LINC01116 promotes the proliferation and invasion of glioma by regulating the microRNA-744-5p-MDM2-p53 axis

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Abstract. Long non-coding RNAs (lncRNAs) have been implicated in the development and progression of tumors. However, the roles and underlying mechanisms of long intergenic non-protein coding RNA 1116 (LINC01116), a member of the lncRNA family, in glioma progression are largely unclear. The expression of LINC01116 and microRNA (miR)-744-5p in glioma tissues and cells was detected by reverse transcription-quantitative PCR. The influences of LINC01116 or miR-744-5p on cell proliferation and invasion were evaluated by Cell Counting Kit-8, colony formation and Transwell assays, and western blotting was used to detect the expression of p53 pathway proteins. A dual-luciferase reporter system was used to locate common binding sites between miR-744-5p and LINC01116 or the 3' untranslated region of E3 ubiquitin-protein ligase Mdm2 (MDM2). RNA immunoprecipitation was used to determine the interactions between RNAs and proteins. Moreover, a xenograft mouse model was constructed to investigate the effects of LINC01116 *in vivo*, followed by a TdT-mediated dUTP nick end labeling assay to determine the degree of apoptosis in nude mouse tumors. LINC01116 was found to be highly expressed in glioma tissues, which was associated with a malignant phenotype. LINC01116 promoted the proliferation and invasiveness of glioma cells, and inhibited the p53 pathway by preserving the expression of MDM2 mRNA via miR-744-5p sponging. Furthermore, a low degree of miR-744-5p expression was observed in glioma tissues, which was negatively associated with the expression of LINC01116. Overexpression of miR-744-5p inhibited the proliferation and invasiveness of glioma cells, which was rescued by LINC01116. Finally, LINC01116 knockdown inhibited tumor growth in nude mice. In conclusion, LINC01116 is aberrantly expressed and promotes the progression of glioma by regulating the miR-744-5p-MDM2-p53 pathway. In future, targeting LINC01116 may therefore be a potential therapeutic approach for patients with glioma.

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Introduction

Glioma is the most common type of malignant primary brain cancer (1), and is characterized by rapid proliferation, high invasiveness, rapid recurrence and chemo- and radiotherapeutic resistance (2). In spite of the advances in microsurgery and combined treatments, the prognosis of patients with glioma remains unsatisfactory (3). This has driven research to investigate the potential mechanisms underlying the tumorigenesis and development of glioma in order to identify novel therapeutic targets.

A number of studies have demonstrated that the non-coding RNAs [microRNAs (miRNAs/miRs), pseudogenes, long non-coding RNAs (lncRNAs) and circular RNAs] play important roles not only in normal biological processes, but also in the development of tumors (4,5). Complex regulatory networks exist between non-coding RNAs, mRNAs and proteins. A commonly accepted theory is the competing endogenous RNA (ceRNA) hypothesis, through which endogenous RNAs containing certain miRNA binding sites competitively bind the same miRNAs to reduce the suppression of targeted mRNAs, thus retaining the expression of target genes (6). Increasing evidence suggests that the ceRNA mechanism is also involved in tumorigenesis (7-9).

lncRNAs, a group of non-coding RNAs >200 nucleotides in length, are involved in various disease processes, including tumorigenesis (10,11). An increasing number of studies have indicated that the aberrant expression of specific lncRNAs is associated with the development of glioma (12). lncRNAs exert their functions through various mechanisms in an extensive

array of biological processes, such as the maintenance of stemness, regulation of cell proliferation, tumor angiogenesis and drug resistance (13). In glioma, the functions of lncRNAs include regulating genome activity, posttranscriptional protein modification and location, as well as encoding functional micro-peptides and acting as intercellular communicators (14). However, the mechanisms by which lncRNAs regulate the biological functions of glioma remain to be elucidated. The present study aimed to demonstrate the role of LINC01116 in glioma and its underlying mechanism. The expression levels of LINC01116 in glioma tissue and cell lines were determined by reverse transcription-quantitative (RT-q)PCR. The effect of LINC01116 on cell proliferation, migration and invasion was assessed by cell viability, Transwell and colony formation assays and tumor xenografts in nude mice. In order to identify the underlying mechanism by which LINC01116 exerts its role, bioinformatics analysis and dual-luciferase reporter assay were performed.

Materials and methods

Cell culture and glioma samples. Glioma (U87MG, A172 and Shg44) and 293T cell lines were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences. The U87MG cell line was a version from American Type Culture Collection (ATCC; RRID:CVCL_0022), the origin of which is unknown. The cell line was authenticated by STR profiling, with a 96.55% match to the ATCC database profile. The HEB normal human astrocyte cell line was purchased from Cobioer; Nanjing Kebai Biotechnology Co., Ltd. All cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C (5% CO_2 and 95% air). A total of 46 fresh glioma samples and 4 normal brain tissue samples (excised from patients with intracerebral hemorrhage) were collected from the Hangzhou First People's Hospital (all specimens were pathologically diagnosed as glioma between January 2010 and October 2019), and stored in liquid nitrogen. The patients of the present study consisted of 27 male patients and 19 female patients. The age range of patients was between 28 and 74 years. Informed written consent was obtained from the patients, agreeing to use their samples for scientific research. All protocols associated with animals or human tissues were approved by the Ethics Committee of Hangzhou First People's Hospital [Hangzhou, China; approval no. 2020(106)-01].

Online cancer database analysis. The expression of LINC01116 in different grades of glioma, as well as their association with overall survival time and the expression of p53 target genes [BAK1, BAX, cyclin-dependent kinase inhibitor 1 (CDKN1A) and growth arrest and DNA damage-inducible protein GADD45 α (GADD45A)] were analyzed using the Gene Expression Profiling Interactive Analysis (GEPIA) platform (http://gepia.cancer-pku.cn/detail.php) (15).

Matrigel invasion assay. Invasion assays were performed using Transwell plates (diameter, 6.5 mm; aperture, 8.0 μm; Corning, Inc.) according to the manufacturer's protocols. Briefly, the filters of the upper chamber were precoated with Matrigel (Becton, Dickinson and Company) at 37˚C for 30 min, and $1x10^4$ glioma cells resuspended in 200 μl serum-free medium were added into the chambers. A total of 650 μl culture medium was added to the lower compartments with 10% FBS as a chemoattractant. Following culture at 37˚C for 24-48 h, the cells in the upper chambers were removed, and cells that had migrated to the lower membrane were fixed with 4% paraformaldehyde (Beijing Solarbio Science & Technology Co., Ltd.) for 20 min and stained with 0.5% crystal violet solution for 30 min at room temperature. The images were scanned by Invitrogen EVOS FL AUTO (Thermo Fisher Scientific, Inc.).

Cell proliferation and colony formation assays. A Cell Counting Kit-8 (CCK-8; cat. no. HY-K0301; MedChem Express) assay was used to assess cell viability according to the manufacturer's protocols. Briefly, the indicated cells were seeded into a 96-well plate at a density of 70-80% cells per well. The culture medium was replaced by serum-free medium and 10 μl CCK-8 solution was added at the indicated times. After incubation for 1 h, the OD values were determined at 450 nm. For the colony formation assay, $1x10^3$ cells per well were seeded into 6-well plates (Corning, Inc.). After 14 days of culture, the colonies (>10 cells) were fixed with 4% paraformaldehyde for 20 min and stained with 0.5% crystal violet solution for 30 min at room temperature. The images were scanned by Scan Wizard EZ (Microtek) and the number of clones was counted using Image J software (National Institutes of Health).

RNA extraction and RT-qPCR. Briefly, total RNA was extracted from tissues and the indicated cells using TRIzol® reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent kit (cat. no. RR047A; Takara Bio, Inc.), and qPCR analyses were conducted according to the manufacturer's protocol using a TB Green® Premix Ex Taq™ II (cat. no. RR820A; Takara Bio, Inc.) with GAPDH and U6 as the internal controls. The relative expression levels were calculated via the $2^{-\Delta\Delta cq}$ method (16). The sequences of the qPCR primers were as follows: LINC01116 forward, 5'-GTTCAAGTGCGT CCGGGTTT-3' and reverse, 5'-CGGACTTCTTTTCCAGGC GG-3'; miR-744-5p forward, 5'-AATGCGGGGCTAGGG CTA-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'; BAK1 forward, 5'-GTCAGAAAGTAGTGTCGCCA-3' and reverse, 5'-ACTTGTAGCGTCAGGACAGC-3'; GADD45A forward, 5'-CTGGGAATTTGGCGACGTAA-3' and reverse, 5'-ATG GATGTAGTCTGGGTGCAG-3'; TP53 forward, 5'-CCAAAT ACTCCACACGCAAAT-3' and reverse, 5'-CCTTCCCAG AAAACCTACCAG-3'; MDM2 forward, 5'-GGCTCTGTG TGTAATAAGGGAGA3' and reverse, 5'-GGACTGCCAGGA CTAGACTTTG-3', GAPDH forward, 5'-AGGAGCGAGATC CCGCCAACA-3' and reverse, 5'-CGGCCGTCACGCCAC ATCTT-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Vector construction and transduction. Small hairpin RNA (shRNA) against human LINC01116 (01116 KD; 5'-CCAAAG GCCCTGAAGTACACAGTTT-3') and corresponding negative control (NC) sequences were purchased from Shanghai GeneChem Co., Ltd., and the sequence was inserted into

a G248 lentiviral vector (Shanghai GeneChem Co., Ltd.). For LINC01116 overexpression (OE) experiments, the indicated cells ($1x10^4$) were infected with lentivirus containing LINC01116-GV358 plasmids (MOI, 10) at 37˚C for 12 h, which were synthesized by Shanghai GeneChem Co., Ltd. All transfections were conducted following the manufacturer's protocols. MDM2 small interfering (si)RNA (MDM2 KD) (cat. no. #AM16708) was acquired from Thermo Fisher Scientific, Inc., and the miR-744-5p mimics (5'-UGCGGG GCUAGGGCUAACAGCA-3') and inhibitor (5'-UGCUGU UAGCCCUAGCCCCCA-3') were designed and synthesized by Shanghai GenePharma Co., Ltd. All transfections were performed using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. The amount of mimics/inhibitor was 2 μg/$2x10^6$ cells.

Western blotting. Briefly, total protein was extracted from cells or tissues by incubation with RIPA lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was measured using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein sample (20 μg/lane) were separated via 10% SDS-PAGE, and subsequently transferred onto PVDF membranes (Roche Diagnostics). After blocking with skimmed milk (5%) for 1 h at room temperature, the membranes were incubated with the following primary antibodies overnight at 4˚C and secondary antibodies for 1 h at RT: Anti-p53 (1:1,000; cat. no. 2527), anti-MDM2 (1:1,000; cat. no. 86934), anti-BAK1 (1:1,000; cat. no. 12105), anti-GADD45A (1:1,000; cat. no. 4632), anti-β-actin (1:1,000; cat. no. 3700), anti-mouse IgG (1:5,000; cat. no. 7076) and anti-rabbit IgG (1:5,000; cat. no. 7074) from Cell Signaling Technology, Inc. After washing with TBS-Tween-20 (0.05%), bound HRP-conjugated antibodies were detected using Western Lightning Plus-ECL reagents (PerkinElmer, Inc.). The protein bands were measured by FluorChem Q (ProteinSimple).

Online cancer database analysis. StarBase (starbase.sysu.edu.cn/) (17) was used to identify the ceRNA network between LINC01116, microRNA-744-5p and MDM2.

Dual-luciferase reporter assay. Fragments of LINC01116 and the MDM2 mRNA 3' untranslated region (UTR) containing miR-744-5p binding sites, and mutated variants of these fragments, were amplified and subcloned into the pGL3 promoter vector (Promega Corporation). The mutated variants were generated using QuickMutation™ Site-Directed Mutagenesis kit (cat. no. D0206; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The indicated plasmids (1 μg) and miRNA mimics or inhibitors (1 μg) were transfected into 293T cells ($2x10^6$) using Lipofectamine 3000. After 48 h, luciferase activity was measured using a Dual-Luciferase Reporter Assay Kit (Promega Corporation) according to the manufacturer's protocols. *Renilla* luciferase was used as the control.

RNA immunoprecipitation (RIP) assay. RIP assays were conducted using the Magna RIP™ RNA-Binding Protein Immunoprecipitation kit (EMD Millipore), following the manufacturer's protocols. The indicated cells ($2x10^7$) were incubated with 500 μl RIP lysis buffer (Beyotime Institute of Biotechnology). Extracts were incubated with 5 μg antibodies against Ago2 (cat. no. 2897; Cell Signaling Technology, Inc.) at 4˚C overnight, followed by incubating with 30 μl Magnetic beads (MedChemExpress; cat. no. HY-K0205) for 6-8 h at 4˚C. Argonaute proteins are involved in the various steps of miRNA-mediated gene silencing by facilitating the formation of micro-ribonucleoproteins complexes with miRNAs (18,19). Rabbit IgG (cat. no. ab172730; Abcam) was used as a negative control. Completes were washed with washing buffer and incubated with proteinase K at 55˚C for 30 min to isolate the RNA- protein complexes from beads. RNA was isolated using TRIzol and then reverse-transcribed into cDNA by PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.) The levels of precipitated RNA were then determined by RT-qPCR as aforementioned.

Tumor xenografts in nude mice. A total of 30 six-week-old female BALB/c nude mice (weight, 14-20 g) were purchased from The Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All mice were kept in a temperature-controlled pathogen-free environment (21˚C) on a 12-h light–dark cycle in accordance with the Guide for the Care and Use of Laboratory Animals. A total of 10 nude mice were randomly divided into two groups for tumor xenografts. The indicated Shg44 cells ($5x10^6$/100 μl in primary DMEM) were subcutaneously injected into the flank of each nude mouse, and tumor volume was recorded every 6 days. The tumor volumes were calculated using the following formula: Tumor volume (mm^3) = (length x $width^2$)/2. After 3 weeks, the mice were sacrificed by cervical dislocation under anesthesia (1% pentobarbital sodium was used for anesthesia at a dose of 50 mg/kg, intraperitoneal injection), then, the tumor samples were collected for further analysis. All animal care and handling procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (20) and were approved by the Ethics Committee of Hangzhou First People's Hospital.

TUNEL staining. TUNEL staining was used to detect apoptosis in the resected tissue samples. Frozen tumor tissue sections (3-5 μm; stored at -20˚C) from the indicated nude mice were processed for TUNEL staining (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Briefly, following fixation by 4% paraformaldehyde for 20-30 min at room temperature, the frozen sections were washed three times with PBS at room temperature (5 min each time). Then, sections were immersed in PBS containing 1% Triton X-100 for 20 min at room temperature. Each sample was incubated with 50 μl TdT enzyme reaction solution (45 μl equilibration buffer, 1 μl biotin-11-dUTP and 4 μl TdT enzyme) for 60 min at RT. Following PBS washing, 50 μl streptavidin-TRITC labeling buffer was added and incubated for 30 min at room temperature. Then, sections were covered with DAPI (1:1,000) for 10 min at RT. Finally, the sections were placed under a cover slip and scanned by Invitrogen EVOS FL AUTO (Thermo Fisher Scientific, Inc.).

Statistical analysis. The results are presented as the mean ± SD and all experiments were repeated at least three times. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc.). Differences between two groups were analyzed

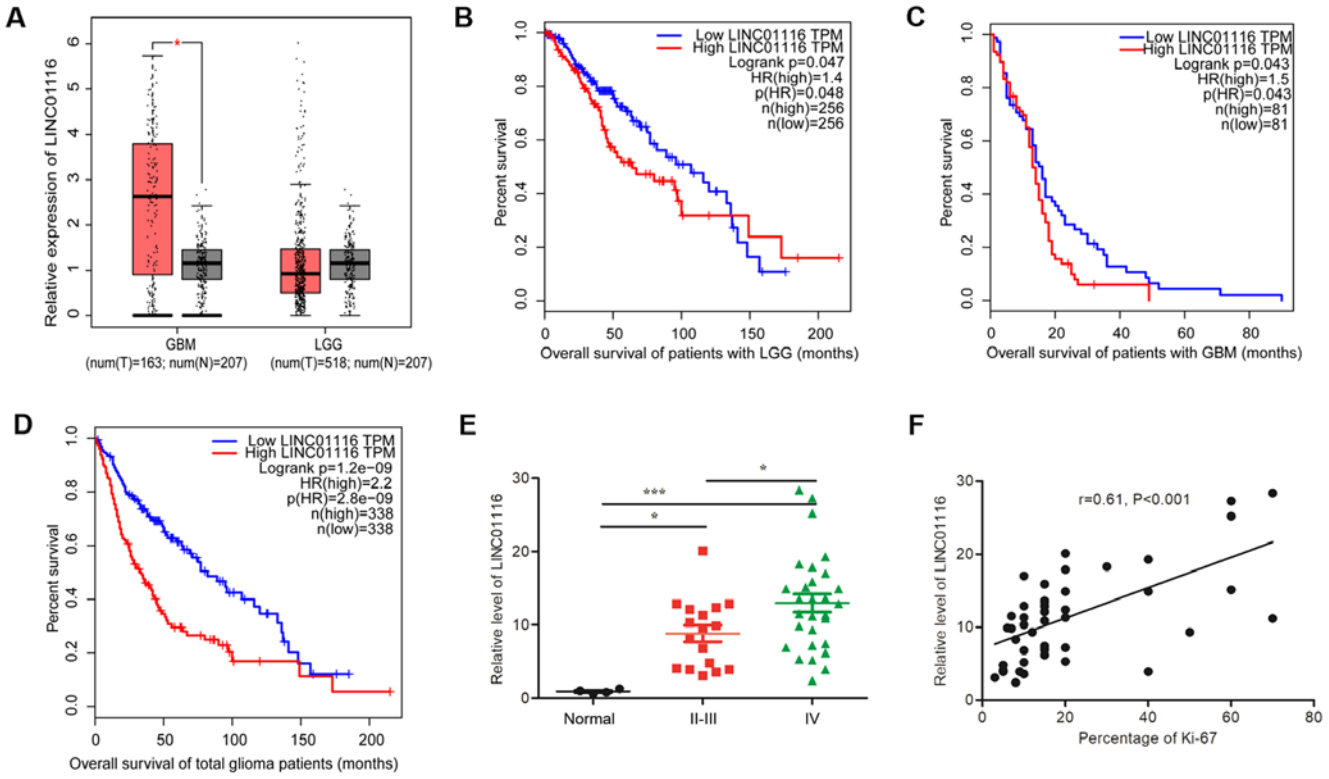

Figure 1. LINC01116 expression is upregulated in glioma and predicts poor prognosis. (A) Relative expression of LINC01116 in GBM and LGG was analyzed using the GEPIA platform. Compared with normal brain tissues, GBM tissues exhibited higher expression levels of LINC01116. (B-D) Overall survival analysis based on LINC01116 expression levels in different grade glioma tissues was also analyzed on the GEPIA platform. Regardless of glioma grade (i.e. total glioma patients), the survival times of patients with high LINC01116 were significantly shorter than those with low LINC01116 expression. (E) Relative expression of LINC01116 in 46 fresh glioma tissues. Both II-III grade gliomas (n=17) and GBMs (n=29) exhibited significantly higher LINC01116 expression levels than normal brain tissues (n=4). (F) Expression of LINC01116 was positively correlated with the percentage of Ki-67-positive cells in glioma tissues (n=46). *P<0.05, ***P<0.001. LINC01116, long intergenic non-protein coding RNA 1116; GBM, glioblastoma; LGG, lower grade glioma; GEPIA, Gene Expression Profiling Interactive Analysis.

using a Student's t-test, and comparisons between two groups were analyzed by a oneway ANOVA, followed by Tukey's post hoc test. Correlations between two sets of data were analyzed using a Pearson's correlation analysis. A log-rank test was used for Kaplan-Meier curves. GraphPad Prism 5.0 software (GraphPad Software, Inc.) was used for data presentation. P<0.05 was considered to indicate a statistically significant difference.

Results

LINC01116 is highly expressed in glioma. To determine the significance of LINC01116 in glioma, its expression and prognostic value were assessed in different grades of glioma [glioblastoma (GBM) and low-grade glioma (LGG)] using the GEPIA website. As shown in Fig. 1A, LINC01116 expression was significantly higher in GBM than in normal brain tissues. Although a similar result was not observed in LGG, there were still a number of these tissues with high levels of LINC01116 expression. In survival analysis, high levels of LINC01116 were found to predict poor prognosis, regardless of the glioma grade (Fig. 1B-D). To confirm whether LINC01116 is associated with a malignant glioma phenotype, its expression was assessed in 46 fresh glioma and 4 normal brain tissue samples. With a deviation from the results of GEPIA analysis, higher expression levels of LINC01116 were observed in low and high grade gliomas than in normal brain tissues (Fig. 1E), and LINC01116 expression was positively correlated with tumor grade and Ki-67 percentage positivity (Fig. 1F).

LINC01116 promotes the proliferation and invasion of glioma. To determine its function in glioma, RT-qPCR was used to assess the expression levels of LINC01116 in three glioma cell lines (U87, A172 and Shg44), compared with normal human astrocyte cells (HEB) (Fig. 2A). According to the expression of LINC01116 in glioma cells, a stable LINC01116 KD cell line was successfully constructed using lentiviral shRNA delivery into Shg44 cells with relative high expression of LINC01116, and stable OE cells were generated by introducing expression plasmids into A172 cells with relative low expression of LINC01116 (Fig. 2B and C). Functional experiments were then performed. Colony formation and cell viability assays revealed that the proliferative capacity of A172 cells was promoted by LINC01116 OE, and attenuated by LINC01116 KD in Shg44 cells (Fig. 2D-F). The results of the Transwell assay revealed that LINC01116 OE increased the invasive ability of A172 cells, while LINC01116 KD decreased that of Shg44 cells (Fig. 2F). These results indicated that LINC01116 may act as an oncogene in glioma.

LINC01116 inhibits the p53 pathway by upregulating MDM2. To investigate the potential tumor promoting mechanisms

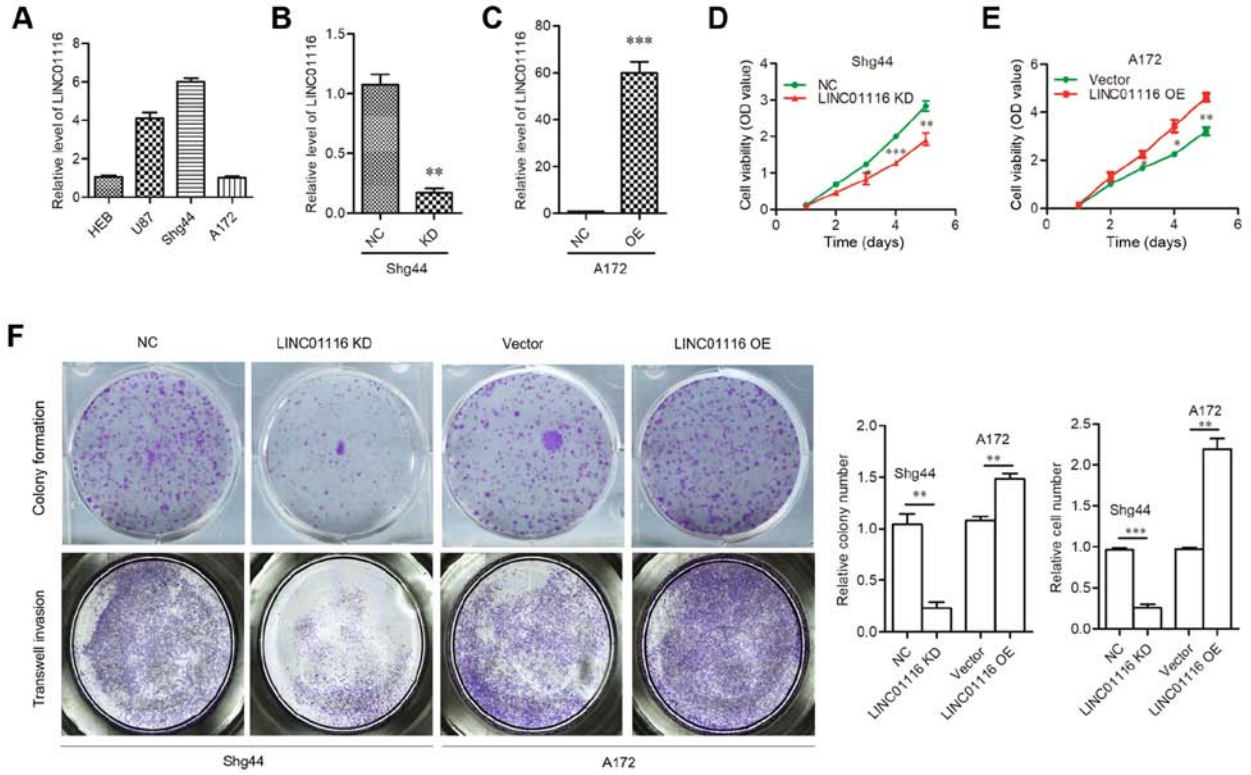

Figure 2. LINC01116 promotes the proliferation and invasiveness of glioma. (A) Relative expression of LINC01116 in HEB and glioma cell lines (U87, A172 and Shg44). Stable (B) LINC01116 KD and (C) LINC01116 OE cell lines were constructed using Shg44 and A172 cells, respectively. Relative LINC01116 expression in stable KD or OE cells was assessed by reverse transcription-quantitative PCR. Cell Counting Kit-8 assays showed that (D) LINC01116 KD decreased Shg44 cell proliferation, whereas (E) LINC01116 OE promoted the proliferation of A172 cells. (F) Effects of LINC01116 KD and OE on the colony formation and invasive abilities of Shg44 and A172 cells respectively. Histograms indicate the relative numbers of colonies and cells. *P<0.05, **P<0.01 and ***P<0.001 vs. NC. LINC01116, long intergenic non-protein coding RNA 1116; KD, knockdown; OE, overexpression; NC, negative control.

of LINC01116, its association with the expression of proliferation- and invasion-related genes was investigated using the GEPIA platform. As shown in Fig. 3A, the mRNA expression levels of BAX, BAK1, CDKN1A and GADD45A were negatively associated with LINC01116 expression in glioma (Fig. 3A). Notably, these genes are all associated with the p53 pathway (21,22). Thus, it was hypothesized that LINC01116 may promote the development of glioma by inhibiting the p53 pathway. To confirm these findings, the two p53-targeted-genes (*BAK1* and *GADD45A*) with the most significant association with LINC01116 were chosen for further assays, combined with *MDM2* and *TP53*. Then, the mRNA expression levels of *BAK1*, *GADD45A*, *MDM2* and *TP53* in LINC01116 OE cells (A172) and LINC01116 KD cells (Shg44) were determined. As shown in Fig. 3B and C, LINC01116 negatively regulated *BAK1* and *GADD45A*, and positively regulated *MDM2* mRNA expression in glioma cells, but had no effect on *TP53* mRNA levels. The protein expression levels of these genes were also determined, and those of BAK1, GADD45A and MDM2 were in accordance with their mRNA expression. However, p53 expression was decreased in LINC01116 OE cells and increased in LINC01116 KD cells (Fig. 3D and E). It was therefore speculated that LINC01116 may inhibit the p53 pathway by upregulating MDM2 in glioma. To verify this hypothesis, p53 protein expression was determined in LINC0111 OE cells following MDM2 KD. As shown in Fig. 3F and G, LINC01116 failed to repress the expression of p53, BAK1 and GADD45A protein in the absence of MDM2. These results indicated that LINC01116 inhibited the p53 pathway in glioma by upregulating MDM2.

LINC01116 upregulates MDM2 by sponging miR-744-5p. One of the mechanisms by which lncRNAs exert their biological functions is by competitively sponging miRNAs, acting as ceRNAs. To investigate whether a ceRNA mechanism exists between LINC01116 and MDM2, the ENCORI platform (17) was used to identify miRNAs that potentially bound both LINC01116 and the 3'UTR of MDM2 mRNA. The miRNAs that could interact with LINC01116 and 3'UTR of MDM2 mRNA simultaneously were analyzed, which led to the identification of miR -744-5p. To determine the association between LINC01116, miR-744-5p and MDM2, miR-744-5p expression was evaluated in A172 LINC01116 OE cells and Shg44 LINC01116 KD cells. LINC01116 was found to negatively regulate the expression of miR-744-5p in A172 and Shg44 cells (Fig. 4A). Next, Shg44 cells were transfected with miR-744-5p mimics and A172 cells were treated with a miR-744-5p inhibitor, RT-qPCR results showed that the relative miR-744-5p expression levels were significantly increased in Shg44 cells treated with miR-744-5p mimics and significantly reduced in A172 cells treated with miR-744-5p inhibitor (Fig. S1A and B). Then, the relative expression of LINC01116 and MDM2 mRNA was determined. As shown in Fig. 4B, miR-744-5p mimics significantly inhibited the expression of LINC01116 and MDM2 mRNA in Shg44 cells, whereas the miR-744-5p inhibitor promoted the expression of LINC01116

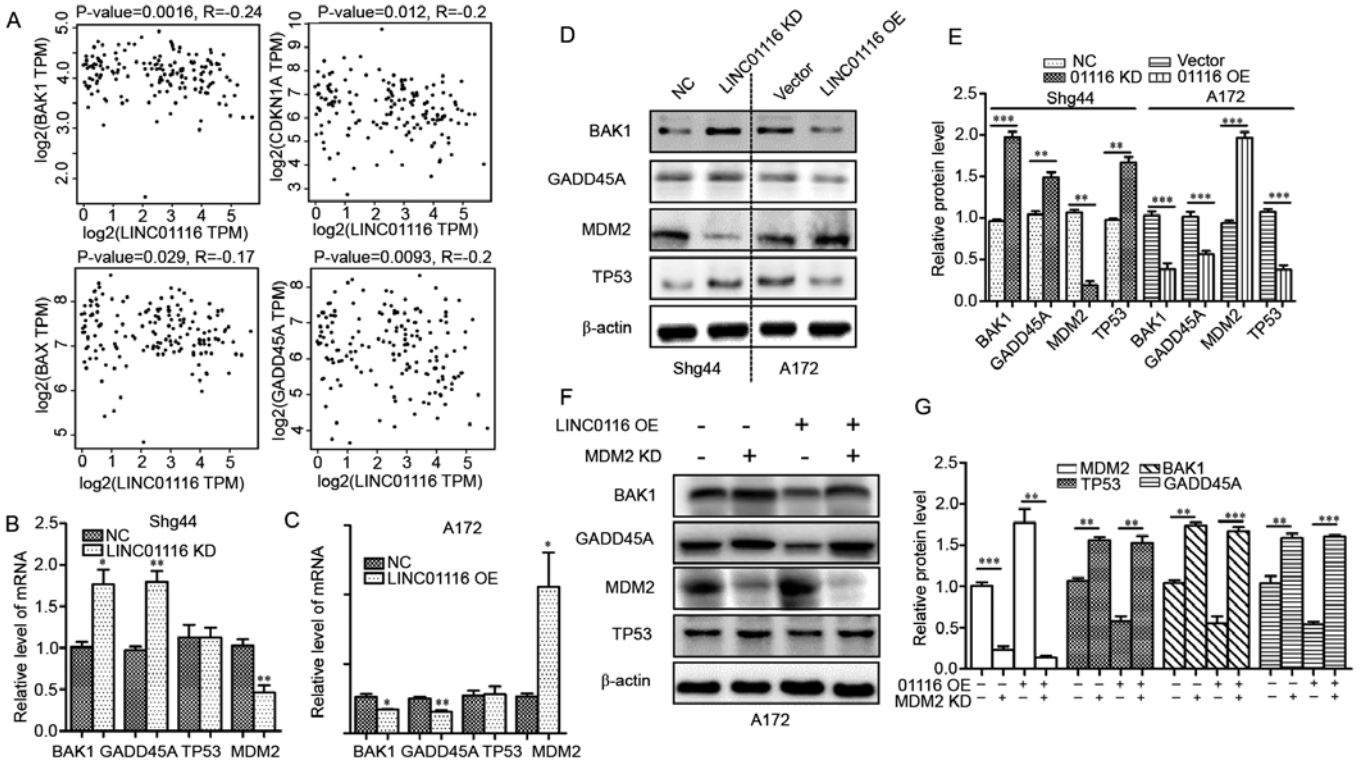

Figure 3. LINC01116 mediates the MDM2-p53 pathway in glioma. (A) Gene Expression Profiling Interactive Analysis revealed that LINC01116 levels negatively correlate with the mRNA levels of p53 target genes *BAK1*, *BAX*, *CDKN1A* and *GADD45A* in GBM tissues (n=81). (B and C) LINC01116 regulated the mRNA expression of *MDM2*, *BAK1* and G*ADD45A*, but not *TP53* in glioma cell lines. (D) Protein expression of MDM2, p53, BAK1 and GADD45A were regulated by LINC01116 in glioma cells. (E) Histogram indicating the relative protein levels in (D). (F) LINC01116 regulated the p53 pathway in A172 cells, which was dependent on the presence of MDM2. (G) Histogram indicating the relative protein levels in (F). *P<0.05, **P<0.01 and ***P<0.001 vs NC. LINC01116, long intergenic non-protein coding RNA 01116; MDM2, E3 ubiquitin-protein ligase Mdm2; KD, knockdown; OE, overexpression; NC, negative control; CDKN1A, cyclin-dependent kinase inhibitor 1; GADD45A, growth arrest and DNA damage-inducible protein GADD45 α.

and MDM2 mRNA in A172 cells. Furthermore, miR-744-5p mimics decreased the expression of MDM2 mRNA and protein promoted by LINC01116 OE in A172 cells, yet the miR-744-5p inhibitor increased the expression of MDM2 mRNA and protein downregulated by LINC01116 KD in the Shg44 cell line (Fig. 4C and D). A dual-luciferase reporter assay was then performed to determine whether miR-744-5p directly binds LINC01116 and the 3'UTR of MDM2. As shown in Fig. 4E, wild-type reporter plasmids were constructed containing the LINC01116 sequence and the 3'UTR of MDM2, as well as the corresponding mutant-type plasmids. The assay results showed that miR-744-5p mimics reduced the luciferase activity of the wild-type, but not the mutant-type plasmids (Fig. 4F and G). In addition, LINC01116 OE reversed the attenuated luciferase activity of the MDM2 wild-type plasmid caused by the miR-744-5p mimics (Fig. 4F). However, miR-744-5p inhibitor did not significantly change the luciferase activity of LINC01116 wild or mutant-type plasmids (Fig. 4G). Finally, a RIP assay was performed to verify whether LINC01116 and miR-744-5p could bind to the same Ago protein. As shown in Fig. 4H, both LINC01116 and miR-744-5p were found to bind Ago2. These results indicated that LINC01116 upregulates MDM2 by sponging miR-744-5p.

miR-744-5p partially reverses the tumor-promoting ability of LINC01116 in glioma. After confirming the existence of a ceRNA mechanism between LINC01116 and miR-744-5p, the expression of miR-744-5p and its correlation with LINC01116 and Ki-67 in glioma were investigated. As shown in Fig. 5A, the expression of miR-744-5p was downregulated in glioma compared with normal brain tissues. Correlation analysis between miR-744-5p and Ki-67 showed that glioma tissues with low miR-744-5p expression displayed higher proliferative ability, although the correlation was weak (r=-0.347) (Fig. 5B). Next, the expression of miR-744-5p was detected in glioma cell lines (Fig. 5C); as a result, Shg44 cells were transfected with miR-744-5p mimics, and A172 cells were treated with an miR-744-5p inhibitor accordingly. As shown in Fig. 5D and E, cell proliferation was inhibited by miR-744-5p mimics and promoted by the inhibitor. Rescue experiments were performed to determine whether miR-744-5p could reverse the tumor-promoting role of LINC01116 in glioma. Firstly, the correlation between LINC01116 and miR-744-5p was analyzed, and the relative expression of miR-744-5p was found to be negatively correlated with that of LINC01116 (Fig. 5F). Rescue experiments revealed that miR-744-5p mimics could reverse the promotion of LINC01116 OE on the proliferative and invasive abilities of A172 cells (Fig. 5G and H). Similar results were obtained in Shg44 cells. Furthermore, findings also suggested that the LINC01116 KD-induced inhibition of proliferation and invasion was reversed by the miR-744-5p inhibitor (Fig. 5I and J).

LINC01116 promotes glioma development in vivo. After partially ascertaining the tumor-promoting mechanisms of

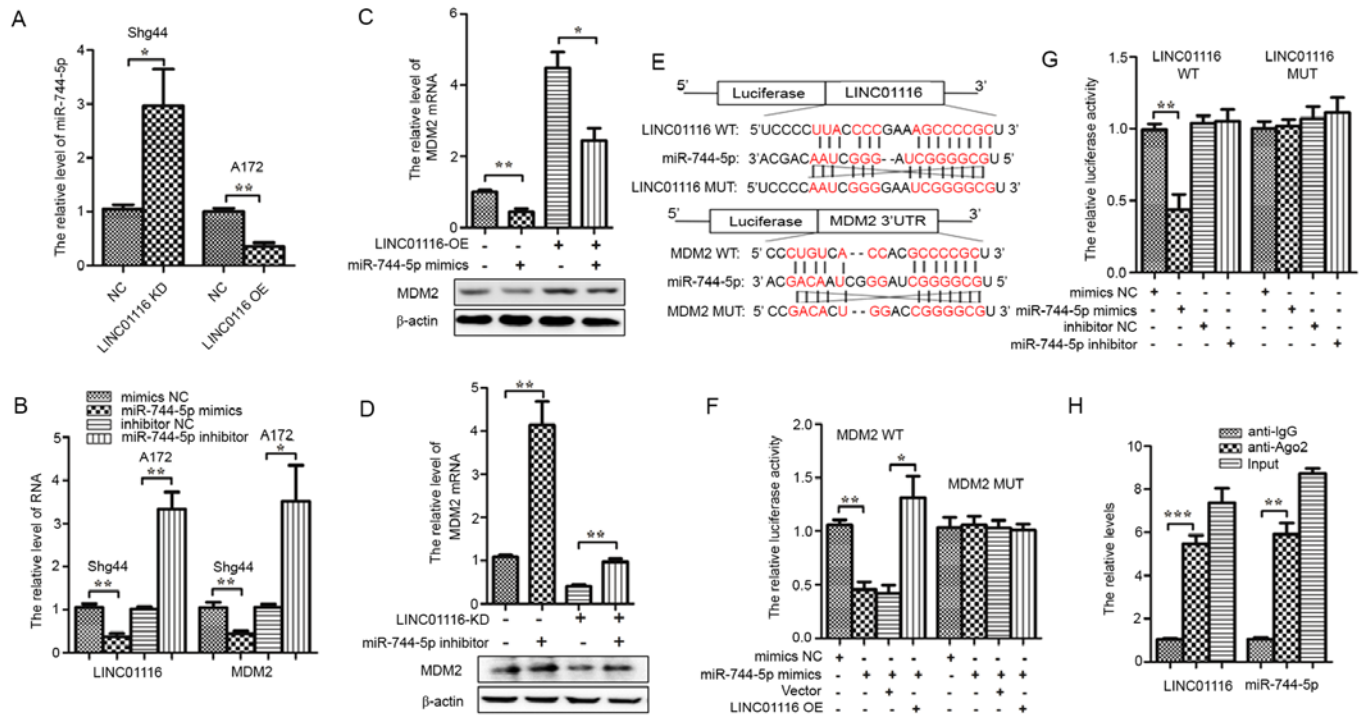

Figure 4. LINC01116 increases *MDM2* mRNA levels by sponging miR-744-5p in glioma. (A) LINC01116 negatively regulated the expression of miR-744-5p in Shg44 and A172 cells. (B) mRNA expression of LINC01116 and *MDM2* were downregulated by miR-744-5p mimics in Shg44 cells, and upregulated by the miR-744-5p inhibitor in A172 cells. (C) LINC01116 OE partially rescued the expression of *MDM2* mRNA and protein downregulated by miR-744-5p mimics in A172 cells. (D) miR-744-5p inhibitors partially rescued *MDM2* mRNA and protein expression downregulated by LINC01116 KD in Shg44 cells. (E) Predicted binding sites between miR-744-5p and LINC01116 or the *MDM2* mRNA 3'-UTR in WT and MUT sequences. (F) miR-744-5p significantly reduced the luciferase activity of WT LINC01116 in 293T cells, but not the MUT sequence, and the reduction in luciferase activity was rescued by LINC01116 OE. (G) Luciferase activity of the WT *MDM2* 3'-UTR, but not the MUT, was significantly reduced by miR-744-5p mimics in 293T cells. However, miR-744-5p inhibitor did not significantly change the luciferase activity of LINC01116 WT or MUTplasmids. (H) RNA immunoprecipitation assays showed that both LINC01116 and miR-744-5p could bind Ago2. *P<0.05, **P<0.01 and ***P<0.001. LINC01116, long intergenic non-protein coding RNA 01116; MDM2, E3 ubiquitin-protein ligase Mdm2; miR, microRNA; KD, knockdown; OE, overexpression; NC, negative control; UTR, untranslated region; WT, wild-type; MUT, mutant.

LINC01116 in glioma, its potential as a therapeutic target was investigated. A tumor xenograft model was established by subcutaneously injecting Shg44 LINC01116 KD cells into nude mice. As shown in Fig. 6A, LINC01116 KD impaired the growth of Shg44 cell tumors. To confirm whether LINC01116 exerted a similar effect on the expression of p53 pathway proteins, the mRNA and protein expression levels of these genes were evaluated in the tumors of nude mice. As predicted, the results were in accordance with those of the *in vitro* cellular experiments (Fig. 6B and C). In addition, TUNEL assay results revealed that LINC01116 KD increased the percentage of apoptotic cells in glioma tissues (Fig. 6D). Collectively, these findings suggested that targeting LINC01116 may be a novel approach for the treatment of glioma.

Discussion

The high mortality rate of glioma is primarily due to its infiltration and migration into a large area of adjacent brain tissue (23). Therefore, verifying the potential mechanisms involved in glioma development is necessary for future therapeutic advancements (2). To date, numerous studies have identified that lncRNAs possess pro- and/or antitumor functions in glioma (24-27), and identifying the regulatory effects of lncRNAs in glioma is a popular area of research.

Previous studies have demonstrated that LINC01116 is highly expressed in several types of cancer (28-30), and that it regulates cancer cell proliferation, migration and chemoresistance via a number of different mechanisms (28,29,31-37). In accordance with these studies, high LINC01116 expression was also observed in glioma in the present study, and was found to be positively associated with glioma grade and proliferative ability. Furthermore, LINC01116 KD inhibited the proliferative and invasive abilities of glioma cells, implying that LINC01116 is involved in the development and progression of glioma. Thus, the potential mechanisms through which LINC01116 is involved in gliomagenesis were investigated.

In a variety of cancer types, including glioma, the tumor suppressor p53 plays a crucial role in the development and progression of tumors, and its mutation or functional inactivation are found in the majority of human cancers (38). p53 acts as 'guardian of the genome' through surveillance and maintenance of genomic stability (39). As a key transcription factor, p53 triggers cell cycle arrest, senescence or apoptosis in response to DNA damage and various oncogenic stimuli (40). However, mutation of the TP53 gene or instability of the p53 protein contributes to tumor progression by producing dysfunctional p53 variants or accelerating p53 degradation (41). Therefore, maintaining the stability and function of p53 is important for cellular homeostasis. In the present study, a

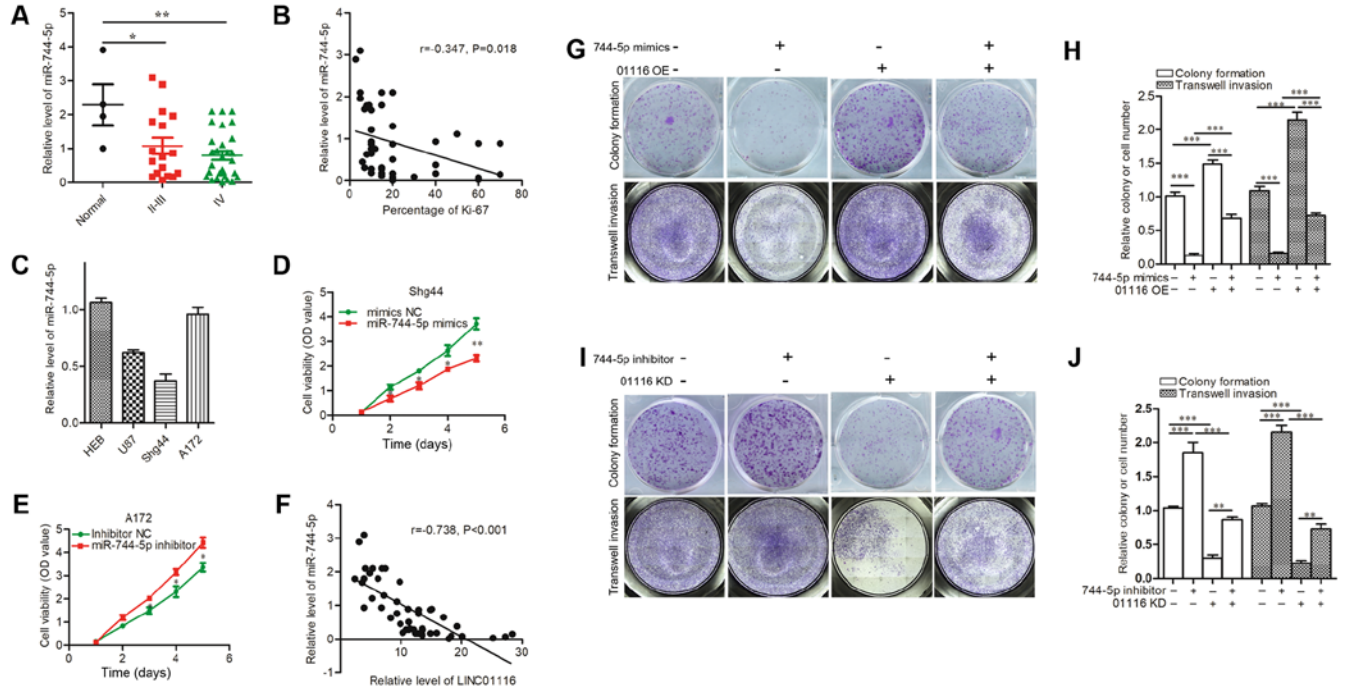

Figure 5. miR-744-5p inhibits the proliferation and invasiveness of glioma and reverses the biological functions of LINC01116. (A) miR-744-5p was significantly downregulated at different glioma grades. (B) miR-744-5p expression was negatively correlated with the percentage of Ki-67 in glioma tissues. (C) Relative levels of miR-744-5p in different glioma cell lines. Cellular proliferation was (D) reduced by miR-744-5p mimics in Shg44 cells, and (E) upregulated by the miR-744-5p inhibitor in A172 cells. (F) miR-744-5p expression was negatively correlated with LINC01116 expression in glioma tissues. (G) Colony formation and invasive ability of Shg44 cells were inhibited by miR-744-5p mimics, which was reversed by OE of LINC01116. (H) Histograms indicate the relative number of colonies and cells. (I) miR-744-5p inhibitor promoted the colony formation and invasive ability of A172 cells and partially restored these behaviors inhibited by LINC01116 KD. (J) Histograms indicate the relative number of colonies and cells. *P<0.05, **P<0.01 and ***P<0.001 vs NC. miR, microRNA; LINC01116, long intergenic non-protein coding RNA 1116; KD, knockdown; OE, overexpression; NC, negative control.

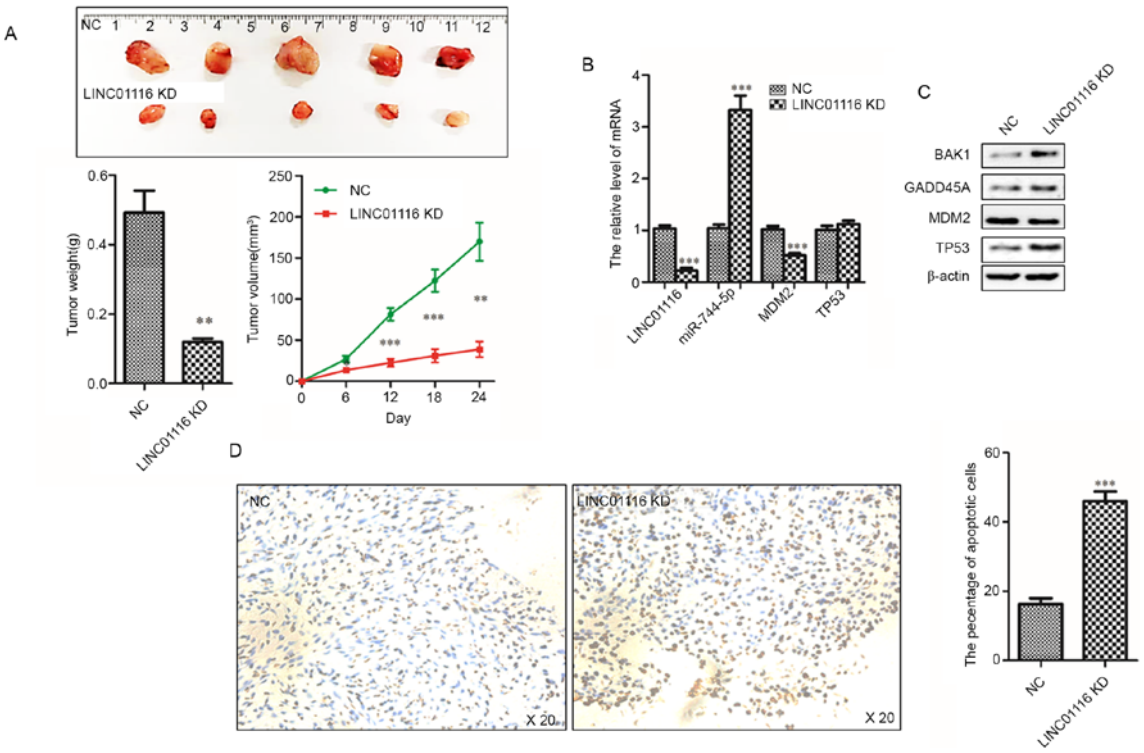

Figure 6. LINC01116 promotes gliomagenesis *in vivo*. (A) LINC01116 KD inhibited the growth of Shg44 cell tumors. Histogram indicating the mean weight of the xenograft tumors (n=5). Line chart of tumor growth curves (n=5). (B) mRNA expression levels of LINC01116, miR-744-5p, *MDM2* and *TP53* in the indicated xenograft tumors. (C) Protein expression of BAK1, GADD45A, MDM2 and p53 in xenograft tumors. (D) TUNEL assays were performed to detect glioma cell apoptosis in the xenograft tumors. Histogram indicating the percentage of apoptotic cells in the xenograft tumors (n=5). Magnification, x20. **P<0.01 and ***P<0.001 vs NC. LINC01116, long intergenic non-protein coding RNA 01116; miR, microRNA; KD, knockdown; NC, negative control; GADD45A, growth arrest and DNA damage-inducible protein GADD45 α; MDM2, E3 ubiquitin-protein ligase Mdm2.

negative association between the expression of LINC01116 and p53 target genes was identified, suggesting that LINC01116 may promote glioma proliferation and invasion by inhibiting the p53 pathway. LINC01116 was also confirmed to inhibit the expression of BAK1 and GADD45A in three glioma cell lines. Notably, LINC01116 regulated the expression of p53 protein, but not p53 mRNA, which impelled the present study to focus on MDM2, an upstream regulator of the p53 pathway. As predicted, LINC01116 positively regulated the protein and mRNA expression of MDM2.

MDM2 functions as an E3 ubiquitin-ligase. It is highly expressed in various malignant tumors and is considered to be a proto-oncogene (42). MDM2 binds the transactivation domain of p53 and induces its degradation through the proteasomal system (43). Additionally, a number of proteins can repress the transcriptional activation of p53 by cooperating with MDM2 (44). In the present study, LINC01116 failed to regulate p53 without MDM2, which suggested that the regulatory role of LINC01116 in the p53 pathway was dependent on MDM2.

Various lncRNAs exert their biological functions via the ceRNA mechanism (45). To verify whether a ceRNA mechanism exists between LINC01116 and MDM2, miRNAs that potentially bind both LINC01116 and the 3'UTR of MDM2 were predicted using the StarBase platform. miR-744-5p was found to be a potential bridge between the regulation of LINC01116 and MDM2. Through further investigation, LINC01116 was found to regulate the mRNA expression of MDM2 by sponging miR-744-5p and promoting its degradation. miRNAs are small endogenous RNAs that control cellular and physiological processes by post-transcriptionally regulating gene expression (46). As miRNAs are crucial regulators of various tumorigenesis-associated genes, research into the functions of miRNA in tumors is expanding (47). Numerous miRNAs have been identified as promising candidate biomarkers for different types of cancer (48). In the present study, miRNA-744-5p was found to be downregulated in glioma tissue samples, and its expression was negatively associated with that of LINC01116. miR-744-5p also inhibited the proliferative and invasive abilities of glioma. Moreover, the pro-oncogenic functions of LINC01116 were attenuated by miR-744-5p OE. Those findings confirmed the presence of a ceRNA mechanism between LINC01116, miR-744-5p and MDM2. Given that targeting non-coding RNAs may be a potential approach to tumor treatment, LINC01116 KD was revealed to inhibit the growth of tumors in a nude mouse xenograft model. This finding suggested that LINC01116 may be a potential target for glioma treatment. Although miR-744-5p was found to link LINC01116 and the MDM2-p53 pathway, there were several limitations of the present study that need further study: i) The nature of the relationship between LINC01116 and p53 target genes (direct or undirect) was not determined; ii) only several p53-targeted genes were tested, and more genes related to the p53 pathway need to be explored in the future; iii) during the *in vivo* study, it was not tested whether miR-744-5p mimics could restore LINC01116-reduced tumor size; and iv) A172 cells with relatively low expression of LINC01116 were chosen for overexpression and Shg44 cells with relatively high expression were chosen for KD, but it would have been more suitable to perform LINC01116 overexpression and KD in the same cell line.

In conclusion, these preliminary data demonstrated that LINC01116 was highly expressed in glioma, which was associated with a malignant phenotype. LINC01116 promoted the proliferation and invasiveness of glioma by sponging miR-744-5p, thus preserving MDM2 expression, which inhibited the antitumor functions of the p53 pathway. Therefore, targeting LINC01116 may be a potential future therapeutic approach for patients with glioma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LJ, CC, WJ, HW, QD and XD performed the experiments. JS and WY designed the study and prepared the manuscript. JS and WY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study received approval from the Ethics Committee of Hangzhou First People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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