MiR-4310 induced by SP1 targets PTEN to promote glioma progression

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MiR-4310 induced by SP1 targets PTEN to promote glioma progression

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Abstract

Background: miRNAs have been reported to be involved in multiple biological processes of gliomas. Here, we aimed to analyze miR-4310 and its correlation genes involved in the tumor progression of human glioma.

Methods: miR-4310 expression levels were examined in glioma and non-tumor brain (NB) tissues. The molecular mechanisms of miR-4310 expression and its effects on cell proliferation, migration, and invasion were explored by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), Transwell chamber, Boyden chamber, and western blot analyses, as well as *in vivo* tumorigenesis in nude mice. The relationships among miR-4310, SP1, and phosphatase and tensin homolog (PTEN) were explored by chromatin immunoprecipitation (ChIP), agarose gel electrophoresis, electrophoresis mobility shift (EMSA), and dual luciferase reporter gene assays.

Results: miR-4310 expression was upregulated in glioma tissues compared to NB. Overexpressed miR-4310 promoted glioma cell proliferation, migration, and invasion *in vitro* and tumorigenesis *in vivo*. Inhibition of miR-4310 was sufficient to reverse these results. Mechanistic analyses revealed that miR-4310 promoted glioma progression through the PI3K/AKT pathway by targeting PTEN. Additionally, SP1 induced the expression of miR-4310 by binding to its promoter region.

Conclusion: miR-4310 promotes the progression of glioma by targeting PTEN and activating the PI3K/AKT pathway meanwhile the expression of miR-4310 is induced by SP1.

Key words: miR-4310, SP1, PTEN, PI3K/AKT signaling, glioma

Background

Glioma is the most common primary intraparenchymal central nervous system (CNS) tumor type. Brain tumors are classified according to the World Health

Organization (WHO) CNS tumor grading system. In the revised 2016 WHO classification of CNS tumors, numerous molecular markers (IDH, 1p/19q codeletion, H3 Lys27Met, and RELA-fusion) are used in combination with histology for pathological diagnosis [1-4]. Although we now have a more accurate diagnosis of glioma，the prognosis for patients with malignant glioma remains very poor, since less than 5% of them have a 5-year relative survival [5].

PTEN is a common tumor suppressor gene. It is a natural inhibitor of the PI3K/AKT pathway. PTEN is frequently mutated in various cancers, including gliomas. Studies have shown that the frequency of PTEN loss of heterozygosity (LOH) in human high-grade gliomas can be up to 70% [6-7]. PTEN plays important roles in the regulation of cell proliferation, apoptosis, and tumor invasion. Clinical findings in high-grade gliomas suggest that PTEN gene alterations are associated with poor prognosis and may influence the response to specific therapies [8-9]. One of the mechanisms involved in the regulation of PTEN dosage occurs through micro-RNAs (miRNAs) [10-11].

miRNAs are short, approximately 20-24 nt, non-coding RNAs that are involved in post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. Several studies have reported that miRNAs are closely related to cancer, including gliomas [12-19]. miR-4310 is a newly discovered miRNA that has been reported to be related to colon cancer [20].

In our study, we performed numerous experiments, aiming to validate that miR-4310 can promote the proliferation, migration, and invasion of glioma cells. Additionally, we intended to elucidate the mechanism underlying the functions of miR-4310, that is, how miR-4310 promotes the activation of the PI3K/AKT pathway by targeting PTEN. Our results indicated that SP1 regulates the expression of miR-4310 by binding to the promoter region of miR-4310. These findings elucidate the molecular mechanisms controlling glioma progression and contribute toward the use of molecular screening markers for targeted therapeutic intervention of glioma.

Methods

Cell culture and sample collection

The human glioma cell line LN229, U87 were purchased from the Chinese Academy of Sciences (Shanghai, China) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Biowest). All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2.

A total of 29 glioma tissues and 9 NB tissues samples were obtained from the Nanfang Hospital of Southern Medical University, Guangzhou, China. For the use of these clinical materials for research purposes, prior consent from patients and approval from the Ethics Committees of Nanfang Hospital were obtained. All specimens had confirmed pathological diagnosis and were classified according to the World Health Organization (WHO) criteria.

Cell transfection

Plasmids were purchased from Vigene Biosciences (Shangdong, China). siRNAs, mimics and inhibitors were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China) (Supplementary table 1). Exponentially growing cells were seeded in a cell culture plate or dish (NEST Biotech Co., Ltd., China) before transfection. Plasmids, mimics and inhibitors were then transfected into cells using Lipofectamine TM 2000 (Invitrogen Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's protocol. Cells were collected 48-72 h after transfection for further experiments.

Lentivirus production and infection

Lentiviral particles encoding hsa-miR-4310 were designed and constructed by GeneChem (Shanghai, China). Cells were infected with lentiviral vector, and the expression of miR-4310 was detected by qPCR.

RNA isolation, reverse transcription, and qPCR

Total RNA was isolated from cells or harvested tissues. cDNA was synthesized using reverse transcription reagents (TaKaRa Bio, Inc., Shiga, Japan), and cDNA was used as a template for amplification using specific primers. The Bio-Rad T100 and Bio-Rad CFX96 detection systems were applied for RT-PCR and QPCR, respectively, according to the manufacturer's instructions. Related primers are shown in

Supplementary table 1.

Western blot analysis

Cell lysates were obtained in lysis buffer, and protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). Proteins were separated by SDS-PAGE and transferred onto polyvinyl difluoride membranes, which were immunoprobed with the corresponding antibodies. The proteins were detected using enhanced chemiluminescence reagent (Millipore, USA). Antibodies against the following proteins were used: ZEB1, N-Cadherin, E- Cadherin, PI3K, p-PI3K, AKT, p-AKT, PTEN, p21, p27, GAPDH, β-actin. Images were captured using a ChemiDocTM CRS+ Molecular Imager (Bio-Rad, Hercules, CA, USA). Antibody information and dilution are shown in Supplementary table 2.

Migration and Invasion Assay

The transwell and boyden assay was used to test cell migration and invasion abilities. Cells were suspended in 100 mL DMEM without serum and seeded into the top chamber of the transwells coated with Matrigel (BD Biosciences, NJ, USA) or left uncoated, and the bottom chambers were filled with 500 mL DMEM supplemented with 10% FBS. The migrated cells were stained with crystal violet and then photographed and quantified by counting the cell numbers in five random fields. All assays were independently performed in triplicate.

Wound Healing Assay

Cells were seeded and grew in 6-well plates until a confluent monolayer was reached, and scratches (wounding) were created using a 10μl pipette tip. Progression of migration was photographed at initiation and 12h after wounding. All experiments were repeated at least three times.

MTT assay

Cell proliferation were determined using the MTT assay. Cells were seeded into 96-well plates at a density of 1000 cells/well and incubated overnight to allow cell adherence. Cell viability was measured using MTT (5 mg/ ml) (Sigma-Aldrich, MO, USA). The absorbance value (OD) of each well was measured at 490 nm.

EdU incorporation assay

EdU incorporation was assessed using an Apollo567 In Vitro Imaging Kit (RiboBio Co., Ltd., Guangzhou, China) according to the manufacturer's protocol. Cells were incubated with 10 μM EdU for 2 h and then fixed with 4% paraformaldehyde. After permeabilization with 0.3% Triton X-100, the cells were stained with Apollo fluorescent dyes and the cell nuclei were stained with 5 μg/ml DAPI. All assays were independently performed in triplicate.

Electrophoretic mobility shift assay (EMSA)

An electrophoretic mobility shift assay was conducted using an EMSA Kit (BersinBio, Guangzhou, China) according to the manufacturer's instructions. Nuclear extracts were obtained from cells, and their concentrations were determined using a BCA assay kit. An EMSA was performed with a reaction mixture containing nuclear extracts and biotin-labeled probes. Competition or supershift assays were performed by adding a 100-fold excess of cold competitors (unlabeled wild-type or mutant probes) or polyclonal rabbit anti-SP1 (Cell Signaling Technology) to the reaction mixture. After electrophoresis and incubation, signals were recorded and analyzed. Related sequences are shown in Supplementary table 1.

Luciferase reporter assays

A fragment of the PTEN 3'-UTR (wild-type 3'-UTR) was amplified. Site-directed mutagenesis (mut) of the miR-4310-binding site or miR-4310 promoter region binding site was conducted using the GeneTailor Site-Directed Mutagenesis System (Invitrogen, Guangzhou, China). The wt 3'-UTR or mut 3'-UTR were cloned into the pENTER vector for luciferase reporter assays. The vector was cotransfected with miR-4310 mimics/inhibitor or the control sequence into cells, and luciferase activity was measured 48h after transfection using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA).

To investigate the effect of SP1 on the transcriptional activity of miR-4310, fragments encoding SP1-binding sites were cloned into the pGL4.1-Basic luciferase reporter vector, and vectors containing mutant SP1-binding sites were also constructed. These vectors and the SP1 plasmid were cotransfected into cells, following which luciferase activity was detected. Related sequences are shown in Supplementary table

1.

In situ hybridization (ISH) and evaluation of ISH staining

Tissue sections were deparaffinized in xylene and rehydrated in a graded alcohol series and distilled water. After treatment with proteinase K at 37 °C for 30 min, the sections were rinsed, fixed and then prehybridized for 2 h. Hybridization was performed with miRCURY miR-4310 digoxygenin-labeled probes designed and synthesized by BersinBio (Guangzhou, China). The chip were then washed and incubated with anti-digoxygenin-HRP Fab fragments for 1 hour at room temperature. Positive miR-4310 staining was observed by adding BM purple alkaline phosphatase substrate (Roche, Basel, Switzerland) according to the manufacturer's instructions.

The intensity of staining was scored on a scale of 0 to 3, in which 0 = negative staining, 1 = weakly positive staining, 2 =moderately positive staining, and 3 = strongly positive. The percentage of staining was estimated on a scale of 0 to 4, in which 0 = none, 1 = positive staining in 1–25% of cancer cells, 2 = positive staining in 26 –50%; 3 = positive staining in 51–75%; and 4 = positive staining in 76 –100%. The immunohistochemical score (IS) was calculated through multiplying the intensity score by the percentage score. Samples with IS between 0 and 1 were classified as Score 0, samples with IS between 2 and 4 were Score 1, samples with IS between 5 and 8 were Score 2, and samples with IS between 9 and 12 were Score 3.[21] Then a score of 0-1 is considered as low expression, and a score of 2-3 is considered as high expression.

Immunohistochemistry (IHC) and evaluation of immunohistochemical staining

Paraffinized sample sections were deparaffinized and dehydrated, and antigen retrieval was then performed in citrate buffer for 3 min. Endogenous peroxidase activity and nonspecific antigens were blocked with 3% H2O2 and goat serum followed by incubation with antibodies overnight at 4 °C. After washing, the sections were incubated with HRP-conjugated secondary antibody and visualized using DAB substrate (Maixin Biotech. Co., Ltd., Fuzhou, China). The evaluation of immunohistochemical staining was scored as ISH staining.

Animal Studies

Animal experimental protocols were approved by The Institutional Animal Ethical Committee, Experimental Animal Center of Southern Medical University, China.

The subcutaneous xenograft mouse model was adopted to evaluate tumor growth, in which 5×10^6 cells in 0.1ml PBS medium were injected into the left-right symmetric flank of 3-4-week-old male BALB/c nu/nu mice. Mice were sacrificed 30 days after cell inoculation, and tumors were excised, weighed, and processed for further experimentation. Tumor size was determined using measurements of the shortest diameter (A) and the longest diameter (B) with a caliper. The volume was calculated using the formula $V = (A^2\times B)/2$.

Statistical analysis

All data were analyzed using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) and Graph Pad Prism 6.0. The data are presented as the means±SDs. Statistical significance was detected using Student's two-tailed t-test for differences between two groups, one-way ANOVA for differences between multiple groups, the general linear model repeated measures variance analysis for differences in tumor growth and MTT assay results. Correlations between gene expression and clinicopathological characteristics were assessed by the chi-square test. Cox regression analysis and Kaplan-Meier survival analysis were used for analyzing the relationship between the variables and patient's survival time.

All statistical tests were two-sided, and a P value of <0.05 indicated statistical significance (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$).

Results

miR-4310 promotes glioma cell proliferation, migration, and invasion *in vitro.*

To explore the roles of miR-4310 in glioma progression, we first compared the expression of miR-4310 between 8 non-tumor brain tissues (NB) and 26 glioma tissues. We found that glioma patients showed higher levels of miR-4310 than NB (Fig.1 A).

To further verify the biological function of miR-4310 in glioma cells, we transfected the U87 and LN229 glioma cell lines with the miR-4310 lentivirus and its negative control (NC). We thus obtained two groups of glioma cell lines that could stably express miR-4310 and their respective negative control cell lines. We named

them U87-LV-NC, U87-LV-miR-4310 and LN229-LV-NC, LN229-LV-miR-4310. We used qPCR to confirm their high miR-4310 overexpression efficiency (Supplementary Fig.1 A-B). This allowed us to use them as tool cell lines in our following studies.

First, we studied the *in vitro* effects of miR-4310 expression on cell proliferation. For this purpose, we applied Edu incorporation and MTT assays on our U87 and LN229 glioma cell lines. Our results revealed that overexpression of miR-4310 promoted significantly cell proliferation, whereas suppression of miR-4310 expression restored the proliferation rate (Fig.1B-C).

Next, we performed Transwell chamber, Boyden chamber, and wound healing assays which showed that overexpressed miR-4310 can promote glioma cell migration and invasion, and this function could be restored by miR-4310 inhibition (Fig.1D-F).

In conclusion, our *in vitro* results showed that miR-4310 can indeed promote the proliferation, migration and invasion on glioma cell lines.

miR-4310 promotes tumorigenesis *in vivo.*

The above results prompted us to perform an *in vivo* tumor formation experiment by subcutaneously injecting U87-miR-4310 or control cells into nude mice. For this purpose, 10 nude mice were used for subcutaneous tumor formation, of which, 9 were successfully tumorigenic and 1 died halfway. After 30 days of implantation, 8 out of the 9 tumorigenic, injected with U87-miR-4310 cells, mice had larger tumor burdens (Fig. 2A) and displayed higher expression of Ki67 and proliferating cell nuclear antigen (PCNA) in tumor tissues relative to controls (Fig. 2B). These results suggested that miR-4310 significantly promoted tumorigenesis *in vivo*.

Biological function of miR-4310 is achieved by activating the PI3K/AKT pathway and EMT-associated genes

The epithelial-mesenchymal transition (EMT) process and the PI3K/AKT signaling pathway are known to have an inseparable relationship to tumor cell proliferation, migration and invasion. Thus, we sought to investigate the expression levels of proteins related to EMT and the PI3K/AKT pathway by western blot. Our results showed that overexpression of miR-4310 upregulated the zinc finger E-box binding homeobox 1 (ZEB1) and N-cadherin, and downregulated E-cadherin (Fig. 1G).

We then examined the effect of miR-4310 on the PI3K/AKT pathway. We found that overexpression of miR-4310 significantly increased the phosphorylation of PI3K and AKT, but not their total protein levels, and downregulated the downstream molecules of PI3K/AKT, p21 and p27 (Fig. 1H). All the above effects were restored in the presence of miR-4310 inhibitor.

The above experimental results show that miR-4310 achieves its biological functions by activating the EMT and the PI3K/AKT signaling pathway.

miR-4310 directly targets PTEN

The biological function of miRNAs is achieved by binding to their target gene mRNA. PTEN is an important factor that antagonizes the PI3K/AKT signaling pathway. Therefore, we supposed that PTEN may be the target gene of miR-4310. miR-4310 could release the antagonistic effect of PTEN on the PI3K/AKT pathway by targeting PTEN, thereby activating this pathway.

We used TargetScan (http://www.targetscan.org/) and predicted two sites of PTEN that could be direct targets of miR-4310 (Fig. 3A). Overexpression of miR-4310 downregulated the protein level of PTEN, but had no effect on the mRNA level (Fig. 3B-C). This indicates that miR-4310 can regulate the translation of the PTEN mRNA. This result was confirmed by immunohistochemistry staining in xenografts derived from U87-LV-NC and U87-LV-miR-4310 cells (Fig. 3D).

To further prove that miR-4310 directly targets PTEN we performed the dual luciferase reporter gene assay. Our results showed that the luciferase activity was abrogated when co-transfected with wild-type PTEN reporter and miR-4310 mimics (Fig. 3E lane 5). This result could be reversed by co-transfection with wild-type PTEN reporter and miR-4310 inhibitor (Fig. 3E lane 6),but was not affected when co-transfected with mutant PTEN reporter and miR-4310 mimics or inhibitor (Fig. 3E lane 14, 15). Moreover, when we singly co-transfected with mutant site 1 or site 2 of PTEN reporter and miR-4310 mimics， we could still observe that luciferase activity was abrogated (Fig. 3E lane 8, 11) but the degree of abrogation was less than that of lane 5 (Fig. 3E). Thus, the results of the dual luciferase reporter gene assay illustrate that

PTEN is the target gene of miR-4310 and both binding sites are active.

Then, understand whether the biological function of miR-4310 is achieved through PTEN, we transiently transfected a PTEN plasmid into the U87-LV-miR-4310 and LN229-LV-miR-4310 cell lines to test whether overexpression of PTEN could inhibit the proliferation, migration and invasion of these cells. The obtained results showed that overexpression of PTEN can inhibit U87-LV-miR-4310 and LN229-LV-miR-4310 proliferation, migration and invasion (Fig. 4A-D). The immunoblotting assay data also confirmed that overexpression of PTEN can affect the EMT and PI3K/AKT associated proteins. (Fig. 4E-F)

Collectively, these results are supporting our initial hypothesis that miR-4310 promotes activation of the PI3K/AKT pathway by targeting PTEN, thus causing the release of the antagonistic effect of PTEN on the signaling pathway.

SP1 induces the expression of miR-4310 by binding to its promoter region.

SP1 is a zinc finger transcription factor that binds to GC-rich motifs of many promoters. The encoded protein is involved in many cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling. According to reports, SP1 plays important role in the tumorigenesis, progression, and drug resistance of gliomas [22-25]. We found that the promoter region of miR-4310 also contains GC-rich fragments. Thus, we supposed that SP1 may be an upstream signaling molecule of miR-4310 and may regulate miR-4310 expression by binding to the promoter region of miR-4310.

We used UCSC (http://genome.ucsc.edu), PROMO (http://alggen.lsi.upc.es/), and JASPA (http://jaspar.genereg.net/) to predict whether SP1 could bind to certain sequences in the promoter region of miR-4310. Two sites were identified that SP1 could bind on: site A (-1508~-1499) and site B (-1939~-1930) (Fig. 5A). We overexpressed SP1 in U87 and LN229 glioma cell lines and used PCR to detect the level of miR-4310. The results showed that the expression level of miR-4310 increased (Fig. 5B). This preliminarily proves that SP1 may act as an upstream factor of miR-4310 to regulate the expression of miR-4310.

We then verified our supposition by chromatin immunoprecipitation (ChIP) and

agarose gel electrophoresis assay on U87 and LN229 cells. Site A and site B sequences were enriched in the anti-SP1 group, indicating that SP1 can bind to site A and site B (Fig. 5B-C). Subsequently, we used electrophoresis mobility shift assay (EMSA) and dual luciferase reporter gene assay to further confirmed this conclusion (Fig.5 D-E). These results too, supported that SP1 could induce the expression of miR-4310 by binding to its promoter region.

Clinical relationship among miR-4310, SP1, and PTEN.

To further clarify the relationship among miR-4310, PTEN, and SP1, we used in situ hybridization (ISH) and immunohistochemistry (IHC) to semi-quantitatively analyze their expression levels in 86 paraffin embedded glioma tissue samples. Among the 52 samples with low expression level of miR-4310, 59.6% (31/ 52) also exhibited high expression of PTEN and 40.4% (21/52) low expression of PTEN, whereas 26.9% (14/52) exhibited high expression of SP1 and 79.1% (38/52) low expression of SP1. Similarly, of the 34 samples with high expression level of miR-4310, 40.5% (15/34) presented with high expression of PTEN and 59.5% (19/34) with low expression of PTEN, whereas 58.8% (20/34) presented high expression of SP1 and 41.2% (14/34) low expression of SP1 (Fig. 6A-B). Our results showed that SP1 has a positive correlation with miR-4310 (Chi-square test, $p=0.003$; Spearman correlation coefficient $\rho=0.319$). Maybe due to the small size of our sample, we were not able to find a significant negative correlation between miR-4310 and PTEN, but they still present a negative tendency to some extent (Chi-square test, $p=0.188$; Spearman correlation coefficient $\rho=-0.152$). The other clinical characteristics of the glioma patients are summarized in Table 1.

miR-4310, SP1, and PTEN are independent prognostic factors for glioma.

According to CGGA (http://www.cgga.org.cn), we analyzed the expression levels of SP1 and PTEN in each glioma grade and performed Kaplan-Meier survival analysis based on SP1 and PTEN expressions. The results of this analysis show that the expression level of SP1 increases with the WHO grade of glioma (Supplementary Fig. 1 C, E). Moreover, the Kaplan-Meier survival analysis showed that high expression of SP1 is associated with poor prognosis of glioma patients (Supplementary Fig. 1 D, F).

Similarly, high expression of PTEN is associated with good prognosis of glioma patients (Supplementary Fig. 1 G-H). We still performed a Kaplan–Meier survival analysis on 86 glioma patients. The result showed that patients with high SP1 expression had poorer OS rates compared with those exhibiting low SP1 expression (Supplementary Fig. 1 I).

Although, neither p value reached significance. In these tissue samples, patients with high miR-4310 expression or PTEN expression tended to have longer or shorter survival times (Supplementary Fig. 1 J-K). We suspected that non-significance was due to small sample size and mutual interference among SP1, PTEN and miR-4310.

Furthermore, we used univariate and multivariate COX regression to analyze whether miR-4310, SP1, and PTEN are independent prognostic factors for glioma. We first used the data from these 86 cases to analyze the relationship between various factors and the overall survival (OS) of glioma patients (Table 2). Age, gender, WHO grades, SP1, PTEN and miR-4310 were included in this study as possible prognostic factors.

The multivariate COX regression results revealed that miR-4310 ($p= 0.002$) and WHO grades ($p<0.0001$) are prognostic factors for glioma. We next performed a survival analysis based on the miR-4310 expression (Fig. 7D), and these results suggested that high expression of miR-4310 is associated with poor prognosis in glioma patients.

In addition, considering that SP1 and PTEN are also common risk factors in clinic, we also used the CGGA-693 database for a further COX regression analysis. Age, gender, WHO grades, SP1, PTEN, IDH mutation, chemotherapy and radiotherapy were included in the study as possible prognostic factors. The results based on CGGA-693 revealed that WHO grades ($p<0.0001$), SP1 ($p=0.006$), PTEN ($p<0.0001$), IDH mutation($p<0.0001$), and chemotherapy ($p=0.004$) are independent prognostic factors for glioma (Table 3). Survival analysis results suggest that high expression of SP1 is associated with poor prognosis in glioma patients. Similarly, high expression of PTEN correlates with benign prognosis in glioma patients. (Fig. 6 D-E)

Discussion

Glioma is a primary brain tumor with poor prognosis. Despite the recent significant advances in the molecular diagnosis of gliomas, their treatment is still in a stagnation stage [26]. Difficulties in the treatment of gliomas are closely related to their invasive growth. Therefore, the study of the aggressive proliferation, migration and invasion growth behavior of glioma cells are a key measure to overcome the difficulties of glioma treatment.

A growing number of reports demonstrate that miRNAs play an important role in the progression of glioma. More and more miRNAs are found to act as tumor suppressor or oncomiRs in gliomas [27-30]. As research progresses, scientists are beginning to tap the huge potential of miRNAs for disease treatment. For example, anti-miR-122 (Miravirsen) is used for hepatitis C therapy in clinical trials; MRX34 (a synthetic miR-34a mimic loaded in liposomal nanoparticles) is in a phase I clinical trial for primary liver cancer and liver metastases [31-36]. Therefore, we believe that miRNAs show great potential for the development of new glioma therapies.

As a newly discovered miRNA, the role of miR-4310 in glioma has not been reported. Through a series of experiments, we found that miR-4310 significant promotes the proliferation, migration, and invasion of glioma cells. This suggests that miR-4310 acts as an onco-miRNA in gliomas. miRNAs act by binding to the target gene's mRNA in order to mediate the degradation of the target gene mRNA or to suppress its translation process. It is well established that PTEN is closely related to the tumorigenesis and progression of gliomas [6-9]. Through Targetscan, we predicted that miR-4310 and PTEN have two binding sites. Next, we used dual luciferase reporter gene experiments to prove that both sites of PTEN can bind to miR-4310 and perform its biological function. The role of PTEN and the PI3K/AKT pathway in glioma has been well reported [37-41]. In our study, we observed that miR-4310 regulated the proteins related to EMT and PI3K/AKT pathway with western blot. That means the function of miR-4310 is achieved by combining PTEN to release the inhibitory effect of PTEN on the PI3K/AKT pathway, thereby promoting the progression of glioma.

SP1 is a zinc finger transcription factor that binds to GC-rich motifs of many promoters. Through bioinformatics analysis, we found that the promoter region of miR-

4310 also has a GC-rich region. A series of experiments proved that SP1 could induce the expression of miR-4310 by binding to its promoter region. Importantly, we revealed that the SP1/miR-4310/PTEN axis activates the PI3K/AKT signaling pathway to promote glioma progression (Fig 7).

Finally, we analyzed 86 paraffin embedded glioma tissue samples and found a positive correlation between miR-4310 and SP1. This is consistent with the conclusions we provided above. Through Kaplan-Meier survival analysis and COX regression analysis we proved that miR-4310, SP1, and PTEN can be used as a risk factor for glioma prognosis. Although limited by sample size, ethnicity, geographical restrictions, and other various factors, there are certain contradictions in our conclusions. But looking at all the data comprehensive, our conclusion is still credible and instructive.

Reportedly, glioma is the most common primary intraparenchymal central nervous system tumors. Patients with glioma have a poor prognosis, especially those with high-grade gliomas. Therefore, exploring deeper the pathogenesis of gliomas and finding new therapy approaches are important to overcome the poor prognosis of this disease. In this study, we explored a new regulatory mechanism as illustrated in our working model in Fig. 7, the SP1/miR-4310/PTEN/PI3K/AKT axis, in glioma. This axis expands the molecular regulatory mechanisms in glioma and improves our understanding of the mechanism of glioma progression. Besides, both SP1 and PTEN play an important role in the tumorigenesis, progression and drug resistance of glioma [42-44]. The discovery of new signal regulation pathways will contribute in the development of new therapies.

Declarations:

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests: The authors declare that they have no competing interests.

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Abbreviations:

CNS: central nervous system

WHO: World Health Organization

PTEN: phosphatase and tensin homolog

LOH: loss of heterozygosity

MiR-4310: microRNA-4310

EMT: epithelial-mesenchymal transition

NB: non-tumor brain tissues

NC: negative control

ZEB1: zinc finger E-box binding homeobox 1

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

ChIP: chromatin immunoprecipitation

EMSA: electrophoresis mobility shift

ISH: in situ hybridization

IHC: immunohistochemistry

DMEM: Dulbecco's modified Eagle's medium

Reference

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Figures

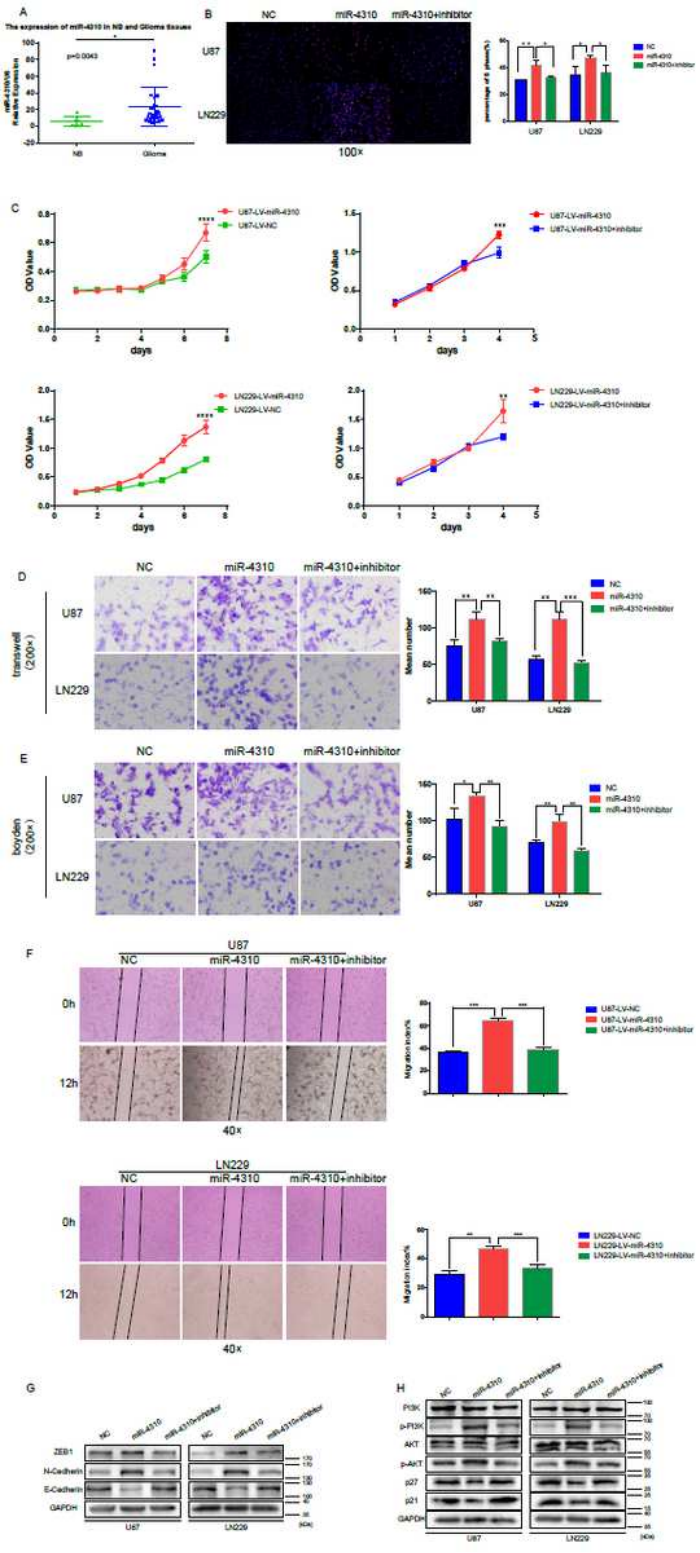

Figure 1

miR-4310 promotes glioma cell proliferation, migration, and invasion by activating PI3K/AKT pathway and promoting EMT progress. (A) The expression of miR-4310 in NB tissues and glioma tissues were examined by qPCR. (B)The proliferative ability of indicated U87 and LN229 cells were evaluated by EdU

assays; (C)The cell viability of indicated U87 and LN229 cells were evaluated by MTT assays. (D-F) The migration ability and invasion ability of U87 and LN229 cells were tested by Transwell assays (D), Boyden assays (E) and wound healing assay (F). (G-H) The expression of PI3K/AKT pathway and EMT related proteins were examined by Western blot. GAPDH were used as a loading control. Data were presented as mean±s.d. NS, no statistical significance, * p□0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

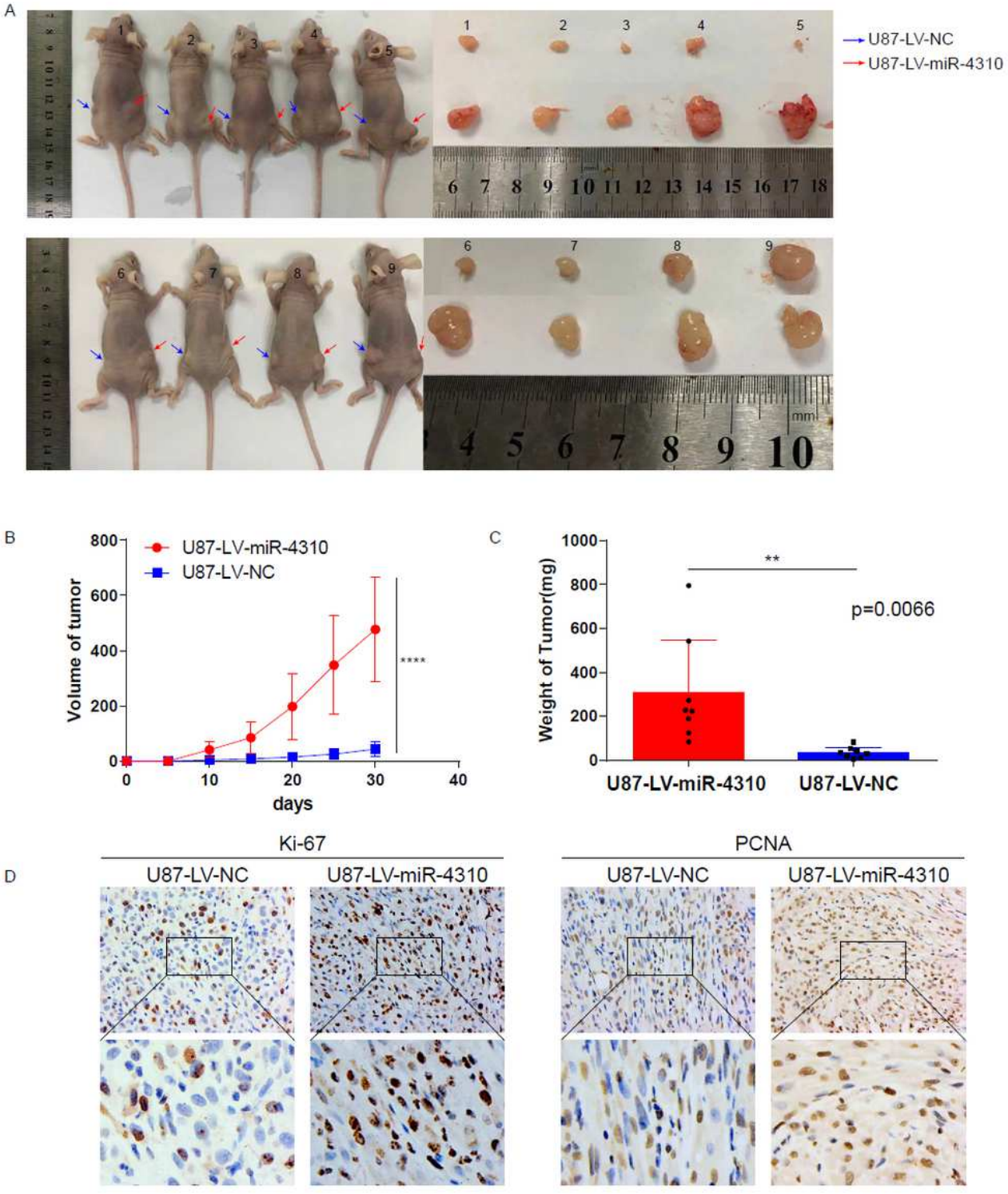

Figure 2

miR-4310 promotes tumorigenesis in vivo. (A) Images of xenograft tumor models injected with U87 cells transfected with miR-4310 lentiviral expression particles or negative control (NC). (B) Tumor volume was measured every five days for each mouse and tumor growth curve was plotted. (C) Xenograft tumors from miR-4310 group and control group were weighed at day 30. (D) Expression of ki-67 and PCNA were detected in xenograft tumors from mice. Data were presented as mean±s.d.. NS, no statistical significance, * p0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

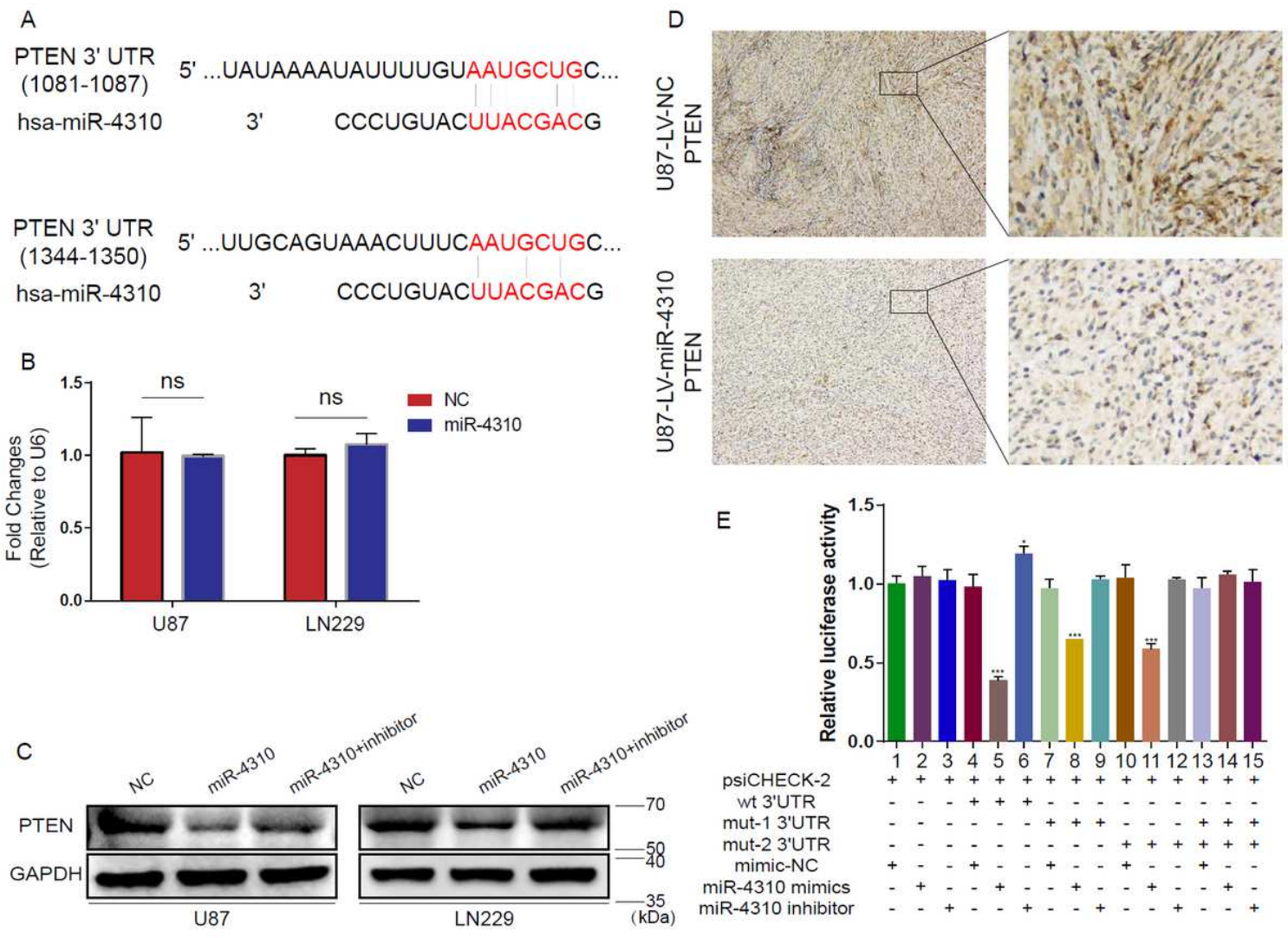

Figure 3

miR-4310 directly targets PTEN. (A) Schematic diagram of putative binding sequences of miR-4310 in the 3'-UTR of PTEN. (B) PTEN mRNA expression was detected by qPCR in miR-4310-overexpressing U87 and LN229 cells. (C) PTEN protein level was detected in U87 and LN229 cells under LV-miR-4310 transfection or co-transfection with miR-4310 inhibitor. (D) PTEN expression was evaluated by IHC staining in xenografts derived from U87-LV-NC and U87-LV-miR-4310 cells. (E) Luciferase reporter assay was used to determine the miR-4310 binding sites in the PTEN 3'-UTR. Data were presented as mean±s.d. NS, no statistical significance, * p0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

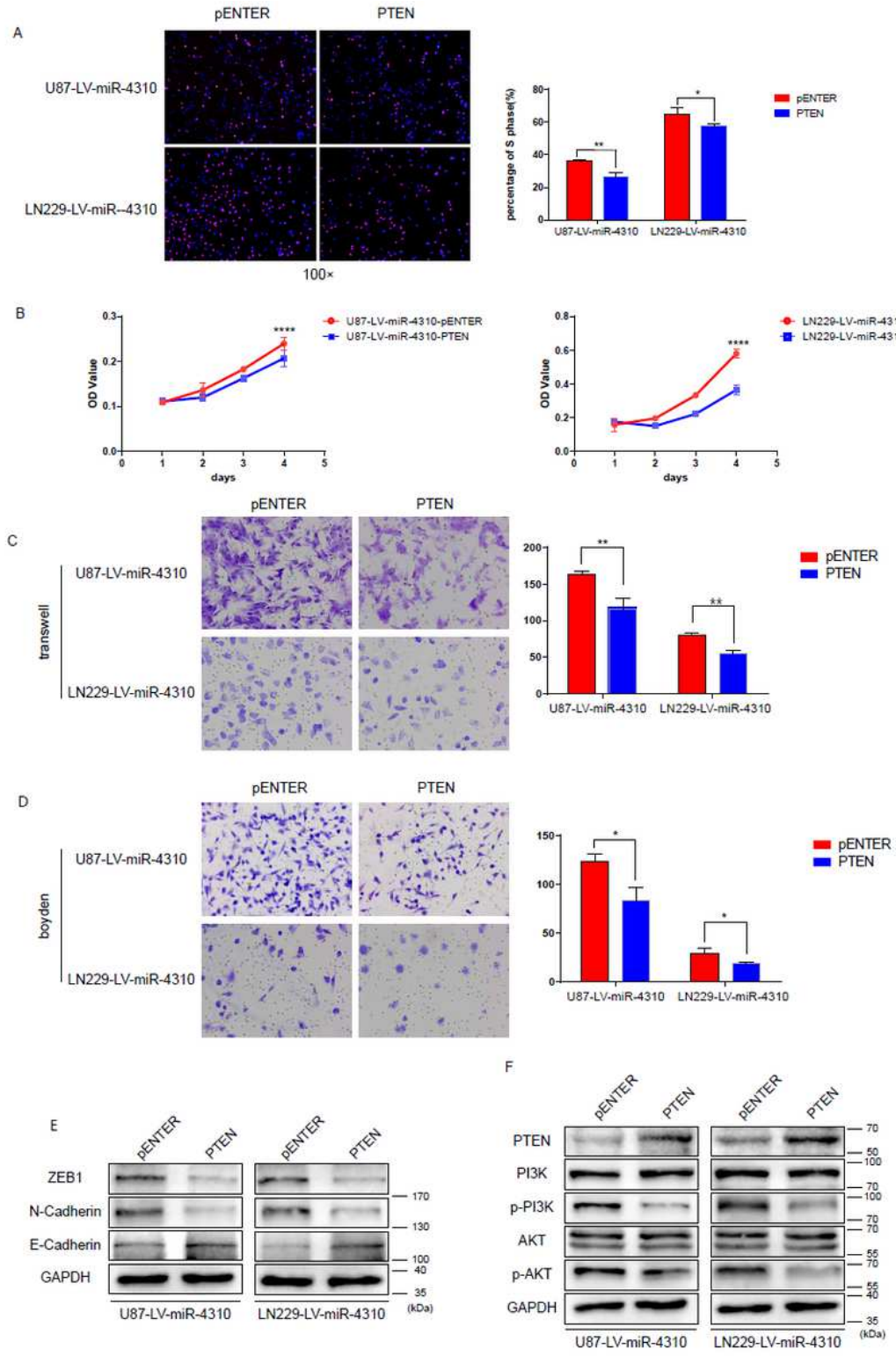

Figure 4

Overexpression of PTEN can reverse the biological functions mediated by miR-4310. (A-B) Proliferative ability of U87 and LN229 cells co-transfected with PTEN plasmid and LV-miR-4310 was examined by EdU assays(A) and MTT assays(B). (C-D) Migration and invasion of U87 and LN229 cells co-transfected with PTEN plasmid and LV-miR-4310 were examined by Transwell assays(C) and Boyden assays(D), respectively. (E-F) Expression of ZEB1, N -Cadherin, E-Cadherin, PI3K, AKT, p-PI3K and p-AKT were

detected by western blot in U87-LV-miR-4310 and LN229-LV-miR-4310 performed after transfection with pENTER plasmid (the control group) and PTEN plasmid (the treatment group) as indicated. GAPDH were used as a loading control. Data were presented as mean±s.d. NS, no statistical significance, * p 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

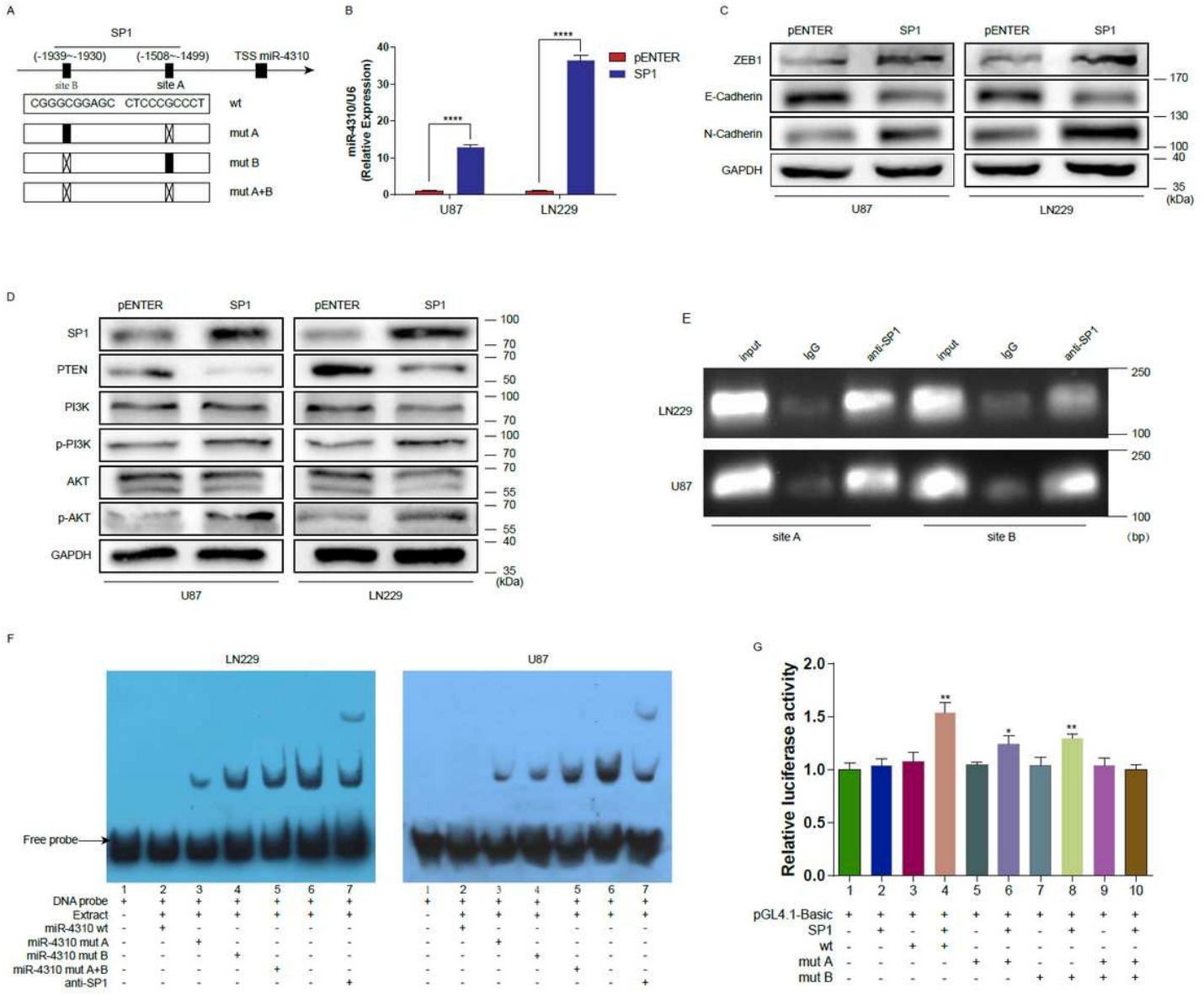

Figure 5

SP1 induces the expression of miR-4310 by binding to its promoter region. (A) Schematic diagram of the promoter regions of miR-4310 with the putative SP1 TFBSs (site A and site B) and the structure of the wild-type (WT) and TFBS mutant (mut A, mut B, and mut A+B) luciferase reporters driven by the promoter. (B) Expression of SP1 was detected by qPCR assays. (C-D) The expression of PI3K/AKT pathway and EMT related proteins were examined by Western blot. GAPDH were used as a loading control. (E) ChIP assay along with PCR and agarose gel electrophoresis showed amplification of SP1-binding sites A and B. (F) Protein-DNA interactions between SP1 and the miR-4310 promoter were determined by

electrophoretic mobility shift assays (EMSA). (G) Luciferase reporter assay was used to determine the binding of SP1 to miR-4310 promoter region. Statistical methods: One-way ANOVA and Dunnett's multiple comparison test. Data were presented as mean±s.d.. NS, no statistical significance, * p□0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

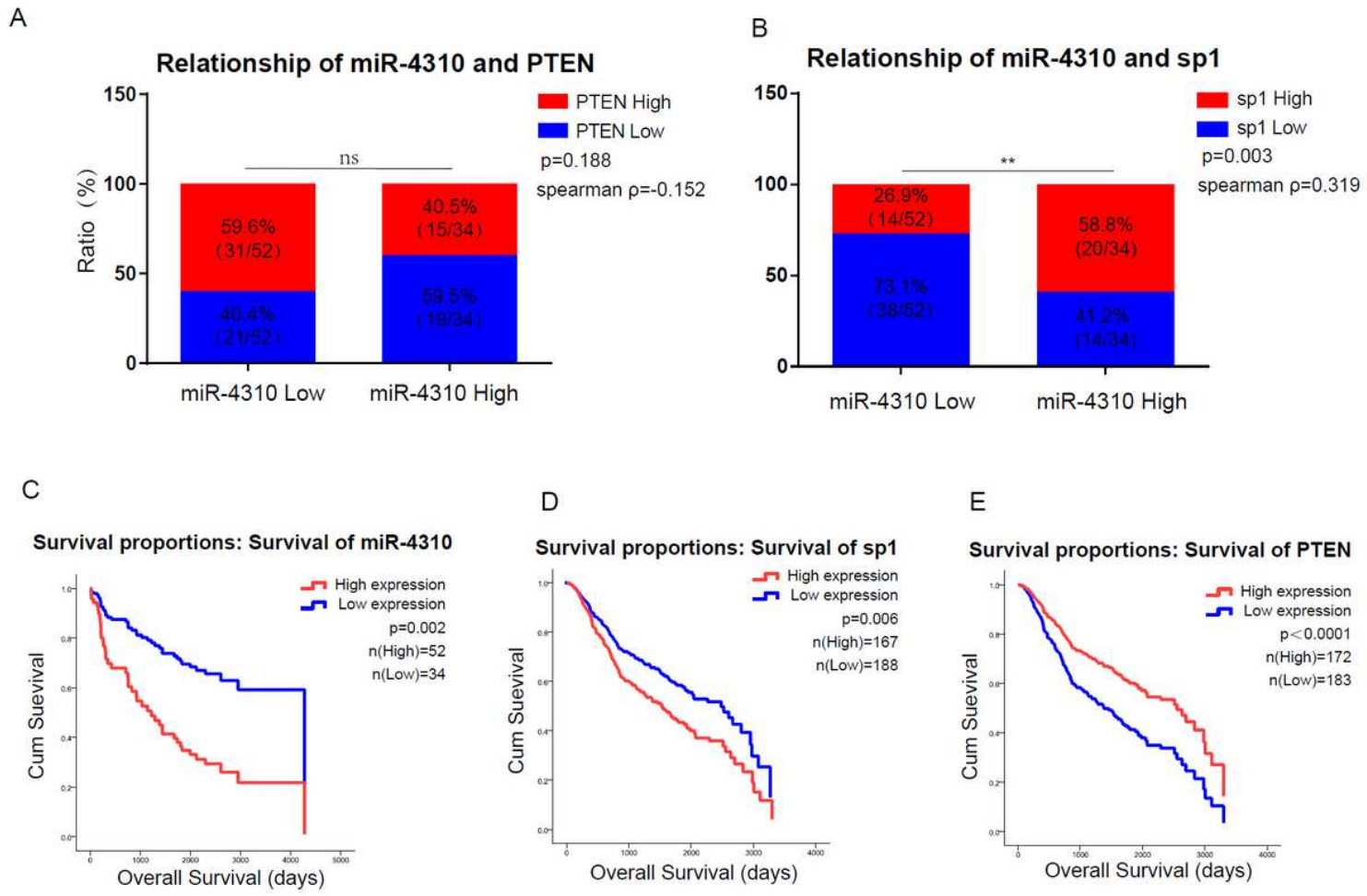

Figure 6

Clinical relationship among miR-4310, SP1, and PTEN. (A) and (B) Correlations between miR-4310 and PTEN, SP1 and miR-4310 expression level scored by ISH or IHC staining were shown. (C-E) Cox regression analysis of miR-4310, PTEN and SP1 expression in glioma cohorts from Nanfang Hospital neurosurgery department (C), CGGA-693 database (D-E). Data were presented as mean±s.d.. NS, no statistical significance, * p□0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

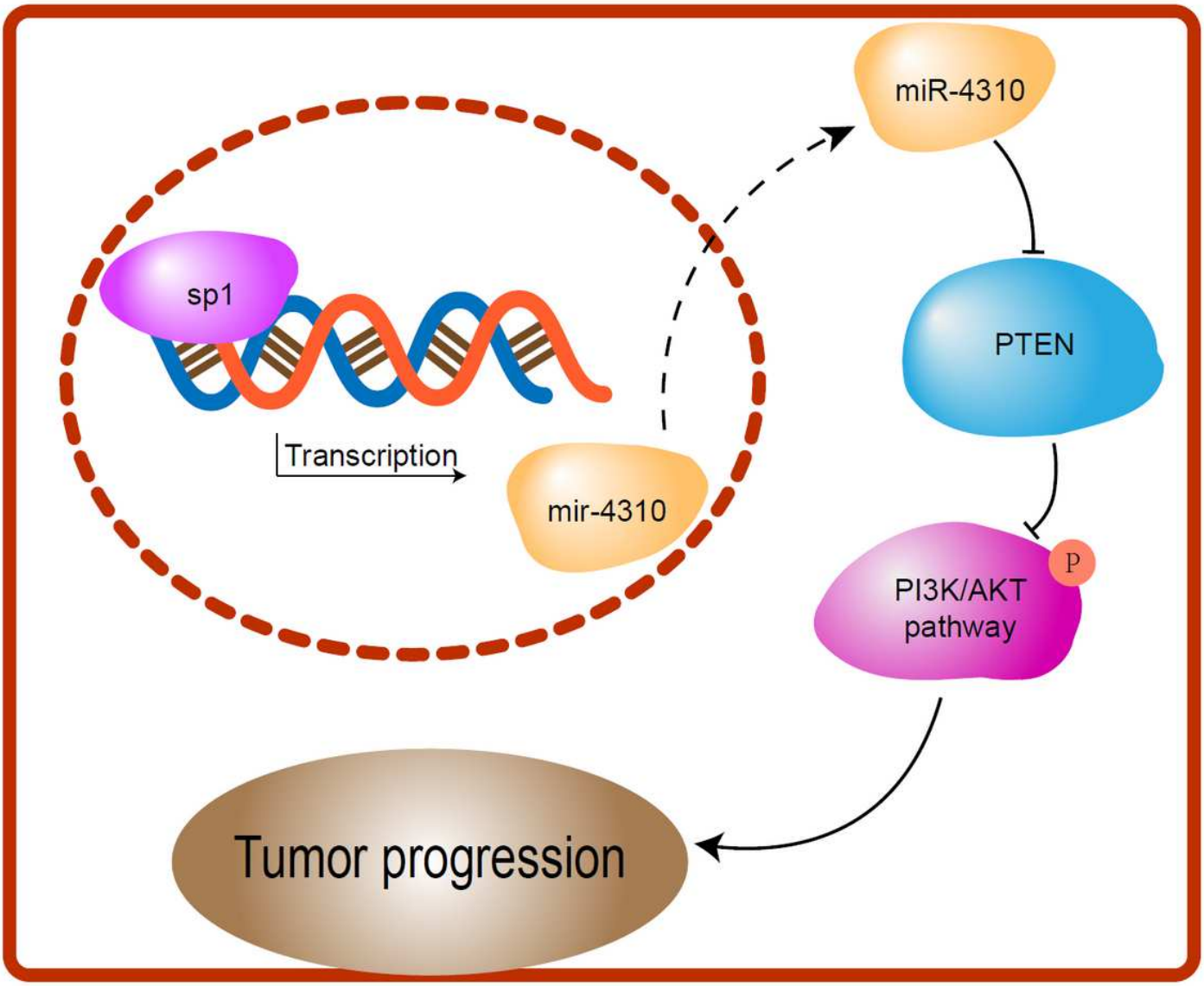

Figure 7

Schematic diagram of SP1/miR-4310/PTEN/PI3K/AKT pathway in glioma. SP1 regulates miR-4310 expression by binding to the miR-4310 promoter region. By targeting PTEN, miR-4310 releases the inhibitory effect of PTEN on the PI3K/AKT pathway and activates the PI3K/AKT pathway, thereby promoting the proliferation, invasion and migration of glioma.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- Table3.pdf

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