

Original Article

Lipoma HMGIC fusion partner-like 3 (LHFPL3) promotes proliferation, migration and epithelial-mesenchymal transitions in human glioma cells

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Abstract: Malignant gliomas, with the highest incidence among the primary malignant brain tumor, are the most feared types of cancers because of its poor prognosis and direct repercussions on quality of life and cognitive function. However, the pathogenesis for malignant glioma is still unclear. Previous studies reported that the expression level of LHFPL3 (lipoma HMGIC fusion partner-like 3) is elevated in malignant glioma tissues when compared with the normal brain tissues which is consistent with our analysis of LHFPL3 expression level of 20 pairs of human clinical glioma tissues. But the functions of LHFPL3 in malignant remain largely unknown. In this study, si-LHFPL3 was used to inhibit LHFPL3 expression level. CCK8 assays showed that si-LHFPL3 suppressed the proliferation of glioma cells; Flow cytometric analysis revealed si-LHFPL3 induced apoptosis and induced G2/M phase arrest in glioma cells. Our study also proved that si-LHFPL3 inhibited the migration of glioma cells examined by transwell migration assays. Further study showed that si-LHFPL3 increased the expression level of E-cadherin and decreased the expression level of N-cadherin, vimentin and snail, which are the biomarkers of epithelial-mesenchymal transitions (EMT). Therefore, our study indicated LHFPL3 may exert its function on proliferation and migration via regulating EMT-related proteins. In summary, this study explained the function of LHFPL3 in human glioma and deepened our understanding of glioma progression.

Keywords: LHFPL3, gliomas, epithelial-mesenchymal transitions

Introduction

Human malignant gliomas, originating from glia, are among the most common primary brain tumors, with 17,000 new diagnoses per year, which are also the most feared types of cancers because of their dismal prognosis and adverse impact on cognitive function and quality of life [1, 2]. Understanding of the mechanisms of malignant glioma and developing biomarkers have significance for the patients. Previous studies revealed that, like most other cancers, the initiation of malignant gliomas is an outcome of the accumulation of genetic alterations, like ectopic expression of RTK, PI3K, MAPK and P53 signal pathways in glioblastoma [3]. However, their action and the exact member required for glioma pathogenesis is still unclear. Further studies are needed to explore the mechanisms of glioma pathogenesis.

According to Vedrana Milinkovic et al.'s study, ectopic expression of LHFPL3 (lipoma HMGIC fusion partner-like 3) is more common in advanced malignant glioma [4]. LHFPL3, a member of LHFPL-like family, is located at the long arm of chromosome 13 and functions as a translocation partner of HMGIC in lipoma [4, 5]. Besides, Nagaishi et al. reported that amplification of LHFPL3 correlated with mesenchymal differentiation in gliosarcoma [6]. However, the function of LHFPL3 still remains largely unknown.

Malignant tumor cell dissemination and metastatic behavior are among the reasons for the great cancer-related mortality. Epithelial tumor cells acquired the malignant state via epithelial-mesenchymal transition (EMT) [7, 8]. Previous reports showed EMT also has important role in malignant glioma [9, 10]. Various

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biomarkers are used to identify EMT [8, 11]. E-cadherin is associated with epithelial cell adhesion and the switching from E-cadherin to N-cadherin is the hallmark of EMT. Snail can suppress E-cadherin expression and regulate a various aspects of EMT. Besides, the process of EMT involves the acquisition of mesenchymal markers such as vimentin.

In the present study, our results showed si-LHFPL3 suppressed proliferation, induced apoptosis and disturbed the distribution of cell cycle in glioma cells. Moreover, si-LHFPL3 decreased the migration ability of glioma cells. Further study proved si-LHFPL3 increased E-cadherin expression level and decreased N-cadherin, vimentin and snail protein level in glioma cells. The results indicated si-LHFPL3 may exert its function on proliferation and migration via these EMT-related proteins in glioma.

In summary, our research suggested LHFPL3 may induce proliferation and migration by regulating EMT process in human glioma. Our findings provided a better insight into the molecular mechanism of malignant glioma and the potential to develop new diagnostic biomarker.

Materials and methods

Clinical specimens

20 paired of glioma and normal brain tissues obtained from the People's Hospital of Zhengzhou University (Zhengzhou, China). This study was approved by the Ethics Committee of the People's Hospital of Zhengzhou University. All the specimens were collected and examined with the approval of the patients.

Cell culture

The human glioma U251, T98G, A172, U87 cell lines and normal human glial cell line HEB used in this study were from cell bank of Shanghai Institute for Biological Sciences. U251, A172 and U87 cells were cultured in complete high-glucose Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA); T98G cells were cultured in Eagle's Minimum Essential Medium (Gibco, Carlsbad, CA, USA); All the medium was supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) and 1% units/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). These cells were cultured at 37°C in 5% CO₂ in a humidified incubator.

SiRNA and transfection

Si-LHFPL3 and NC were purchased from GenePharma (Shanghai, China) and transfected into U87 cells using Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA, USA). U87 cells were seeded into 6-well plates (2 × 10⁵ cells per well) and incubated overnight in 2 ml of medium. SiRNA were diluted in 250 ul of Opti-MEM (Gibco, Carlsbad, CA, USA) and mixed gently. 250 ul Opti-MEM was mixed gently with 4 ul Lipofectamine 2000 and incubated for 5 min at room temperature. After 5 min, the diluted siRNA was mixed gently with the diluted Lipofectamine 2000 and incubated for 20 min. The 6-well plates was replaced with Opti-MEM (1.5 ml per well). The mixture was added into the 6-well plates and incubated for 4~6 h in incubator. After 4~6 h, the mixed solution was removed and added 2 ml normal medium into the 6-well plates.

RNA extraction, reverse transcription (RT)-polymerase chain reaction (PCR) and real-time PCR

Total RNA was isolated using Trizol reagent (Takara, Otsu, Shiga, Japan). Reverse transcription (RT)-polymerase chain reaction (PCR) was performed by DBI PrimeScript RT Reagent Kit. Quantitative real-time RT-PCR (qRT-PCR) analysis was conducted using DBI SYBR Premix Ex. The primers used in this study were as follow: LHFPL3 forward: 5'-ACCAACTATGTGCGGAACCTCG-3' and reverse: 5'-TCCACGCCGTCGCCTAT-3'; β-actin forward: 5'-ACCACACCACACATTTCTCATAC-3' and reverse: 5'-ACTGTCTCCTTCATCGTTCCA-3'. The conditions for all the real-time PCR reactions were as follows: 94°C for 2 min, followed by 40 cycles of amplification (94°C for 20 sec, 58°C for 20 sec, 72°C for 20 sec). β-actin was used as an internal loading control.

Western blot

Total protein samples were prepared by RIPA buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 and 50 mM Tris-HCl, pH 7.6) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and PMSF (Beyotime Biotechnology, Shanghai, China) and quantified by BCA protein assay kit (Thermo Scientific). After separation on 10% polyacrylamide gels and transfer to PVDF mem-

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brane (Millipore, Billerica, MA, USA) by a 300 mA current, the membranes were blocked overnight in 4°C and then incubated with the following antibodies anti-LHFPL3 (1:5000, Ya Ji Biological Technology, Shanghai, China), anti-E-cadherin (1:50, Abcam, Cambridge, UK), anti-N-cadherin (1 µg/ml, Abcam, Cambridge, UK), anti-vimentin (1:1000, Abcam, Cambridge, UK), anti-snail (1 µg/ml, Abcam, Cambridge, UK) and anti-GAPDH (1:500, Abcam, Cambridge, UK) for 1 h in room temperature. After washing by TBST buffer (Beyotime Biotechnology, Shanghai, China) and incubation with goat anti-rabbit horseradish peroxidase-conjugated (1:20000, BOSTER) for 40 min at room temperature, chemiluminescence was used to visualize the immune-complexes. GAPDH was used as an internal loading control.

CCK8 assay

The viability of the U87 cells was examined by CCK8 assay. U87 cells were seeded into 96-well plates (1×10^4 cells per well) and incubated overnight in 100 µl of medium. Then, the cells were transfected with si-LHFPL3 or NC by lipotamin 2000 reagent according to the manufacturer's instruction. After culture for 24 h, 48 h, 72 h, respectively, the medium was wiped off and 100 µl of CCK8 solution (10 µl of CCK8 liquid dissolved in 100 µl medium) was added to each well and incubated 4 h at the incubator. The absorbance at 450 nm was tested using a BioTek SYNFRGY4 microplate reader (Thermo Fisher Scientific, multiscan MK3).

Flow cytometric analysis (cell cycle and apoptosis)

U87 cells were seeded into 6-well plates (5×10^5 cells per well) and incubated overnight in 1 ml medium. Then, the cells were transfected with si-LHFPL3 or NC by lipotamin 2000 reagent according to the manufacturer's instruction. After transfection for 48 h, the cells were harvested and washed with PBS. Cell cycle and apoptosis were performed, respectively. For cell cycle analysis, the cells were fixed with 70% ethanol. The fixed cells were treated with 300 µl propidium iodide (Multi Sciences, Hangzhou, China) and the stained cells were immediately examined by FACS cytometer (BD Biosciences, Franklin Lakes, NJ, USA). For apoptosis analysis, the cells were resuspended into 200 µl

apoptosis staining solution containing 200 µl binding buffer, 2 µl propidium iodide and 1 µl Annexin V-FITC (Multi Sciences). After stained for 15 min in darkness at room temperature, the stained cells were detected by FACS cytometer.

Transwell migration assay

U87 cells transfected with NC or si-LHFPL3 were cultured in medium without FBS for 24 h. Then 2×10^4 cells in 100 µl DMEM with 0.2% BSA were seeded into each transwell cell culture inserts (Costar Corporation, USA) and placed 700 µl of DMEM medium with 10% FBS in the lower compartment. After incubation for 48 h, The cells in the upper surface of the membrane were removed, and the migrated cells were fixed with 4% paraformaldehyde for 20 min and stained with Crystal violet solution (Sigma-Aldrich, USA) for 5~10 min. The inserts with the migrated cells were coverslipped with mounting and photographed by microscope (Olympus).

Immunohistochemistry

The expression level of LHFPL3 in human clinical brain tissues was examined by a standard immunohistochemistry protocol for paraffin-embedded tissue sections. The sections were deparaffinized in xylene and rehydrated in series of graded alcohols. After incubation in 3% H₂O₂ for 15 min at room temperature, the sections were blocked with a blocking solution containing 10% normal rabbit serum in PBS for 1 h and then incubated with anti-LHFPL3 (1:50) for 1 h. Subsequent to washing with PBS and incubation with biotinylated secondary antibody followed by streptavidin (Vectastain Elite ABC kit, Vector laboratories, Burlingame, Ca). The slides were incubated with diaminobenzidine reagent (Ector laboratories, Burlingame, Ca). The slides were photographed by microscope. Brown-color represents the expression level of LHFPL3.

Statistical analysis

Student's test was used to the analysis of data in this study. Values are expressed as the mean ± SD. P < 0.05 was considered statistically significant. All the experiments in this study were totally repeated for three or more trials.

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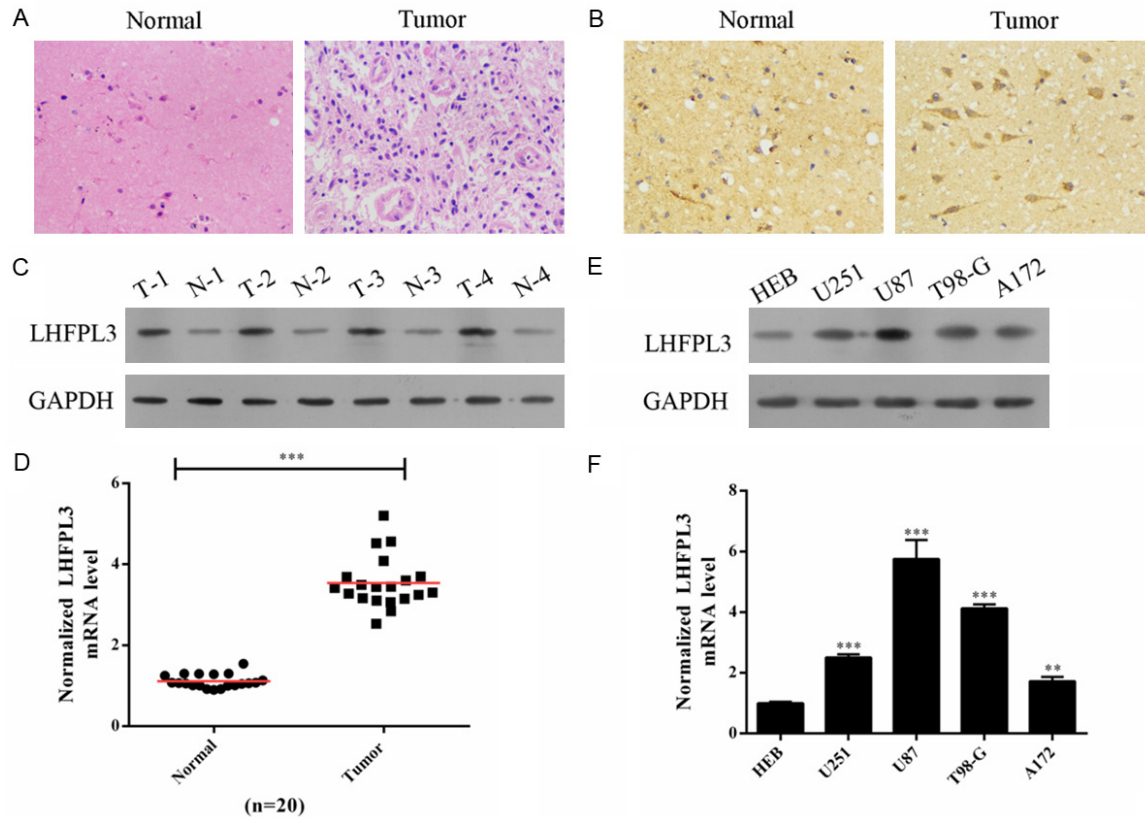


Figure 1. Expression of LHFPL3 in human glioma tissues and cell lines. (A) The human glioma tissues were stained by HE staining; (B-D) The expression level of LHFPL3 in human glioma tissues was examined by immunohistochemistry (B), western blot (C) and real-time PCR (D), LHFPL3 expression level in glioma tissues increased compared with the matched normal tissues, for western blot, GAPDH was used as internal control, for real-time PCR, β -actin was used as internal control, $n=20$, $***P < 0.001$; (E, F) Western blot (E) and real-time PCR (F) analysis of LHFPL3 expression level in the normal human brain glial cell line (HEB) and glioma cell lines (U251, U87, T98-G, A172), HEB was used as control, $**P < 0.01$ and $***P < 0.001$.

Results

Expression of LHFPL3 in human glioma tissues and cell lines

To investigate the function of LHFPL3 in glioma, firstly, we evaluated the expression levels of LHFPL3 in human glioma tissues and cell lines.

We collected 20 pairs of clinical human glioma tissues and the adjacent normal brain tissues. The observation of the HE staining showed, compared with the adjacent normal brain tissues, glioma nucleus increased greatly and there was accumulation tendency, the atypia and split image elevated in the clinical human glioma tissues (**Figure 1A**). The results of immunohistochemistry and western blot showed that LHFPL3 increased at protein level in human clinical glioma tissues compared with the matched normal tissues adjacent tissues

(**Figure 1B** and **1C**), and consistently, the data of real-time PCR also proved LHFPL3 expression in human glioma tissues was also elevated at mRNA level (**Figure 1D**). Additionally, we examined LHFPL3 expression level in one normal human glial cell line (HEB) and four glioma cell lines (U251, U87, T98-G and A172) by western blot and real-time PCR, HEB was used as a control cell line. We found that the glioma cell lines displayed higher endogenous LHFPL3 expression level than the normal human glial line (**Figure 1E** and **1F**). Overall, these results proved that LHFPL3 expression level increased in glioma and indicated that LHFPL3 may play an important role in glioma.

si-LHFPL3 suppressed the proliferation of glioma cells

In order to explore the function of LHFPL3 in glioma, chemically synthesized si-LHFPL3 was

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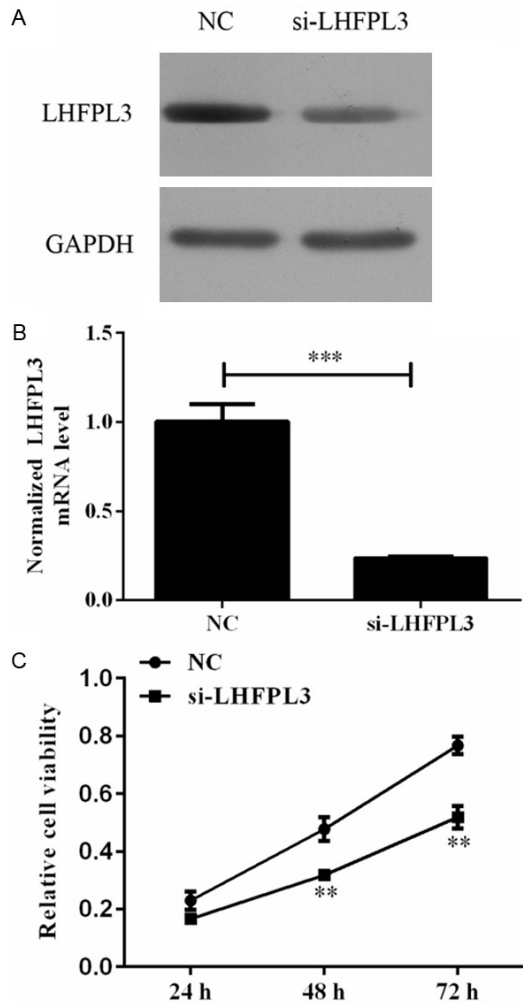


Figure 2. si-LHFPL3 suppressed the proliferation of glioma cells. (A, B) Validation of the action of si-LHFPL3 on LHFPL3 expression level by western blot (A) and real-time PCR (B) subsequent to transfection; (C) CCK8 assays revealed that si-LHFPL3 suppressed proliferation of glioma cells; ** $P < 0.01$ and *** $P < 0.001$ (vs NC).

transfected into U87 cells. LHFPL3 expression level was tested via western blot and real-time PCR, respectively. The results showed that si-LHFPL3 significantly inhibited LHFPL3 expression at protein and mRNA level (Figure 2A and 2B). These results also suggested that the si-LHFPL3 can be used to investigate the function of LHFPL3 in glioma cells. The function of si-LHFPL3 on proliferation was identified by CCK8 assays. The data showed si-LHFPL3 significantly suppressed proliferation of U87 cells compared with the control (group of NC as control), (Figure 2C).

si-LHFPL3 induced apoptosis, disturbed distribution of cell cycle and suppressed migration of glioma cells

To further investigate the function of LHFPL3, flow cytometer was used to detect the apoptosis and cell cycle of U87 cells transfected with si-LHFPL3 (or NC). Flow cytometric analysis showed U87 cells transfected with si-LHFPL3 had a significantly higher rate of apoptosis compared with the cells transfected with NC (Figure 3A) and si-LHFPL3 induced G2/M phase arrest (Figure 3B). The effect of si-LHFPL3 on migration was examined by transwell migration assay and the data showed LHFPL3 siRNA markedly inhibited the migration of glioma cells (Figure 3C).

si-LHFPL3 regulated EMT-related proteins in glioma cells

Previous reports showed EMT have important function in malignant glioma and LHFPL3 correlates with mesenchymal differentiation. Therefore, continuously, we investigated the function of si-LHFPL3 on EMT-related proteins. According to previous reports, E-cadherin, N-cadherin, vimentin and snail are the biomarkers of EMT. Hence, we tested the effect of si-LHFPL3 on the expression level of E-cadherin, N-cadherin, vimentin and snail by western blot and the observation revealed the expression level of E-cadherin increased whereas N-cadherin, vimentin and snail expression decreased in U87 cells transfected with si-LHFPL3 (Figure 4). The result suggested LHFPL3 have an effect on EMT.

Discussion

Malignant gliomas, tumors originating from glia, are the most common and feared types of cancers, with the annual incidence of 5.26 per 100,000 population [12-15]. According to the degree of undifferentiation, aggressiveness and anaplasia, the World Health Organization (WHO) classification system classifies gliomas into 4 grades [16, 17] and malignant gliomas includes WHO grade III and grade IV tumors. Despite of the advance in surgery, radiotherapy and chemotherapy, the prognosis of malignant glioma is still very poor [18-21]. So the enhancement of biology understanding of these lethal cancers is necessary and urgent. Previous

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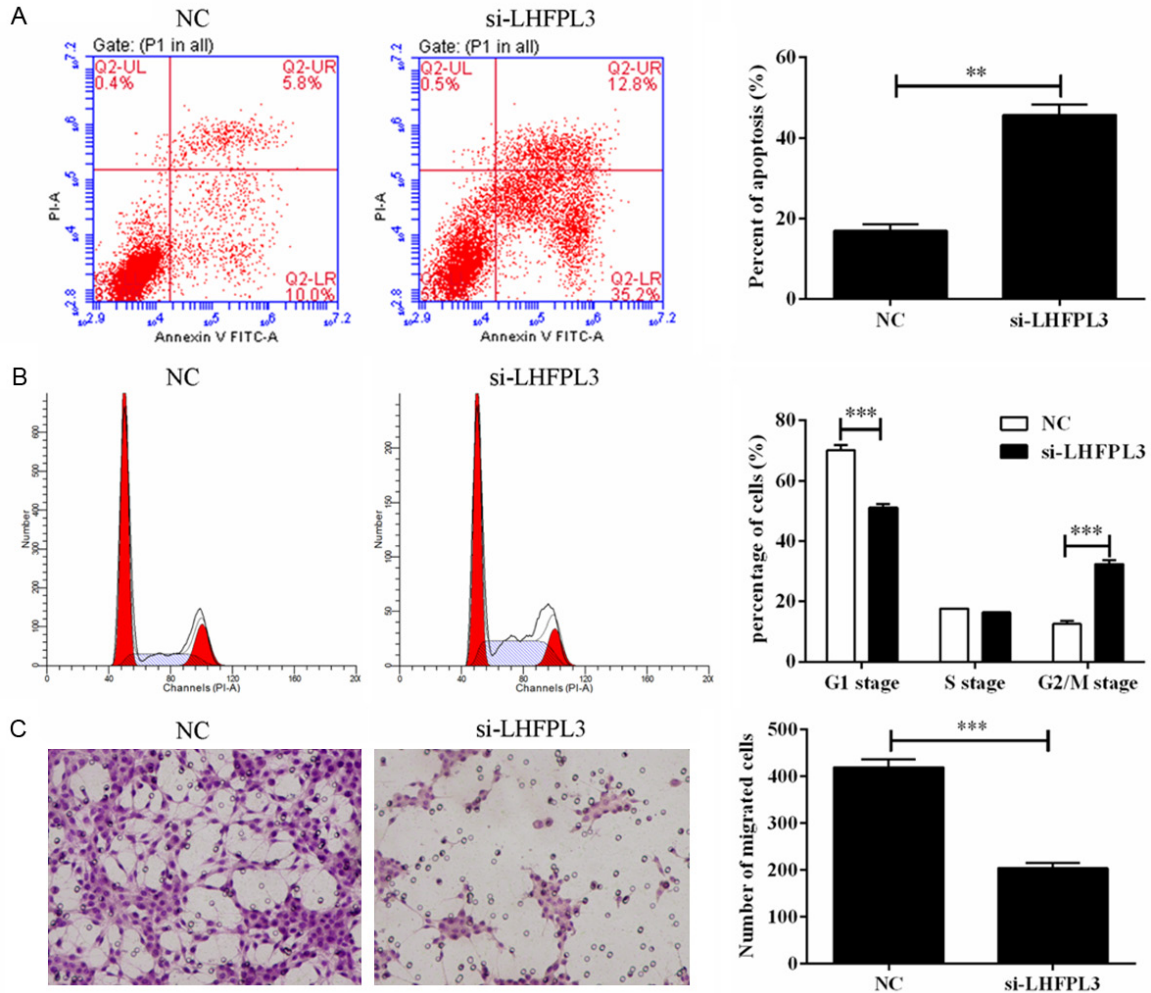


Figure 3. si-LHFPL3 induced apoptosis, disturbed distribution of cell cycle and suppressed migration of glioma cells. A. Representative micrographs (left) and quantification of apoptosis (right) in glioma cells, si-LHFPL3 increased apoptosis rate of glioma cell; B. Representative micrographs (left) and quantification of cell cycle distribution (right) in glioma cells, si-LHFPL3 induces G2/M arrest; C. Representative micrographs (left) and quantification (right) of migrated glioma cells, transwell migration assays showed that si-LHFPL3 decreased migrated cells compared with the control group (NC); * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (vs NC).

studies suggested that, multistep processes, like sequential and cumulative genetic alterations, are involved in the pathogenesis of malignant glioma and malignant glioma may be the consequence of these genetic alterations. According to genome-association researches, susceptibility variants of 11q23.3 (PHLDB1), 8q24.21 (CCDC26), 7p11.2 (EGFR), 9p21.3 (CDKN2BAS), 5p15.33 (TERT) and 20q13.33 (RTEL) have been proved involved in malignant gliomas [22-24]. However, the exact number and the action remain unclear. Further studies are needed.

In the study of Vedrana Milinkovic et al., they analyzed 30 human glioma samples by AP-PCR

fingerprinting and found the specific changes in LHFPL3 which might be the characteristic of primary glioblastoma [4]. Alterations of LHFPL3 (lipoma HMGIC fusion partner-like 3) are more common in samples with high level of genomic instability, as well as in grade IV gliomas [25]. In the present study, we detected 20 pairs of human clinical glioma tissues and the results showed that higher expression level of LHFPL3 in glioma tissues compared with in the matched normal adjacent tissues (Figure 1A-D). Besides, we also analyzed the expression level of LHFPL3 in one normal human glia cell line (HEB) and four human glioma cell lines (U251, U87, T98-G and A172) and the data showed that glioma cell lines displayed higher endoge-

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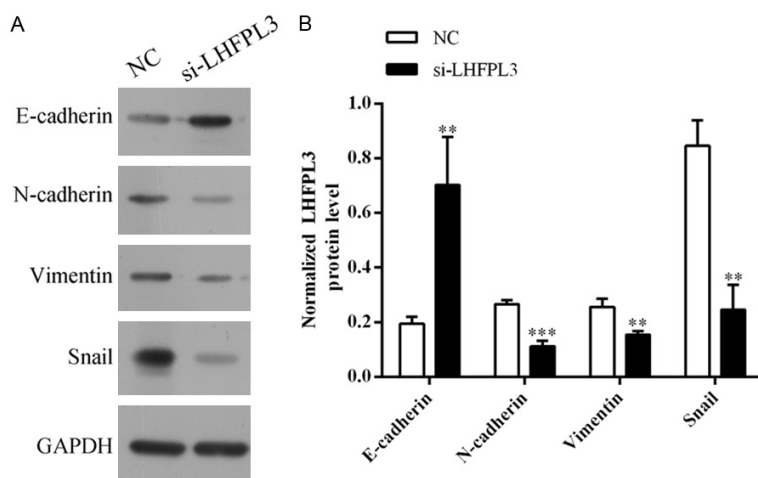


Figure 4. si-LHFPL3 regulated EMT-related proteins in glioma cells. Western blot analysis showed Lsi-LHFPL3 increased the expression level of E-cadherin and decreased the protein level of N-cadherin, vimentin and snail compared with the control (NC), representative photographs (A) and quantification (B), $**P < 0.01$ and $***P < 0.001$ (vs NC).

nous LHFPL3 expression level than the normal human glial line (Figure 1E and 1F). These results indicated that LHFPL3 may exert important action in malignant glioma.

Some previous work revealed that, the amplification of LHFPL3 associated with mesenchymal differentiation in gliosarcoma [6] and multiple nucleotide substitutions in LHFPL3 can significantly reduce the overall survival of the glioma patients [4]. Here, we used si-LHFPL3 as the tool to investigate the function on malignant glioma. Our results showed that si-LHFPL3 suppressed proliferation (Figure 2C), induced apoptosis and increased distribution of cell cycle in G2/M phase in U87 cells (Figure 3A and 3B). Moreover, transwell migration assays showed si-LHFPL3 inhibited migration in U87 cells (Figure 3C). Hence, these results suggested that LHFPL3 promoted proliferation and migration in U87 cells.

Epithelial-to-mesenchymal transition (EMT) is a process of multiple steps which reflects the change from epithelial phenotype to mesenchymal phenotype. Commonly, EMT correlates with tumor dissemination and metastasis. According to previous studies, EMT involves in glioma progression. Glioma patients with mesenchymal character frequently have shorter overall survival and diseases free survival, due to their higher aggressive dissemination and

motility. E-cadherin, N-cadherin, vimentin and snail are all biomarkers of EMT. LHFPL3 may correlate with mesenchymal differentiation in gliosarcoma. Therefore, we detected the effect of si-LHFPL3 on the EMT biomarkers. Our data showed LHFPL3 siRNA elevated E-cadherin expression level and reduced N-cadherin, vimentin and snail protein level (Figure 4). The results suggested LHFPL3 may play important role in EMT of glioma.

In summary, we proved LHFPL3 have the function of promoting the proliferation and migration via regulating EMT processes in malignant glioma.

Our findings advance the understanding of biology mechanism for glioma progression and provide one potential biomarker candidate for glioma diagnosis.

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Disclosure of conflict of interest

None.

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