients with GC. The results of this study revealed that the LETM1 knockdown suppresses GC cell proliferation, migration, and invasion, and facilitates GC cell apoptosis. Conversely, the overexpression of LETM1 enhances cell proliferation, migration, and

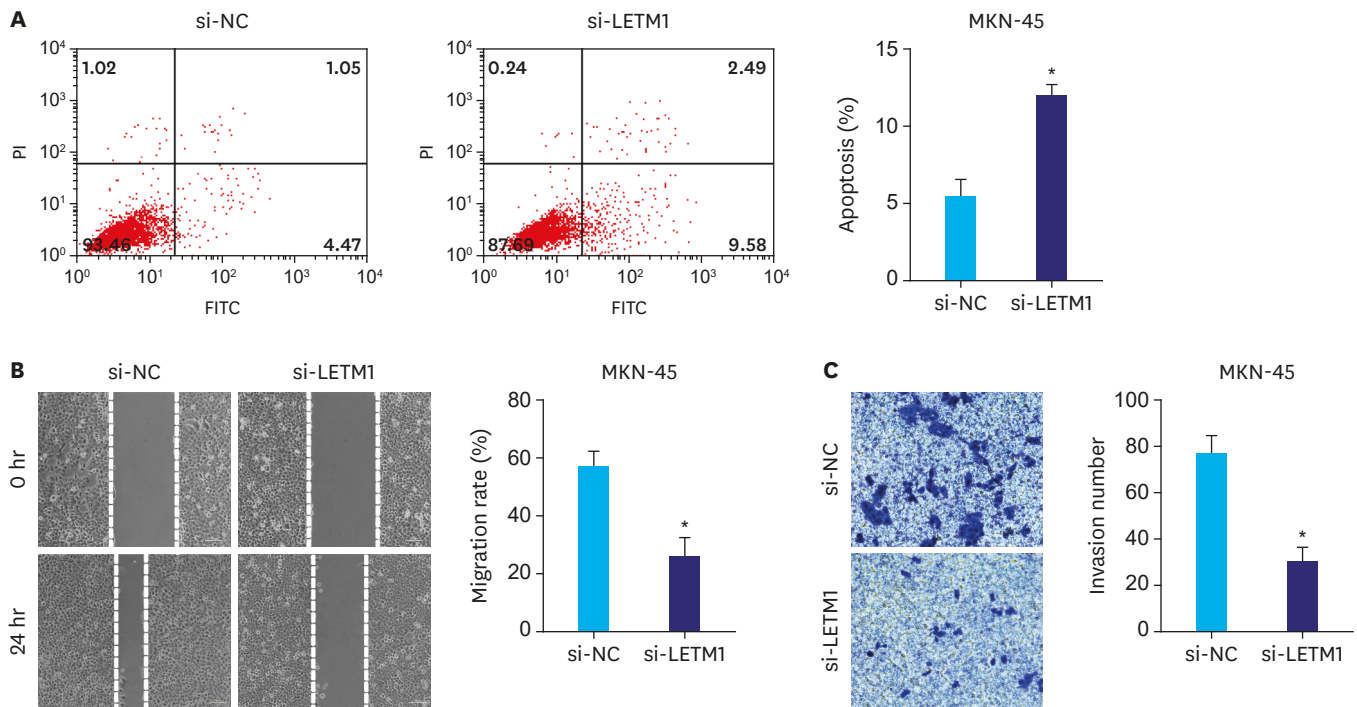


Fig. 3. LETM1 knockdown facilitates cell apoptosis and inhibits cell migration and invasion. (A) The apoptosis of si-LETM1-transfected and si-NC-transfected MKN-45 cells was analyzed by flow cytometry. (B) Wound healing assays were performed to assess the cell migration ability. The images were captured at 24 hours post-scratching (magnification: 100×). (C) The MKN-45 cells were subjected to Transwell invasion assay after siRNA transfection. The cells were fixed and stained using 1% crystal violet. The representative images are shown (magnification: 200×). Data are presented as mean±standard deviation. LETM1 = leucine zipper-EF-hand containing transmembrane protein 1; si-LETM1 = siRNA targeting LETM1; si-NC = negative control for the si-LETM1 group; PI = propidium iodide; FITC = fluorescein isothiocyanate. *P<0.01.

invasion, and decreases GC cell apoptosis. The results of this study also indicated that LETM1 exerts its biological functions through the PI3K/Akt signaling pathway. These findings indicate that LETM1 plays a major role in the tumorigenesis and progression of GC.

Several studies have reported that several oncogenes and tumor suppressor genes are involved in the occurrence and development of GC [28,29]. LETM1, which is markedly upregulated in multiple human cancers, predicts poor prognosis and functions as an oncogene [30-32]. In bladder cancer and renal cell carcinoma, the knockdown of LETM1 inhibits cell proliferation, migration, and invasion through the suppression of the Wnt/ β -catenin signaling pathway [30,32]. In breast cancer, the enhanced protein expression level of LETM1 was associated with lymph node metastasis, poor differentiation, and late clinical stage. Additionally, enhanced LETM1 protein expression level was an independent poor prognostic factor for patients with breast cancer [31]. In non-small cell lung carcinoma, Piao *et al.* [33] reported that LETM1 expression was correlated with the lymph node metastasis and the clinical stage and that LETM1 may facilitate the invasion or metastasis of non-small cell lung carcinoma cells. Consistent with these findings, the findings of this study demonstrated that LETM1 may act as an oncogene to promote the malignant biological behaviors of GC. In contrast to the findings of Piao *et al.*, Hwang *et al.* reported that LETM1 suppresses lung cancer cell growth in vitro and in vivo by inducing the destruction of mitochondria in the lung cancer cells via ATP depletion and AMPK activation [22]. The reason for this contradiction is unclear and may be related to the heterogeneity of the tumor, which must be explored in future studies.

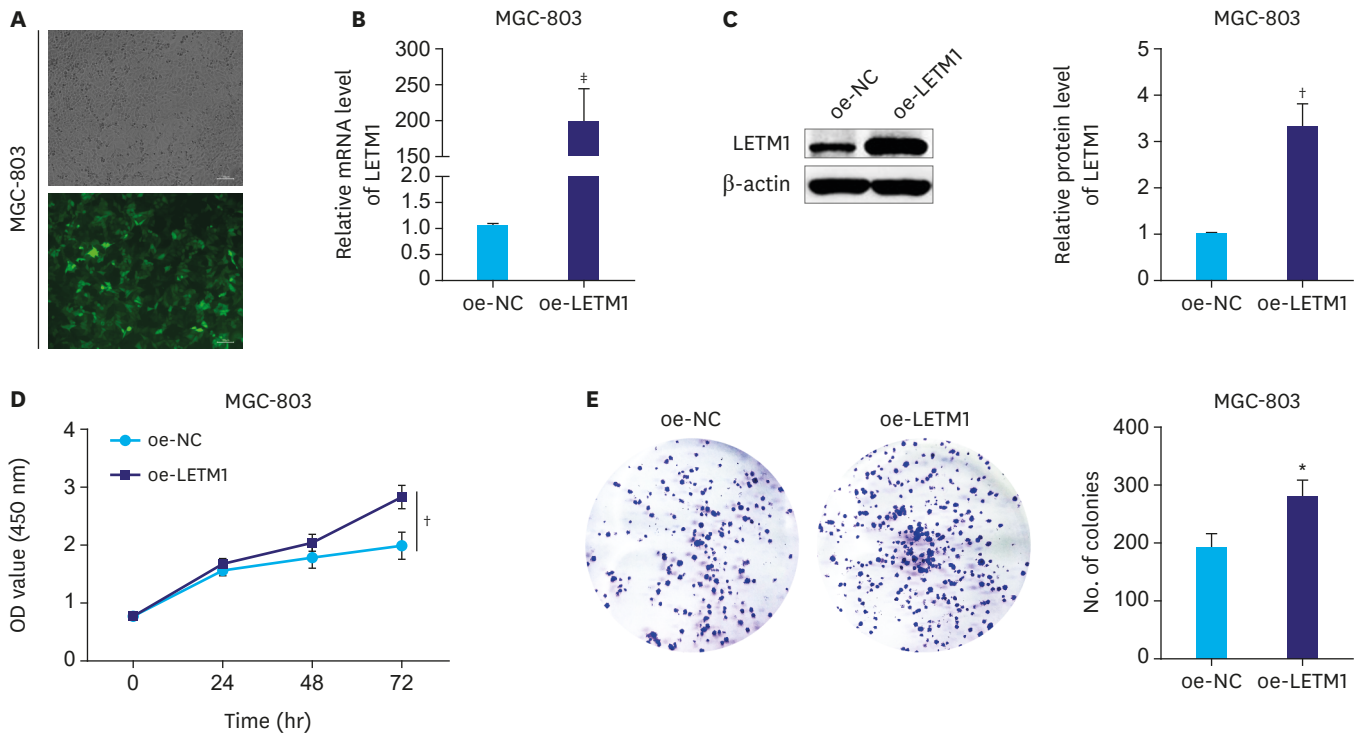


Fig. 4. Overexpression of LETM1 promotes cell proliferation and viability. (A) The transfection efficiency after 48 hours of transfection (magnification: 100×). (B) The mRNA expression level of LETM1 in the oe-LETM1-transfected and oe-NC-transfected MGC-803 cells was analyzed by qRT-PCR. (C) The protein expression level of LETM1 in the oe-LETM1-transfected and oe-NC-transfected MGC-803 cells was analyzed by western blotting. (D, E) CCK-8 assays and colony formation assays were performed to measure the proliferation capacity and viability of oe-LETM1-transfected and oe-NC-transfected GC cells. Data are presented as mean±standard deviation.

LETM1 = leucine zipper-EF-hand containing transmembrane protein 1; oe-LETM1 = LETM1 overexpression group; oe-NC = negative control for the oe-LETM1 group; qRT-PCR = quantitative real-time polymerase chain reaction; CCK-8 = Cell Counting Kit-8; OD = optical density.

*P<0.05; †P<0.01; ‡P<0.001.

The aberrant activation of the PI3K/Akt signaling pathway facilitates tumorigenesis and cancer progression [34,35]. The phosphorylation of Akt, which is an important node in the PI3K signaling pathway, has a pivotal role in tumors that are dependent on the PI3K/Akt axis activity [35]. In this study, the knockdown of LETM1 inhibited the expression of PI3K and p-Akt but did not affect the total amount of Akt. This indicated that LETM1 might exert its functions by activating the PI3K/Akt signaling pathway in the GC cells. Particularly, the function of LETM1 is dependent on the phosphorylation level of Akt after the activation of PI3K protein and not on the total amount of Akt. Consistent with our findings, both Yang and Piao [15,23] reported that the expression of LETM1, which was evaluated by immunohistochemical staining, was markedly associated with p-Akt protein in colorectal adenocarcinoma and esophageal squamous cell carcinoma. These findings suggest that the knockdown of LETM1 exerts tumor-suppressive effects by regulating the PI3K/Akt signaling pathway in GC.

Additionally, the knockdown of LETM1 increased the expression of p53 protein, which is well characterized as a tumor suppressor [36], and thereby inhibits malignant progression. In contrast, the overexpression of LETM1 decreased the expression of p53 protein to promote malignant phenotype. The mutation of p53 and the resultant inactivation of p53 allow evasion of tumor cell death and promote rapid tumor progression, while the restoration of normal function of p53 can trigger tumor cell death and tumor elimination [37]. LETM1 regulates the expression of p53 protein through the phosphorylated Akt because the activated Akt can increase ubiquitination and degradation of p53 protein [38].

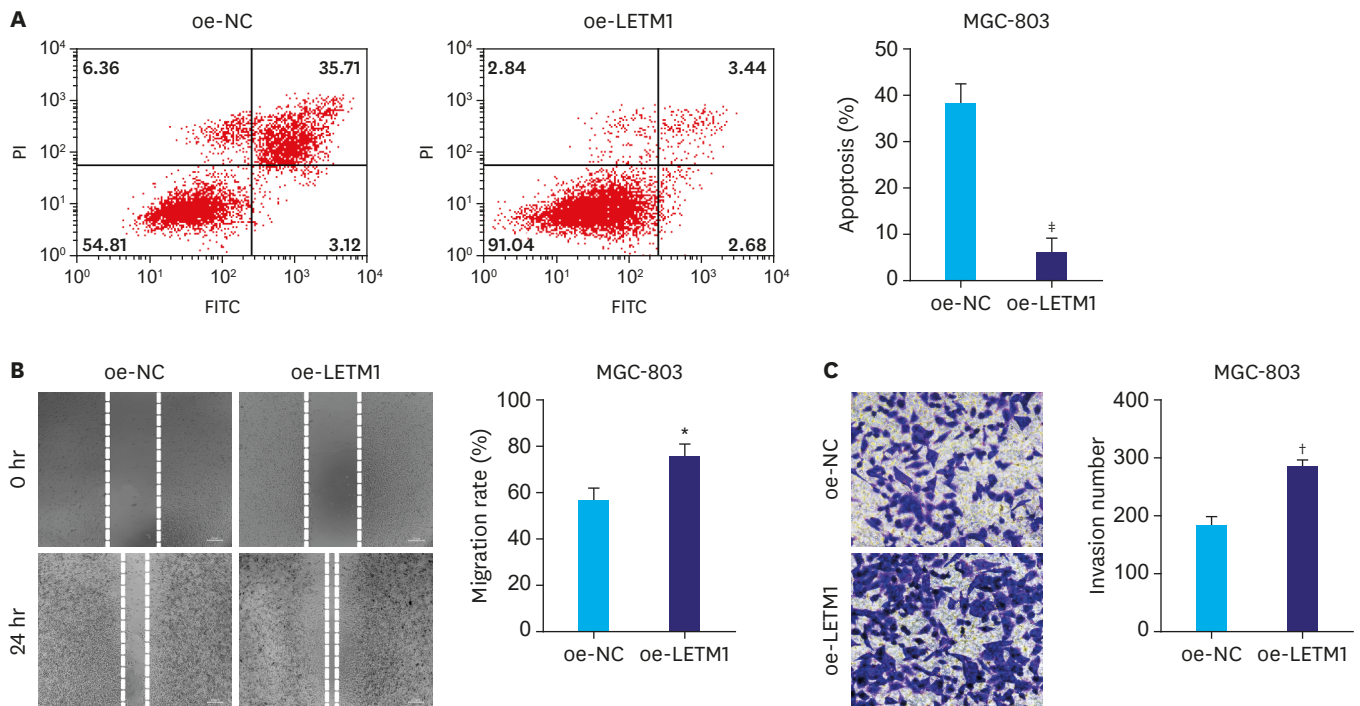


Fig. 5. Overexpression of LETM1 suppresses cell apoptosis and promotes cell migration and invasion. (A) The apoptosis of oe-LETM1-transfected and oe-NC-transfected MGC-803 cells was analyzed by flow cytometry. (B) Wound healing assays were performed to evaluate the migration of oe-LETM1-transfected and oe-NC-transfected MGC-803 cells. The images were captured at 24 hours post-scratching (magnification: 100×). (C) The oe-LETM1-transfected and oe-NC-transfected MGC-803 cells were subjected to Transwell invasion assays. The cells were fixed and stained using 1% crystal violet. The representative images are shown (magnification: 200×). Data are presented as mean±standard deviation. LETM1 = leucine zipper-EF-hand containing transmembrane protein 1; oe-LETM1 = LETM1 overexpression group; oe-NC = negative control for the oe-LETM1 group; PI = propidine iodide; FITC = fluorescein isothiocyanate. *P<0.05; †P<0.01; ‡P<0.001.

This explains the association of expression of PI3K and p-Akt protein with decreased p53 protein expression upon LETM1 overexpression in GC.

In conclusion, these results reveal that the knockdown of LETM1 inhibits GC cell proliferation, migration, and invasion, which are reversed upon LETM1 overexpression, via the activation of the PI3K/Akt signaling pathway. Therefore, LETM1 may be a promising therapeutic target for GC.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136:E359-E386. [PUBMED](#) | [CROSSREF](#)
2. Van Cutsem E, Sagaert X, Topal B, Haustermans K, Prenen H. Gastric cancer. *Lancet* 2016;388:2654-2664. [PUBMED](#) | [CROSSREF](#)
3. Suerbaum S, Michetti P. *Helicobacter pylori* infection. *N Engl J Med* 2002;347:1175-1186. [PUBMED](#) | [CROSSREF](#)
4. Wang F, Meng W, Wang B, Qiao L. *Helicobacter pylori*-induced gastric inflammation and gastric cancer. *Cancer Lett* 2014;345:196-202. [PUBMED](#) | [CROSSREF](#)
5. Oliveira C, Pinheiro H, Figueiredo J, Seruca R, Carneiro F. Familial gastric cancer: genetic susceptibility, pathology, and implications for management. *Lancet Oncol* 2015;16:e60-e70. [PUBMED](#) | [CROSSREF](#)

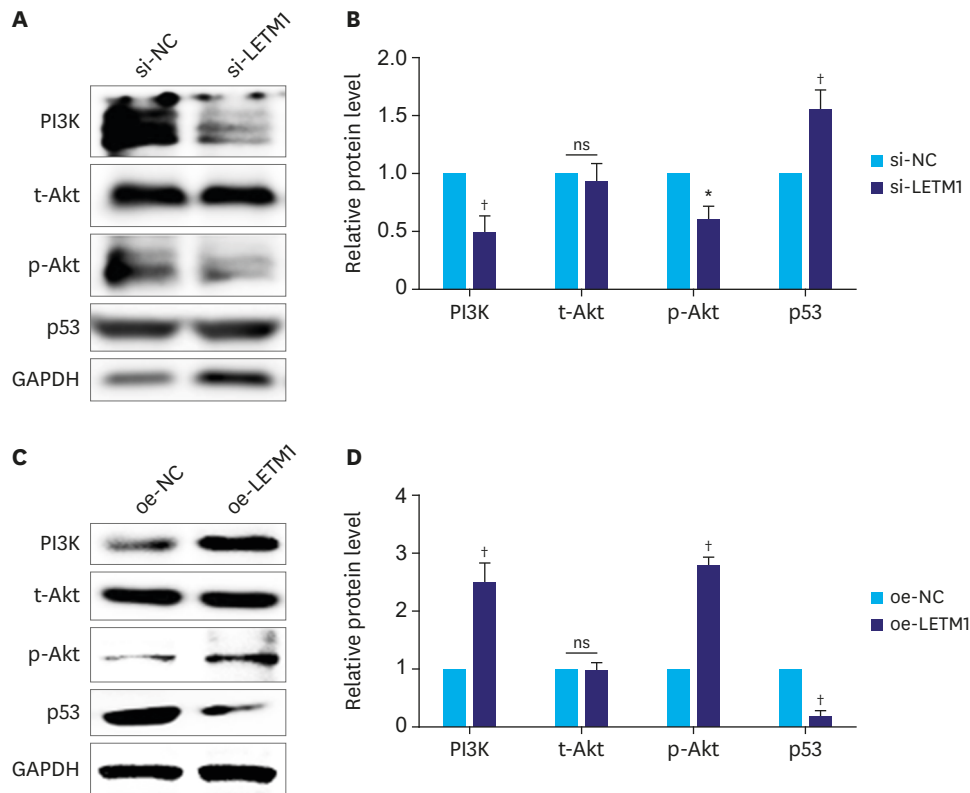


Fig. 6. LETM1 exerts its function via the PI3K/Akt signaling pathway. (A, B) The protein expression levels of PI3K, t-Akt, p-Akt, and p53 in the si-LETM1-transfected and si-NC-transfected MKN-45 cells were measured by western blotting. (C, D) The protein expression levels of PI3K, t-Akt, p-Akt and p53 in the oe-LETM1-transfected and oe-NC-transfected MGC-803 cells were detected by western blotting. Data presented as mean±standard deviation. LETM1 = leucine zipper-EF-hand containing transmembrane protein 1; PI3K = phosphatidylinositol 3-kinase; Akt = protein kinase B; t-Akt = total protein kinase B; p-Akt = phosphorylated protein kinase B; ns = no significance; si-LETM1 = siRNA targeting LETM1; si-NC = negative control for the si-LETM1 group; oe-LETM1 = LETM1 overexpression group; oe-NC = negative control for the oe-LETM1 group; GAPDH = glyceraldehyde-3-phosphate dehydrogenase. *P<0.05; †P < 0.01.

- Wu CW, Hsiung CA, Lo SS, Hsieh MC, Chen JH, Li AF, et al. Nodal dissection for patients with gastric cancer: a randomised controlled trial. *Lancet Oncol* 2006;7:309-315. [PUBMED](#) | [CROSSREF](#)
- Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006;12:354-362. [PUBMED](#) | [CROSSREF](#)
- Dassen AE, Lemmens VE, van de Poll-Franse LV, Creemers GJ, Breninkmeijer SJ, Lips DJ, et al. Trends in incidence, treatment and survival of gastric adenocarcinoma between 1990 and 2007: a population-based study in the Netherlands. *Eur J Cancer* 2010;46:1101-1110. [PUBMED](#) | [CROSSREF](#)
- Hsieh HL, Tsai MM. Tumor progression-dependent angiogenesis in gastric cancer and its potential application. *World J Gastrointest Oncol* 2019;11:686-704. [PUBMED](#) | [CROSSREF](#)
- Hart L, Rauch A, Carr AM, Vermeesch JR, O'Driscoll M. LETM1 haploinsufficiency causes mitochondrial defects in cells from humans with Wolf-Hirschhorn syndrome: implications for dissecting the underlying pathomechanisms in this condition. *Dis Model Mech* 2014;7:535-545. [PUBMED](#) | [CROSSREF](#)
- Li Y, Tran Q, Shrestha R, Piao L, Park S, Park J, et al. LETM1 is required for mitochondrial homeostasis and cellular viability (Review). *Mol Med Rep* 2019;19:3367-3375. [PUBMED](#) | [CROSSREF](#)
- Piao L, Li Y, Kim SJ, Byun HS, Huang SM, Hwang SK, et al. Association of LETM1 and MRPL36 contributes to the regulation of mitochondrial ATP production and necrotic cell death. *Cancer Res* 2009;69:3397-3404. [PUBMED](#) | [CROSSREF](#)

13. Piao L, Li Y, Kim SJ, Sohn KC, Yang KJ, Park KA, et al. Regulation of OPA1-mediated mitochondrial fusion by leucine zipper/EF-hand-containing transmembrane protein-1 plays a role in apoptosis. *Cell Signal* 2009;21:767-777.
[PUBMED](#) | [CROSSREF](#)
14. Chen L, Yang Y, Liu S, Piao L, Zhang Y, Lin Z, et al. High expression of leucine zipper-EF-hand containing transmembrane protein 1 predicts poor prognosis in head and neck squamous cell carcinoma. *BioMed Res Int* 2014;2014:850316.
[PUBMED](#) | [CROSSREF](#)
15. Yang Z, Ni W, Cui C, Qi W, Piao L, Xuan Y. Identification of LETM1 as a marker of cancer stem-like cells and predictor of poor prognosis in esophageal squamous cell carcinoma. *Hum Pathol* 2018;81:148-156.
[PUBMED](#) | [CROSSREF](#)
16. Elmenier FM, Lasheen DS, Abouzid KA. Phosphatidylinositol 3 kinase (PI3K) inhibitors as new weapon to combat cancer. *Eur J Med Chem* 2019;183:111718.
[PUBMED](#) | [CROSSREF](#)
17. Ye B, Jiang LL, Xu HT, Zhou DW, Li ZS. Expression of PI3K/AKT pathway in gastric cancer and its blockade suppresses tumor growth and metastasis. *Int J Immunopathol Pharmacol* 2012;25:627-636.
[PUBMED](#) | [CROSSREF](#)
18. Yu HG, Ai YW, Yu LL, Zhou XD, Liu J, Li JH, et al. Phosphoinositide 3-kinase/Akt pathway plays an important role in chemoresistance of gastric cancer cells against etoposide and doxorubicin induced cell death. *Int J Cancer* 2008;122:433-443.
[PUBMED](#) | [CROSSREF](#)
19. Kobayashi I, Semba S, Matsuda Y, Kuroda Y, Yokozaki H. Significance of Akt phosphorylation on tumor growth and vascular endothelial growth factor expression in human gastric carcinoma. *Pathobiology* 2006;73:8-17.
[PUBMED](#) | [CROSSREF](#)
20. Newton AC, Trotman LC. Turning off AKT: PHLPP as a drug target. *Annu Rev Pharmacol Toxicol* 2014;54:537-558.
[PUBMED](#) | [CROSSREF](#)
21. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002;2:489-501.
[PUBMED](#) | [CROSSREF](#)
22. Hwang SK, Piao L, Lim HT, Minai-Tehrani A, Yu KN, Ha YC, et al. Suppression of lung tumorigenesis by leucine zipper/EF hand-containing transmembrane-1. *PLoS One* 2010;5:e12535.
[PUBMED](#) | [CROSSREF](#)
23. Piao L, Feng Y, Yang Z, Qi W, Li H, Han H, et al. LETM1 is a potential cancer stem-like cell marker and predicts poor prognosis in colorectal adenocarcinoma. *Pathol Res Pract* 2019;215:152437.
[PUBMED](#) | [CROSSREF](#)
24. Chen L, Zhang K, Ye C, Chen S, Cao Y, Zhang Y. The expression of LETM1 in gastric cancer and its correlation with prognosis. *J Anhui Med Univ* 2019;54:1430-1434.
25. Uhlen M, Zhang C, Lee S, Sjöstedt E, Fagerberg L, Bidkhori G, et al. A pathology atlas of the human cancer transcriptome. *Science* 2017;357:eaan2507.
[PUBMED](#) | [CROSSREF](#)
26. Szász AM, Lániczky A, Nagy Á, Förster S, Hark K, Green JE, et al. Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients. *Oncotarget* 2016;7:49322-49333.
[PUBMED](#) | [CROSSREF](#)
27. Zhang Y, Zhang R, Luo G, Ai K. Long noncoding RNA SNHG1 promotes cell proliferation through PI3K/AKT signaling pathway in pancreatic ductal adenocarcinoma. *J Cancer* 2018;9:2713-2722.
[PUBMED](#) | [CROSSREF](#)
28. Kim C, Mulder K, Spratlin J. How prognostic and predictive biomarkers are transforming our understanding and management of advanced gastric cancer. *Oncologist* 2014;19:1046-1055.
[PUBMED](#) | [CROSSREF](#)
29. Puneet, Kazmi HR, Kumari S, Tiwari S, Khanna A, Narayan G. Epigenetic mechanisms and events in gastric cancer-emerging novel biomarkers. *Pathol Oncol Res* 2018;24:757-770.
[CROSSREF](#)
30. Xu J, Huang B, Li S, Zhang X, Xie T, Xu Y. Knockdown of LETM1 inhibits proliferation and metastasis of human renal cell carcinoma cells. *Oncol Lett* 2018;16:6377-6382.
[PUBMED](#) | [CROSSREF](#)
31. Li N, Zheng Y, Xuan C, Lin Z, Piao L, Liu S. LETM1 overexpression is correlated with the clinical features and survival outcome of breast cancer. *Int J Clin Exp Pathol* 2015;8:12893-12900.
[PUBMED](#)

32. Huang B, Zhang J, Zhang X, Huang C, Hu G, Li S, et al. Suppression of LETM1 by siRNA inhibits cell proliferation and invasion of bladder cancer cells. *Oncol Rep* 2017;38:2935-2940.
[PUBMED](#) | [CROSSREF](#)
33. Piao L, Yang Z, Feng Y, Zhang C, Cui C, Xuan Y. LETM1 is a potential biomarker of prognosis in lung non-small cell carcinoma. *BMC Cancer* 2019;19:898.
[PUBMED](#) | [CROSSREF](#)
34. Burris HA 3rd. Overcoming acquired resistance to anticancer therapy: focus on the PI3K/AKT/mTOR pathway. *Cancer Chemother Pharmacol* 2013;71:829-842.
[PUBMED](#) | [CROSSREF](#)
35. Polivka J Jr, Janku F. Molecular targets for cancer therapy in the PI3K/AKT/mTOR pathway. *Pharmacol Ther* 2014;142:164-175.
[PUBMED](#) | [CROSSREF](#)
36. Soussi T, Wiman KG. Shaping genetic alterations in human cancer: the p53 mutation paradigm. *Cancer Cell* 2007;12:303-312.
[PUBMED](#) | [CROSSREF](#)
37. Bykov VJ, Eriksson SE, Bianchi J, Wiman KG. Targeting mutant p53 for efficient cancer therapy. *Nat Rev Cancer* 2018;18:89-102.
[PUBMED](#) | [CROSSREF](#)
38. Ogawara Y, Kishishita S, Obata T, Isazawa Y, Suzuki T, Tanaka K, et al. Akt enhances Mdm2-mediated ubiquitination and degradation of p53. *J Biol Chem* 2002;277:21843-21850.
[PUBMED](#) | [CROSSREF](#)