INTRODUCTION

Glioma is the most common type of primary intracranial tumors and is highly lethal due to its pathogenetic location, high invasiveness, and poor prognosis (Gu et al., 2015). According to WHO classification, glioma can be divided into I, II, III, and IV grade levels, and malignancy degree increases with grade. Glioblastoma, classified as Class IV according to its biological
behavior, represents the highest degree of malignancy, and the average one-year survival rate of its patients is only 46% (Allen, Huang, & Clarke, 2014). Currently, the most commonly applied method to treat gliomas is surgery, with adjuvant therapies like radiotherapy, chemotherapy, medicine, etc. Nevertheless, overall treatment effect is still not ideal, especially for those diagnosed with high-grade gliomas. A large number of studies have shown that among patients with glioma, even after standard combination therapy, the five-year survival rate is still poor. Therefore, it is urgent to identify sensitive biomarkers to treat glioma.

Recent studies have indicated that long noncoding RNAs (lncRNA) have important biological functions via regulating several important processes (Rossi & Antonangeli, 2014). Previous studies have shown that many lncRNAs exhibit cellspecific expressions and subcellular localization, and that their alterations may lead to various human diseases, such as prostate cancer, colon cancer, breast cancer, bladder cancer, liver cancer, brain tumor, etc. (Chen, Yao, Wang, & Liu, 2017; Gloss & Dinger, 2016; J. Li, Xue, et al., 2017; Prensner et al., 2013; Tracy et al., 2018; Wu et al., 2017; Zhang, Su, Lu, & Wang, 2013). lncRNA-H19 (OMIM accession number: 103280; HGNC: 4713) is one of common lncRNAs, and has been observed in endoderm and mesoderm-derived tissues (Necsulea et al., 2014). It seats in conservative gene cluster, locating on human 11p15.5. (Monnier et al., 2013). lncRNA-H19 (OMIM accession number: 103280; HGNC: 4713) is one of common lncRNAs, and has been observed in endoderm and mesoderm-derived tissues (Necsulea et al., 2014). It seats in conservative gene cluster, locating on human 11p15.5. (Monnier et al., 2013). lncRNA-H19 (OMIM accession number: 103280; HGNC: 4713) is one of common lncRNAs, and has been observed in endoderm and mesoderm-derived tissues (Necsulea et al., 2014). It seats in conservative gene cluster, locating on human 11p15.5. (Monnier et al., 2013). lncRNA-H19 (OMIM accession number: 103280; HGNC: 4713) is one of common lncRNAs, and has been observed in endoderm and mesoderm-derived tissues (Necsulea et al., 2014). It seats in conservative gene cluster, locating on human 11p15.5. (Monnier et al., 2013). lncRNA-H19 (OMIM accession number: 103280; HGNC: 4713) is one of common lncRNAs, and has been observed in endoderm and mesoderm-derived tissues (Necsulea et al., 2014). It seats in conservative gene cluster, locating on human 11p15.5. (Monnier et al., 2013). lncRNA-H19 (OMIM accession number: 103280; HGNC: 4713) is one of common lncRNAs, and has been observed in endoderm and mesoderm-derived tissues (Necsulea et al., 2014). It seats in conservative gene cluster, locating on human 11p15.5. (Monnier et al., 2013). lncRNA-H19 (OMIM accession number: 103280; HGNC: 4713) is one of common lncRNAs, and has been observed in endoderm and mesoderm-derived tissues (Necsulea et al., 2014). It seats in conservative gene cluster, locating on human 11p15.5. (Monnier et al., 2013). lncRNA-H19 (OMIM accession number: 103280; HGNC: 4713) is one of common lncRNAs, and has been observed in endoderm and mesoderm-derived tissues (Necsulea et al., 2014). It seats in conservative gene cluster, locating on human 11p15.5. (Monnier et al., 2013). lncRNA-H19 (OMIM accession number: 103280; HGNC: 4713) is one of common lncRNAs, and has been observed in endoderm and mesoderm-derived tissues (Necsulea et al., 2014). It seats in conservative gene cluster, locating on human 11p15.5. (Monnier et al., 2013).

All experimental protocols were approved by Heping Hospital Affiliated to Changzhi Medical College. Glioma Cell U251 and U87-MG were purchased from Beijing Union Medical College Hospital. The cells were cultured in mixed medium DMEM containing 10% of fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C with 5% of CO2.

2.1.1 | Construction of recombinant lentivirus

Small interfering RNA (siRNA) sequence targeting lncRNA-H19 (NCBI reference sequence: NR_002196.2) was 5′-GACGTGA CAAGCAGGACAT-3′. A scramble fragment 5′-GCAGATAGGTAGGCGTTAT-3′ was used as negative control that had no significant homology to any human gene sequences. Stem-loop oligonucleotides (TTCAGAGAGA) were synthesized and cloned into lentivirus-based vector LV3, and resulting plasmids were named as LV3-si-H19 and LV3-NC, respectively. Lentivirus packaging system including recombinant LV3-si-H19 plasmid or LV3-NC together with two packaging plasmids (psPAX2 and pMD2.G) was co-transfected into cells using Lipofectamine 2000 (Invitrogen). Then, lentiviral particles were harvested from the media 48 hr after transfection, from centrifuged supernatant (4000 g, 10 minutes, 4°C), and lentiviral particles were purified with 0.45 mm cellulose acetate filters. The titer of concentrated lentivirus was determined via dilution, adopting fluorescent microscopy.

2.1.2 | Lentivirus infection

Cells were cultured in growth medium to 30% confluence at the time of transduction (2 x 10⁵ cell/ml cells in 6-well plates), and then, transfected with LV3-si-H19 and LV3-NC at multiplicity of infection (MOI) of 1. Cells were harvested 72 hr after transduction and analyzed with FACS to sort and collect green fluorescent protein (GFP)-positive ones, which could be distinguished easily from untransfected ones by green fluorescent under fluorescence microscope.

2.1.3 | Lentivirus infection

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2.1.4 | Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from cells with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Those total RNA samples with an OD A260/A280 ratio close to 2.0, which indicated that RNA was pure, were reversely transcribed into cDNA. Real-time PCR reactions using SYBR green (invitrogen) were run on Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems). Real-time PCR primers for lncRNA-H19

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and beta-actin were designed and synthesized by Dingguo Changsheng Biotech. Primer sequences were as follows: lncRNA-H19 forward 5′- CGGTCACTTTTGGTTA-3′ and reverse 5′-GGAGGGTGTCTGCTTC-3′, β-actin forward 5′-ATCATGTTTGAGACCTTCAACA-3′ and reverse 5′-CATCTCTTGCTC GAAGTCCA-3′.

2.1.5 Cell proliferation analysis

Cell growth curves were obtained by measuring cell viability with Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer’s instruction. After transduction for 72 hr, the cells in test groups were plated at a final concentration of 3,000 cells/well into 96-well culture plates for 5 days in a 37°C, 5% of CO2 incubator. Then, the number of viable cells was measured on the first, second, third, fourth, and fifth days. At each time point, CCK8 (5 mg/ml) was added into each well and incubated at 37°C for 2 hr. Absorbance was measured at 450 nm using Microplate reader. Each group was measured in triplicate.

2.1.6 Cell cycle analysis

The cells in test groups were seeded into 6-well culture plates (5 × 10^5) and harvested at 72 hr through centrifugation at 1200 rpm for 5 min. After washing twice with precooled DPBS, the cells were fixed in 70% of alcohol overnight at 4°C, and then, pelleted via centrifugation to remove stationary liquid. After that, the cells were washed twice using DPBS, and 100 μL of RNaseA (1 mg/ml) was added into each tube before additional incubation of 30 min at 37°C. At the end, propidium iodide (PI, Sigma) was added into each well, and incubated in dark at room temperature. Cell cycle distribution was analyzed using flow cytometer (FACSCalibur, BD).

2.1.7 Apoptosis assay

Seventy-two hours after transfection, apoptotic U87-MG and U251 cells were labeled with annexin V-phycoerythin (PE), mixed gently and incubated at 2–8°C for 15 min in dark. Then, 5 μl Annexin V-PE/7-AAD was added to each sample and incubated at 2–8°C for 5 min in dark. Then, fluorescence-activated apoptotic cells were categorized employing flow cytometer (BD FACSVantage™ SE, BD Biosciences Co., Franklin Lakes).

2.1.8 Wound healing and cell transwell migration assays

Wound healing assay: Cells (5 × 10^4) were seeded on a 6-well plate and cultured for 72 hr. When cultured cells covered the bottom of the plate, a scratch was made on cell monolayer with a 200 μl pipette tip. U251 was monitored with a microscope every 24 hr. U87-MG was monitored with a microscope every 6 hr. Cell migration was determined via the rate of cells filling the scratched area. Normalized wound area was calculated in the software PS.

Transwell assay: After incubated for 72 hr, 5 × 10^4 cells were plated in 6-well transwell chamber in 3 ml complete medium. After 48 hr, the cells were washed twice with 1× PBS, and then, membranes were removed gently with a razor blade and rinsed. The cells that migrated through the membrane and attached to the bottom of the membrane were fixed and stained with hematoxylin. After washed for 2 min with water, new water was used to soak the membrane for 10 min. Mounted with neutral gum, under microscopy, cells in six randomly selected visual fields were counted. Tests were repeated via three independent experiments.

2.1.9 Tumor sphere formation assay

Cells (3 × 10^2 cells / well) were seeded in ultralow adhesion 96-well, 3–6 wells each group. The cells were continuously incubated in incubator. To prevent cell adhesion, the 96-well was shaked twice a day, add medium was added to each well every 2–3 days. Cell balls were observed under light microscope. Cell spheres were photographed and those greater than 50 μm were counted.

2.1.10 Animal model

Animal breeding and procedures were conducted in line with the Animal Care and Use Committee guidelines of our hospital. A total of 30 male rats were used to conduct in vivo experiments. The rats were divided into three groups, with 10 in each group. 5 × 10^5 U251 cells (in 5 μL PBS) which were infected by LV3-si-H19 were inoculated into the right stratum (lateral: 2 mm; anterior to the bregma: 0.5 mm; depth: 3 mm) of the animals via a small animal stereotactic frame (RWD Life Science, Shenzhen, China). LV3-NC-infected U251 served as negative control, while U251 cells without infection were employed as untransfected controls.

2.1.11 Tumor growth evaluation

After injection, tumor growth in animal model was observed using bioluminescence imaging technique and recorded every 5 days. Based on tumor size, tumor volume was calculated according to the following formula: tumor volume = a × b^2 × 0.5, a: the lengthy diameter; b: the short diameter. Sixty-day growth curve for tumor was plotted to
estimate the effects of lncRNA-H19 expression on glioma growth.

Sixty days after tumor implantation, five rats in each group were sacrificed to isolate tumor specimens, and tumor inhibition rate was calculated. Tumor inhibition rate = [(the average tumor weight of control group - the mean weight of LV3-si-H19 group)/the average weight of control group] × 100%. Additionally, the expression level of lncRNA-H19 in tumor specimens was also estimated using qRT-PCR.

2.1.12 | Survival estimation based on rat model

The rest five rats in each group were used for survival analysis. Rats in transfected group and control group faced similar conditions, and the survival time of each group was recorded to estimate the effects of H19 expression on the survival of glioma.

2.1.13 | Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM). Differences between two groups were compared using student's t test, and those among three groups were analyzed with ANOVA. Statistical analysis was performed using SPSS version 13.0 (SPSS Inc.). Results were considered statistically significant when $p < 0.05$.

3 | RESULTS

3.1 | Lentivirus-mediated siRNA inhibited the expression of lncRNA-H19 in U251 and U87-MG cells

U251 and U87-MG cell lines were transfected with LV3-si-H19 and LV3-NC, with the highest transfection efficiency at 100%, which was determined via detecting the expression of green fluorescent protein (GFP) 72 hr after infection (Figure 1a). QRT-PCR analysis showed that lncRNA-H19 expression level was significantly lower in LV3-si-H19 group than in LV3-NC group and untransfected group (Figure 1b,c).

3.2 | lncRNA-H19 knockdown inhibited cell proliferation

The proliferation of U251 and U87-MG cells transfected by LV3-si-H19 was detected. LV3-NC group and untransfected group were used as controls, and experiment was repeated five times. OD value was recorded once a day for 5 days adopting microplate reader, and data were presented in Table 1. For U251 cells, OD values were roughly same to those for untransfected group, negative control group and LV3-si-H19 interference group on the first and second days. OD values were increased, following cultured for 3 d, 4 d, and 5 d, along with number increased of the cultured cells, OD value of each group was also increased, and LV3-si-H19 group's OD values were significantly lower than those for the untransfected group and the LV3-NC group at the same time. For U87-MG cells, OD values were roughly same to those for the three group on the first day, without significant difference. After cultured for 2 d, 3 d, 4 d, and 5 d, OD value of each group was increased, and LV3-si-H19 group's OD values were significantly lower than those for the untransfected group and the LV3-NC group. lncRNA-H19 silencing inhibited U251 and U87-MG cell proliferation in a time-dependent manner, as shown in Table 1 and Figure 2.

3.3 | lncRNA-H19 knockdown induced cell cycle arrest

Flow cytometric analysis showed that the proportion of cells in G2/M phase was markedly increased in LV3-si-H19 group compared with LV3-NC group and untransfected group, but the proportion of cells in G0/G1 and S phases was markedly decreased in LV3-si-H19 group compared with LV3-NC group and untransfected group. The result indicated that lncRNA-H19 knockdown could significantly inhibit cell cycle progression of U251 and U87-MG cells and arrest cell cycle in G2/M phase (Figure 3).

3.4 | lncRNA-H19 knockdown affected cell apoptosis

Apoptosis was assessed via flow cytometry, and the results indicated that knocking down H19 expression had no effect on apoptosis rate of glioma cells, compared with LV3-NC group and untransfected group (Figure 4).

3.5 | lncRNA-H19 knockdown decreased the migration of glioma cells

Wound healing assays and Transwell assays were used to explore the effect of lncRNA-H19 interference on the migration of glioma cells. The results of wound healing assay displayed that reduced lncRNA-H19 expression blocked cell migration of U251 (Figure 5a,b) and U87-MG (Figure 5c,d). For U251, lncRNA-H19 knockdown had no significant effect on cell migration.
migration at 24 hr, but at 48 hr, inhibiting lncRNA- H19 expression could significantly affect the migration of glioma cells compared to control groups. For U87-MG cells, lncRNA- H19 knockdown showed no significant effect on cell migration at 6 hr, compared with control groups. Significant inhibitory effect emerged in LV3-si-H19-transfected group compared with the untransfected group and the LV3-NC group at 12 hr hour. But after 24 hr, the three groups were all healed. We further investigated cell migration using transwell assay, and the result showed that lncRNA-H19 knockdown could inhibit U251 cell and U87-MG cell migration (Figure 5e, f), and that inhibition rate was up to 39.17% and 32.14%, respectively.
3.6 | Tumor sphere formation assay

Formed spheres were counted for all visions. Furthermore, U251 / U87-MG cells formed standard sphere (marked as N_C1), the remaining data (labeled N_Nx) compared with N_C1. The effect of IncRNA-H19 knockdown on sphere formation ability of U251 and U87-MG cells was analyzed using the following formula: inhibition rate = (N_C1-N_Nx) / N_C1 × 100%.

**FIGURE 2** Effects of IncRNA-H19 knockdown on proliferation U251 and U87-MG cells. Growth curves of U251 cell (a) and U87-MG cell (b) with three treatments (untransfected control, LV3-NC transfected group, and LV3-si-H19-transfected group) determined by CCK-8 assay. *p < 0.05, versus untransfected group; **p < 0.01, versus untransfected group; ***p < 0.01, versus negative control group.

**FIGURE 3** Effect of IncRNA-H19 knockdown on the cell cycle progression in the U251 and U87-MG cells. (a) The cells were analyzed by flow cytometry. (b) The statistic analysis of IncRNA-H19 knockdown effect on the cell cycle progression.
The amount of sphere formation was significantly reduced in LV3-si-H19-transfected group, compared with the LV3-NC group and untransfected group (Figure 6a), and inhibition rate of LV3-si-H19-transfected group was up to 57.5% (U251) and 47.5% (U87-MG), respectively (Figure 6b).

3.7 Tumor growth in vivo

In the current study, the rats were used to estimate the effects of H19 expression on glioma progression in vivo. Tumor growth in each group is displayed in Figure 7a. Compared to controls, tumor growth in LV-si H19 group was obviously slow. In control group, tumor was observed at 30 days after implantation, while tumor in LV-si H19 group could be obviously detected at 45 days after cell injection. Sixty days after injection, the average volume was $8.70 \pm 0.25 \text{ mm}^3$ in U251 injected group and $9.10 \pm 0.19 \text{ mm}^3$ in U251 LV-NC group, when tumor volume was significantly lower in LV-si H19 group ($2.25 \pm 0.12 \text{ mm}^3$, $p < 0.01$ for both). Tumor volume between untransfected control and negative control groups had no significant differences ($p > 0.05$; Figure 7b).

Sixty days after injection, the rats were killed to isolate tumor specimens and tumor weight was estimated. The average weight in LV-si H19 group was significantly smaller than that in control groups (LV-si H19 group: $0.44 \pm 0.12 \text{ g}$, U251...
FIGURE 5  Effect of IncRNA-H19 knockdown on glioma cell's migration ability. (a–d) Wound healing assay was used to evaluate the migration of both U251 and U87-MG after silencing IncRNA-H19, (×100). E–F. Transwell migration assay was used to evaluate the migration of both U251 and U87-MG after silencing IncRNA-H19. *$p < 0.01$, versus untransfected control group; **$p < 0.01$, versus negative control group.
group: 1.40 ± 0.09 g, LV-NC group: 1.46 ± 0.23 g, \( p < 0.01 \) for both). However, there was no difference between untransfected control and negative control groups (\( p = 0.687 \); Figure 7c).

We also investigated the expression profiles of \( H19 \) mRNA in each group. Compared to control groups, the expression levels of \( H19 \) mRNA in U251 LV-si H19-transfected group were obviously decreased (\( p < 0.01 \) for both). The untransfected control and negative control groups exerted no obvious differences in expression patterns of \( H19 \) mRNA (\( p = 0.230 \); Figure 7d).

In addition, the mean survival time of the rats was also compared between the study groups. The mean survival time was 110.40 ± 8.41 days in rats injected with U251 LV-si H19 cells, and 68.20 ± 9.28 days and 69.40 ± 11.30 days in untransfected control and negative control groups, respectively. The survival time of LV-si H19 group was significantly longer than that of the control groups (\( p < 0.01 \) for both). The untransfected control and negative groups showed similar survival time (\( p = 0.611 \); Figure 7e).

4 | DISCUSSION

LncRNAs are involved in X chromosome silencing, chromatin modification, transcriptional regulation, small RNA processing, and other important regulatory processes (Rossi & Antonangeli, 2014). It is confirmed that lncRNA plays an important regulatory role in a number of diseases especially tumors, and their dysregulated expression is observed in many human tumors (Gloss & Dinger, 2016). Moreover, previous studies have proven that lncRNA-H19 could act as a tumor promoter or suppressor in different human cancers (Hua et al., 2016; Shi et al., 2014; Zhu et al., 2014; Zhuang et al., 2014). Colnot et al. (Yang et al., 2017) reported that the probability of lncRNA-H19-deficient mice suffering intestinal polyps was significantly higher than wild type in human colorectal cancer transplant mouse model. Ariel et al. (Hua et al., 2016) confirmed that the upregulation of lncRNA-H19 was considered as an early sign of bladder cancer recurrence. Zhuang M et al. (Zhuang et al., 2014) found that lncRNA-H19 expression was significantly increased in gastric carcinoma and cell lines. Upregulated lncRNA-H19 could promote the proliferation of gastric cancer cells, but downregulated lncRNA-H19 might enhance the apoptosis of gastric cancer cells. Shi Y et al. (Shi et al., 2014) studied biological function of lncRNA-H19 expression in glioma and underlying mechanisms, and found that lncRNA-H19 expression was related to glioma’s grade. The expression of lncRNA-H19 and its derivatives miR-675 was higher in advanced grade glioma than low-grade ones.

In this study, we introduced si-H19 into glioma cells using lentiviral vector (Cambon et al., 2017; Fazio et al., 2017), and investigated biological function of lncRNA-H19 in glioma.

\[ \text{FIGURE 6} \quad \text{Tumor sphere formation result, (x100).} \quad * p < 0.01, \text{versus untransfected control group;} \quad ^{\#} p < 0.01, \text{versus negative control group.} \]
Proliferation is a fundamental characteristic of tumor cells, so we tested the effect of lncRNA-H19 on proliferation activity of glioma cells. After lncRNA-H19 interference vector introduced into U251 and U87-MG cells, cell proliferation was detected via CCK8 assay, and the results showed that interfering lncRNA-H19 gene inhibited the proliferation of U251 and U87-MG cells, which inversely proved that lncRNA-H19 gene might promote tumor cell proliferation.

To determine the effect of lncRNA-H19 on cell cycle distribution of U251 and U87-MG cells, we detected cell cycle using flow cytometry. Cell cycle distribution changed significantly after lncRNA-H19 gene knockdown. Specifically, cell cycle progression was blocked, and a large number of cells were arrested in G2/M stage. This phenomenon also indicated that lncRNA-H19 gene could promote the proliferation of U251 and U87-MG cells, which was in line with previous findings.

Sphere formation is one method for identifying self-renewal ability of tumor stem-like cells (Chao, Kan, Lu, & Chien, 2015). Iacopino et al., (2014) isolated tumor stem cells from three glioma cell lines L1, U87-MG, and U373 using ultralow-balling adhesion method. We also isolated tumor stem-like cells from U251 and U87-MG in our previous experiment. The present study suggested that lncRNA-H19 could regulate the self-renewal of glioma cells, and this was the first time to report the involvement of lncRNA-H19 in sphere-formation functions of U251 and U87-MG.

In addition, glioma animal models were constructed via injecting U251 cells in our study. Tumor volume and weight in U251 LV-si-H19 groups were significantly lower compared to control groups. Moreover, survival time of these rats were obviously prolonged. The data revealed that knocking down lncRNA-H19 expression could inhibit glioma growth and improve the survival of glioma.
The conclusion was in line with that from our in vitro experiments. lncRNA-H19 might be a potential therapeutic target in treating glioma. However, there were still several limitations in the present study. First, clinical value of IncRNA-H19 in glioma was not explored in our study. Due to the limited study period, the numbers of glioma cases in our study was not large enough to guarantee the significance of IncRNA-H19 in clinic. Second, potential mechanisms underlying carcinogenic action of IncRNA-H19 in glioma were not explored. Pull-down technique may be helpful to identify the target of IncRNA-H19 in glioma, and proteomic analysis may provide more exact information on the mechanisms of IncRNA H19 functioning in glioma. Further well-designed studies will be performed to address those mentioned issues.

In conclusion, IncRNA-H19 plays a promoting role in glioma progression via enhancing cell proliferation, cell cycle, cell migrations, and sphere-forming function of glioma cell lines. IncRNA-H19 may be a potential biomarker in the diagnosis and treatment of glioma in clinic.

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None.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION
P.L., X.H., and H.W. conceived and designed the experiments, analyzed the data, and wrote the paper. G.Y. and L.S. performed the experiments. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this article.

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