

Long Non-Coding RNA LINC00663 Promotes Gliomagenesis and Progression

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Abstract

Background: Glioma is the most frequent primary malignant brain tumor, characterized by high morbidity, high mortality and dismal prognosis. Numerous analyses have revealed the abnormal expression of long non-coding RNAs (lncRNAs) in glioma cells. This study aims to explore its role in glioma development and prognosis.

Methods: The gene expression in cell lines were measured by qRT-PCR. The role of LINC00663 in glioma was confirmed by CCK8, EdU assay, transwell and western blot as well as by in vivo experiments. Besides, Pearson's correlation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were also performed as needed.

Results: Firstly, data from our preliminary work (NSFC NO. 81572474) showed that LINC00663 might be largely implicated in glioma. Meanwhile, LINC00663 upregulation confirmed in glioma predicted poor clinical outcomes. Functionally, LINC00663 knockdown restrained cell proliferation, migration and invasion in vitro. Mechanistic investigations validated that LINC00663 silencing decreased AKT activation in glioma cells.

Conclusions: LINC00663 promotes glioma development and progression through regulating AKT pathway, suggesting LINC00663 as a probable target for glioma treatment.

Introduction

Glioma, characterized by high morbidity and high mortality, arises from glial or precursor cells and represents 75% of malignant intracranial tumors in adults [1, 2]. Currently, even with maximal safe resection followed by adjuvant radiotherapy and/or chemotherapy, glioma remains rarely curable due to the rapid proliferation, extensive invasion, gene expression changes, signaling pathway disorders and drug resistance [3, 4]. The dismal prognosis of glioma patients also originates from poor understanding of molecular pathogenesis, sensitive therapeutic monitoring tools and deficiency of novel effective therapies [5]. Therefore, it is crucial to elucidate the underlying molecular mechanisms involved in glioma development and progression, which requires us to explore a new lncRNA that may contribute to the diagnosis and prognosis of patients.

lncRNAs are a novel kind of regulatory RNA with no or limited protein-coding potential, which accounts for 98% of the human transcriptome [6, 7]. As lncRNAs interact with DNA, proteins, and other RNAs to exert their functions in many biological processes, their aberrant expressions are implicated in various cancers [8, 9]. To date, many studies have demonstrated that lncRNAs play important roles in the biological development and progression of cancer, such as cell proliferation, migration, EMT formation, angiogenesis and modulation of signaling pathways [10–13]. Taken together, lncRNAs may become promising candidates for glioma diagnosis, prognosis and therapeutic strategies. Nevertheless, there are still many unillustrated lncRNAs whose functions and underlying mechanisms remain elusive.

Long intergenic non-coding RNA 663 (LINC00663, also known as LOC284440) is a long non-coding RNA and localized in chromosome band 19p13.11. Previous findings showed that LINC00663 was differentially expressed in various cancer cell lines and healthy human tissues, suggesting a possible role of LINC00663 cancer pathogenesis [14, 15]. However, the role of LINC00663 in glioma has not been investigated before. Herein, we explored the biological function of LINC00663 in glioma development and progression, and later investigated the potential mechanism, which might inspire us to find an effective treatment target for glioma.

Materials And Methods

Human cells culture

Human glioma cell lines U87 MG, U251 and A172 as well as normal human umbilical vein endothelial cells (HUVECs) were obtained from the Cell Resource Center of Peking Union Medical College (Beijing, China). Cells were incubated in DMEM medium supplemented with 10% FBS (GIBCO, Carlsbad, USA) at 37 °C in 5% CO₂.

Microarray dataset

The public diffuse infiltrating gliomas microarray gene profiling dataset GSE4412 was downloaded from the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>). RNA sequencing data of cancer tissue samples were obtained from Chinese Glioma Genome Atlas (CGGA, <http://www.cgga.org.cn/>) [16, 17]. The cut-off value between high and low LINC00663 expression was set as the expression level of a median sample.

RNA isolation and qRT-PCR

Total RNA was obtained from tissues and cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Thereafter, PrimeScript™ RT reagent kit (Takara Biotechnology, Dalian, China) was used to synthesize cDNA and remove genomic DNA, followed by carrying out SYBR Green quantitative PCR (qPCR). The relative expression was normalized to GAPDH and fold expression changes were calculated with $2^{-\Delta\Delta Ct}$ method. Primers used in this study are listed as follows: LINC00663, 5'-GCTTG TAGCCCTTTCTTTTGG-3' and 5'-AGTCCCTTCTGCCTATGACCCT-3'; GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-TGGTGAAGACGCCAGTGGA-3'.

Cell transfection

U87 and U251 cells were separately transfected with specific shRNAs against LINC00663 (sh-LINC00663#1#2) and the negative control (NC), as well as pcDNA3.1/LINC00663 and the empty pcDNA3.1 vector (all from GenePharma, Shanghai, China). Cell transfection with plasmid was performed using jetPRIME transfection reagent (Polypus-transfection, France) when they reached approximately 60–80% confluence.

Cell counting kit 8 (CCK8) assay

Cells were seeded in 96-well plates (8,000 cells/well), transfected with shRNA, then 10 μ L of CCK-8 solution (Dojindo, Japan) was added and incubated at 37°C for another 2 h. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad,).

EdU assay

Cell proliferation was analyzed by EdU assay Kit (Ribobio, Guangzhou, China). Transfected cells were incubated with EdU (50 μ M) at 37°C for 2 h. Subsequently, cells were fixed by 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and dyed by Apollo Dye Solution. Nucleic acid was stained using Hoechst 33342. The proportion of cells that incorporated EdU was determined by a fluorescent microscope (Carl Zeiss, Jena, Germany).

Transwell assay

The chemotactic motility of cells was estimated using 24-well transwell chambers (Corning Incorporated, Corning, NY, USA). Serum-starved cells were seeded in the upper chamber (without Matrigel for cell migration assay) or on the top side of the membrane pre-coated with Matrigel (BD, Franklin Lakes, USA) (for cell invasion ability assay). DMEM with 20% FBS was added in the lower chamber as a chemoattractant. After 24 h incubation, cells were fixed in 4% paraformaldehyde and dyed using crystal violet. Finally, invaded or migrated cells were counted under a microscope (Carl Zeiss, Jena, Germany).

Western blotting

Total proteins were extracted with RIPA lysis buffer, separated by SDS-PAGE, and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Membranes were sealed with 5% bovine serum albumin for 2 h and incubated overnight with primary antibodies for p-Akt, Akt, p-mTOR, mTOR, and GAPDH from Cell Signaling Technology (Denver, CO, USA). Secondary antibodies were added for cultivation for 1 hour. The amount of protein was detected by the chemiluminescence detection system.

AKT inhibition assay

The AKT inhibitor MK-2206 (an allosteric inhibitor of p-AKT) was obtained from Selleck Chemicals (Houston, Texas, USA). For experiments, cells were seeded at a density of 5×10^5 in 6 cm dishes. The next day, MK-2206 (diluted from a stock solution formulated in DMSO) was added to cultures at a concentration of 0.1 μ M. After 24 h exposure, the cells were collected and lysed for western blotting assays.

Statistical analysis

Statistical analyses were performed using SPSS 26.0 software (SPSS Inc., Chicago, IL, USA). Survival analysis was performed using the Kaplan–Meier method and gene correlation analysis were conducted by Pearson's correlation analysis method. Data were presented as the mean \pm S.E.M. from at least three independent experiments and compared using the Student's t-test or one-way ANOVA. P-value of 0.05 or less was regarded as significance.

Results

LINC00663 expression was upregulated in glioma and positively correlated with poor prognosis

In this study, data from our preliminary work (NSFC NO. 81572474), LINC00663 was screened out and preferentially expressed in glioma tissues compared with normal brain tissues (Fig. 1A). Besides, its expression was associated with the grade malignancy of glioma (Fig. 1B). However, there is no difference in the transcriptional level of LINC00663 between WHO I and WHO II or WHO III and WHO IV grades (data were not shown). Expression profiles acquired from the GEO database also confirmed our conclusion (Fig. 1C). Given the above, we hypothesized that LINC00663 might implicate in glioma.

Also, we detected the endogenous expression of LINC00663 in various glioma cell lines (U87MG, U251, A172) and HUVECs. As expected, LINC00663 expression was significantly upregulated in glioma cells when compared with HUVECs (Figure1D). Moreover, survival analysis demonstrated that glioblastoma patients with higher LINC00663 levels had shorter overall survival times than those with lower levels, according to the GEO dataset GSE4412 (Fig. 1E). All these findings indicated that LINC00663 was upregulated and resulted in a poor prognosis of glioma patients.

Knockdown of LINC00663 expression impeded proliferation, migration and invasion of glioma cells in vitro

To explore the functional relevance of LINC00663 in glioma, we interfered with endogenous LINC00663 expression in U87 and U251 cells by transfection with shRNAs (sh-LINC00663#1 and sh-LINC00663#2) (Fig. 2A). The most efficient shRNA was selected for further experiments. A vector construct containing the full-length LINC00663 was developed and assessed for LINC00663 expression (Fig. 2B). The effect of overexpressing LINC00663 was not investigated, because the survival of glioma cells with transient LINC00663 overexpression was strongly affected (data not shown).

CCK8 assay revealed that knockdown of LINC00663 greatly attenuated cell viability of these two glioma cells compared to the non-targeting control (Fig. 2C and D). Likewise, EdU staining assay manifested that down-regulation of LINC00663 suppressed DNA synthesis and cell proliferation potential, as the percent of EdU-positive cells were dramatically reduced in U87 and U251 cells transfected with shLINC00663 (Fig. 2E and F). These data indicated that LINC00663 might be involved in the regulation of cell proliferation.

Tumor metastasis is the leading cause of cancer malignancy and recurrence. To evaluate whether depletion of LINC00663 could influence cell migration and invasion, transwell assays were performed. Results presented that depletion of LINC00663 resulted in a suppression of motility and invasion capacity in U87 and U251 cells (Fig. 2G and H). In summary, LINC00663 aggravated proliferation, migration and invasion of glioma cells.

LINC00663-positive-associated genes are mainly enriched in the AKT-mTOR pathway

To investigate the potential mechanisms of LINC00663 in glioma, Pearson correlation analysis was performed in 422 samples with primary glioma RNAseq database from CGGA, and a cluster ($|r| > 0.5$, $p < 0.01$) of LINC00663-associated genes was obtained (Fig. 3A). Next, to investigate which pathways were significantly dysregulated in glioma groups with LINC00663 expression, Fisher's exact test was used to identify 51 pathways that included AKT-mTOR signaling (Fig. 3B). Furthermore, the expression level of AKT and mTOR had a significant positive correlation with LINC00663 in the CGGA data set (Fig. 3C). These analyses indicated that LINC00663 may be associated with AKT-mTOR signaling.

Downregulation of LINC00663 decreased AKT activation in vitro

Studies have found that AKT signal transduction cascades are overly activated in the vast majority of glioma that a pathway crucial for driving tumorigenesis and reducing survival times in patients [18]. Correlation analysis implied that the expression of LINC00663 was associated with AKT and mTOR. Herein, to investigate whether LINC00663 affected proliferation, migration, and invasion of glioma cells by regulating AKT signaling.

To further consolidate our results, the protein level of AKT, phosphorylated AKT, mTOR and phosphorylated mTOR were determined by western blotting in LINC00663 conditioned cell models. AKT phosphorylation and activation were greatly inhibited in U87 cells and slightly in U251 cells transfected with shLINC00663, while total AKT and its downstream p-mTOR and mTOR were unaffected in both cells (Fig. 4A), which was consistent with the result in groups under MK2206 (AKT inhibitor) treatment (Fig. 4B). Interestingly, the decreased gene expression of mTOR as found by RNA sequencing data of cancer tissue samples was not confirmed by Western blot. Instead, overexpression of LINC00663 mildly upregulated the expression of p-AKT in U251 cells (Fig. 4C). As a result, we supposed that LINC00663 might contribute to gliomagenesis via activating AKT pathway, which deserves a closer look in the future.

Discussion

Glioma still harbors a high frequency of recurrence and a bleak outcome. Redundant activation of AKT is the inherent characteristics of glioma, which is frequently associated with tumorigenesis and outcomes [19]. Furthermore, emerging evidence has indicated that some aberrant expression of lncRNAs contributes to tumor development, pathogenesis, and survival in glioma [20]. Sequencing of the human genome shows that 70% of the human genome is transcribed into RNA yielding many thousands of ncRNAs, but the overall study of lncRNAs in brain tumors remains lag behind [21]. For example, the roles of few lncRNAs (such as HOTAIR, MALAT1 and CRNDE) have been well studied in gliomas [22–24].

The present study systematically explored LINC00663 expression in clinical glioma specimens and the effects of LINC00663 silencing on cultured glioma cells. qRT-PCR results showed that was upregulated in glioma tissues and cells, and closely related to tumor degree. Further evidence for the adverse impact of LINC00663 expression on glioma progression was found upon analysis of GEO dataset GSE4412, which showed a significant, inverse correlation between LINC00663 levels and survival rates. The results described above strongly prove the oncogenic role of LINC00663. Furthermore, after knocking down LINC00663 expression significantly blocked the proliferation, migration and invasion capacity of glioma cells. Nevertheless, the regulatory mechanisms of LINC00663 are still unknown.

Next, we sought to identify the candidate molecular mechanism by which LINC00663 regulated downstream effectors in glioma. Here, we analyzed the coexpression genes network in CGGA dataset and discovered that AKT-mTOR pathway was affected by LINC00663. It is well known that PTEN is one of the most frequently altered genes in gliomas and loss of its function increases levels of PI(3,4,5)P3 and leads to activation of AKT pathway [25]. Hyper-activation of the AKT pathway is frequently found in glioma and plays a pivotal role in the regulation of tumor cell survival, growth, motility, angiogenesis and metabolism [26]. Multitudes of studies have demonstrated the importance of PI3K in the activation of AKT, while evidence suggests that AKT activation can proceed by the PI3K independent manner [27]. Several lncRNAs (LINK-A, lncRNA OIP5-AS1, MALAT1 and LINC00470) promote the over activation of AKT signaling [28–31]. In this study, Western blot analysis results confirmed AKT activity was apparently suppressed in U87 cells but slightly in U251 cells with LINC00663 knockdown, without affecting mTOR in the AKT pathway, which was worthy of attention in the future. We demonstrated the inhibitory effects of LINC00663 knockdown on the AKT signaling pathway, which is frequently activated in glioma.

A few limitations should be addressed here. Firstly, we did not check the effects of overexpressing LINC00663 on the proliferation, migration and invasion, because the survival of glioma cells with transient LINC00663 overexpression was strongly affected (data not shown). Secondly, an inconsistent conclusion was obtained in different two glioma cell lines, presenting a relationship to p53 gene mutation, further investigations are needed. In addition, it is necessary to perform brain xenograft experiments to confirm the role of LINC00663 on glioma growth in vivo.

In summary, the attenuation of LINC0066 significantly suppressed the proliferation, migration, and invasion of glioma cells, and these effects correlated with reduced AKT activity. LINC00663 can serve as both a growth-promoting lncRNA and a potential therapeutic target to overcome gliomas.

Abbreviations

lncRNA

Long non-coding RNAs; LINC00663:Long intergenic non-coding RNA 663; KEGG:Kyoto Encyclopedia of Genes and Genomes; HUVECs:Normal human umbilical vein endothelial cells; GEO:Gene Expression Omnibus; CGGA:Chinese Glioma Genome Atlas; NC:Negative control; CCK8:Cell counting kit 8

Declarations

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Authors' contributions

YJW designed, supervised the experimental work and critically revised the manuscript. MCP performed the experiments, performed all statistical analyses, and drafted the manuscript. JRS contributed to design, data acquisition, interpretation and critically revised the manuscript. SQY, HM and CNH conducted the experiments. All authors gave their final approval and agreed to be accountable for all aspects of the work.

Competing Interests

The authors declare that no conflicts of interest are disclosed in this study.

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Availability of data and materials

The processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that no conflicts of interest are disclosed in this study.

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Figures

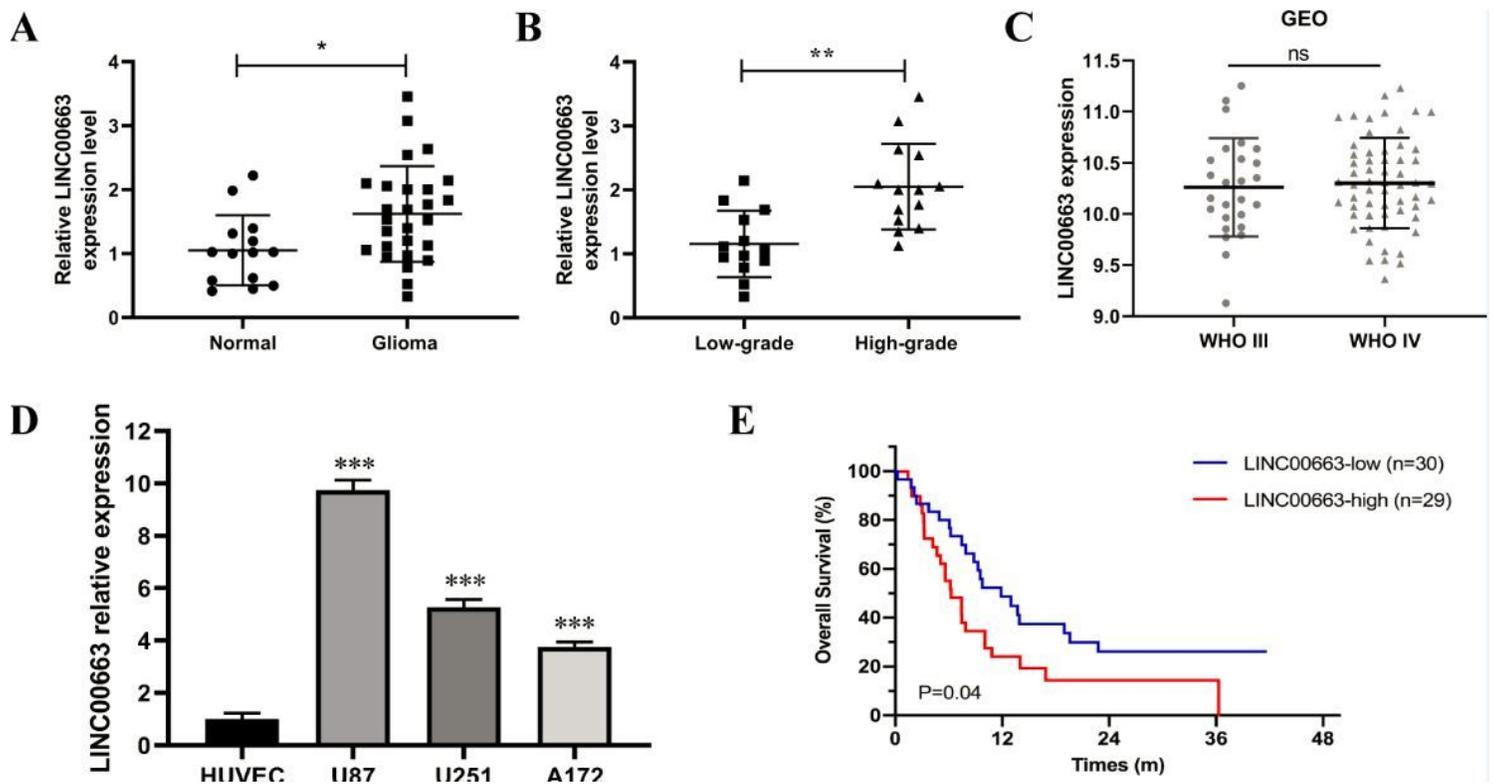


Figure 1

LINC00663 expression levels in glioma and correlated with poor outcomes in glioma patients. (A) LINC00663 expression levels in glioma tissues (n=27) and normal brain tissues (n=14). (B) LINC00663 expression in different grades of glioma tissues (Grade I and II, n =14; Grade III and IV, n = 13). (C) LINC00663 expression in tumor samples classified by tumor grade according to the GEO dataset GSE4412. (D) LINC00663 expression in human GBM cell lines and HUVECs. (E) Kaplan-Meier survival curves of patients with high and low LINC00663 expression in GBMs according to the CGGA database. Data represented as the mean \pm S.E.M from three independent experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

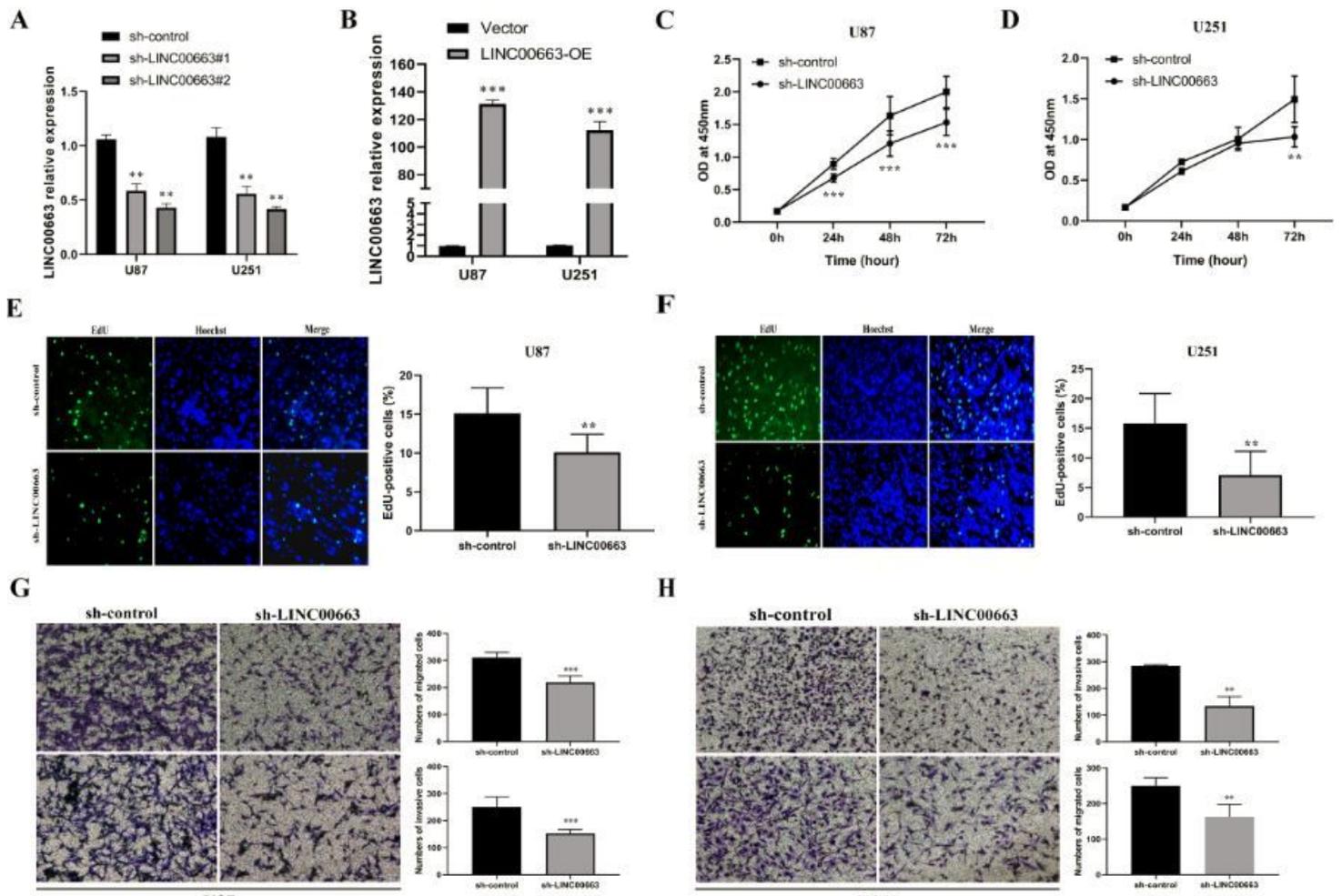


Figure 2

Effects of LINC00663 on proliferation, migration and invasion. (A, B) The transfection efficiency was indicated by qRT-PCR analysis. (C, D) The cell viability in U87 and U251 cells was detected by CCK8 assay. (E, F) EdU assay was used to evaluate the cell proliferation. Representative images for EdU-positive cells (green) and Hoechst-stained nuclei (blue) are shown in E and F. (G, H) Transwell assays were performed to evaluate the migration and invasion of U87 and U251 cells transfected with sh-LINC00663 or sh-control. Data are presented as mean \pm SD of three independent experiments; ** $p < 0.01$, *** $p < 0.001$.

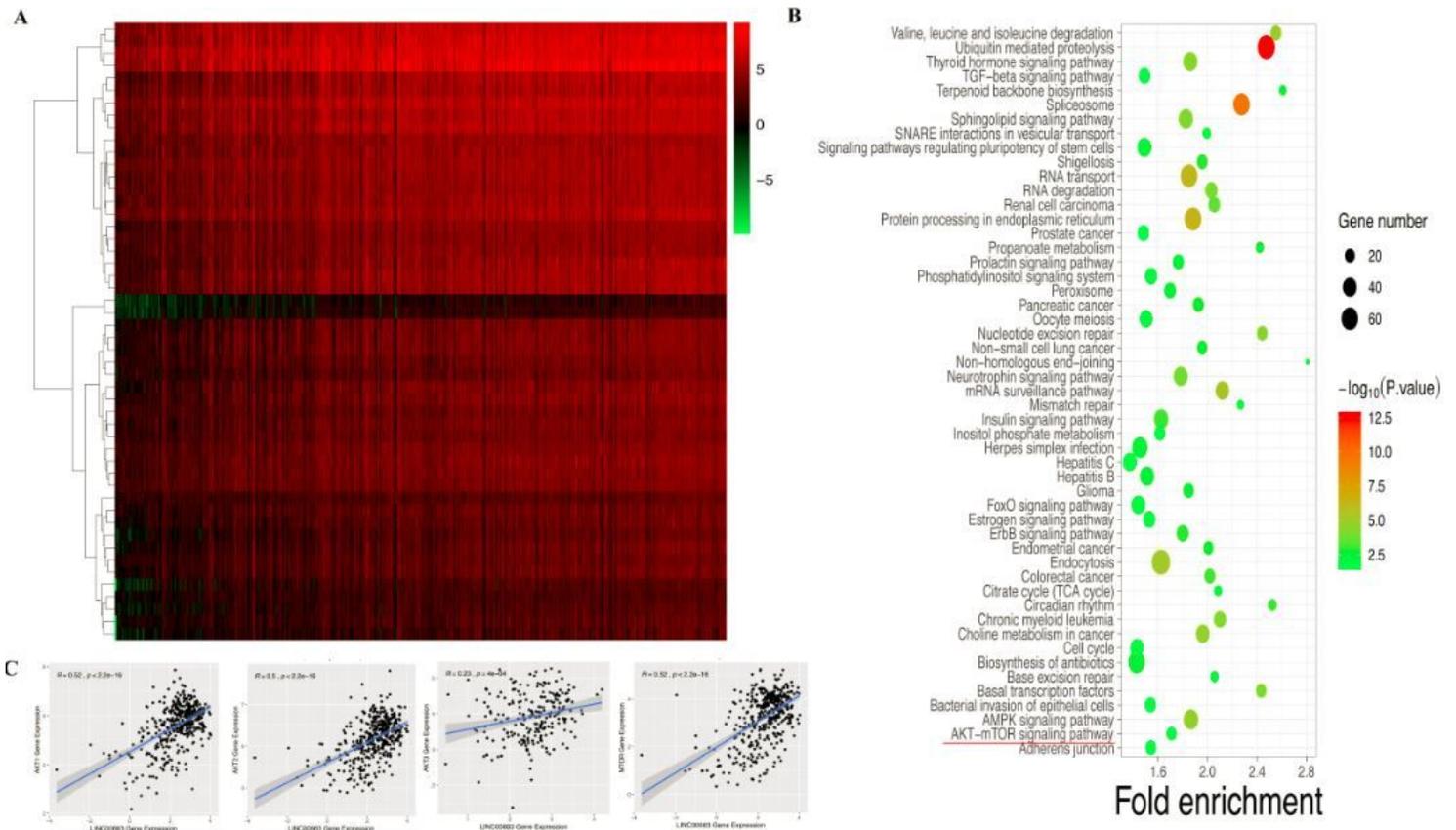


Figure 3

LINC00663-associated genes are mainly enriched in the PI3K-AKT pathway. (A) Heatmaps of LINC00663-associated genes in 422 glioma tissues sorted by the level of LINC00663 expression in CGGA dataset. (B) Pathway analysis was performed using the LINC00663 positive associated genes in CGGA dataset. (C) Correlation between the expression of AKT or mTOR and LINC00663 in gliomas was determined using CGGA dataset.

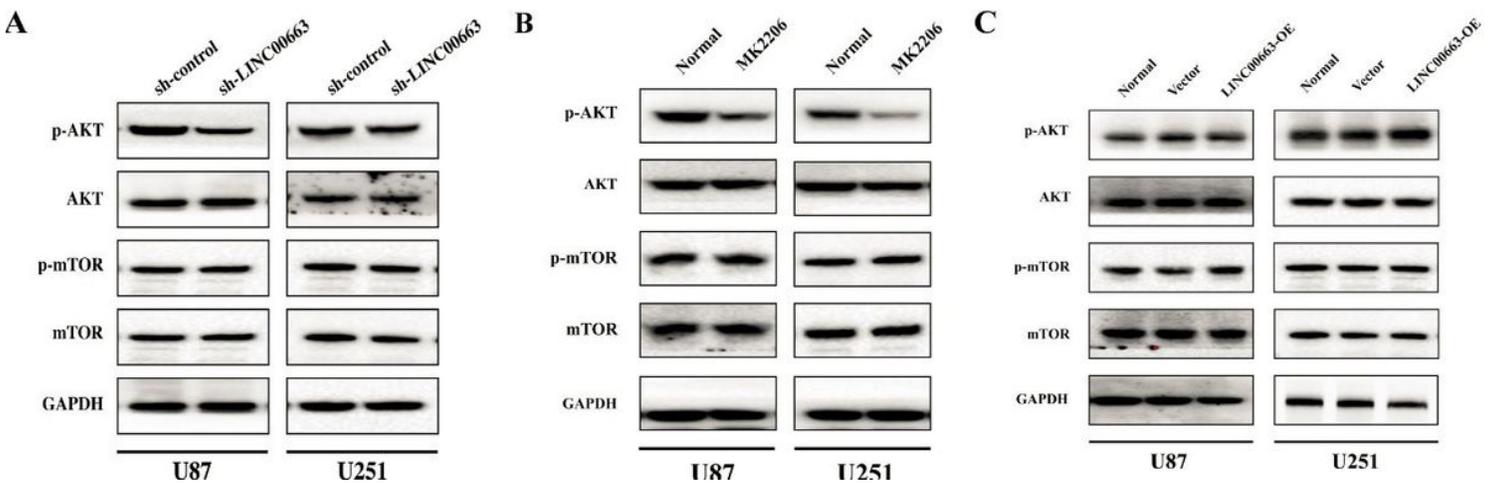


Figure 4

Regulation effects of LINC00663 on the AKT signaling transduction. (A, B, C) Total protein was used to detect the AKT, p-AKT, mTOR and p-mTOR level. GAPDH was used as a control in Western blot analysis. Data are presented as mean \pm S.E.M. of three independent experiments; *** $p < 0.001$.