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Increased level of *H19* long noncoding RNA promotes invasion, angiogenesis, and stemness of glioblastoma cells

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OBJECTIVE Increased levels of *H19* long noncoding RNA (lncRNA) have been observed in many cancers, suggesting that overexpression of *H19* may be important in the development of carcinogenesis. However, the role of *H19* in human glioblastoma is still unclear. The object of this study was to examine the level of *H19* in glioblastoma samples and investigate the role of *H19* in glioblastoma carcinogenesis.

METHODS Glioblastoma and nontumor brain tissue specimens were obtained from tissue obtained during tumor resection in 30 patients with glioblastoma. The level of *H19* lncRNA was detected by real-time quantitative reverse transcription polymerase chain reaction. The role of *H19* in invasion, angiogenesis, and stemness of glioblastoma cells was then investigated using commercially produced cell lines (U87 and U373). The effects of *H19* overexpression on glioblastoma cell invasion and angiogenesis were detected by in vitro Matrigel invasion and endothelial tube formation assay. The effects of *H19* on glioblastoma cell stemness and tumorigenicity were investigated by neurosphere formation and an in vivo murine xenograft model.

RESULTS The authors found that *H19* is significantly overexpressed in glioblastoma tissues, and the level of expression was associated with patient survival. In the subsequent investigations, the authors found that overexpression of *H19* promotes glioblastoma cell invasion and angiogenesis in vitro. Interestingly, *H19* was also significantly overexpressed in $CD133^+$ glioblastoma cells, and overexpression of *H19* was associated with increased neurosphere formation of glioblastoma cells. Finally, stable overexpression of *H19* was associated with increased tumor growth in the murine xenograft model.

CONCLUSIONS The results of this study suggest that increased expression of *H19* lncRNA promotes invasion, angiogenesis, stemness, and tumorigenicity of glioblastoma cells. Taken together, these findings indicate that *H19* plays an important role in tumorigenicity and stemness of glioblastoma and thus could be a therapeutic target for treatment of glioblastoma in the future.

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KEY WORDS long noncoding RNA; *H19*; invasion; angiogenesis; stemness; glioblastoma; mouse; oncology

Malignant gliomas are the most common type of primary malignant brain tumor, and more than half of all gliomas are glioblastomas (Grade IV astrocytoma), one of the most aggressive and lethal types of brain tumor. Glioblastoma cells easily infiltrate into the normal cerebral cortex, ultimately resulting in the death of the patient. Well-defined risk factors for glioblastoma include radiation exposure and certain genetic syndromes.[21] Several molecular and genomic datasets have recently been generated that have allowed identification of at least 4 subtypes of glioblastoma: classical, mesenchymal, proneural, and neural.[14] Previous studies in glioblastoma ge-

ABBREVIATIONS FBS = fetal bovine serum; HCC = hepatocellular carcinoma; HUVEC = human umbilical vein endothelial cell; lncRNA = long noncoding RNA; ncRNA = noncoding RNA; RT-qPCR = real-time quantitative reverse transcription polymerase chain reaction; SD = standard deviation.

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nome characterization have identified somatic changes, including mutation of *PTEN, TP53, EGFR, PIK3CA, PIK3R1, NF1, RB1, IDH1,* and *PDGFRA*, but the functional consequences of most of these alterations are still unknown.[5,8,20,23] The median survival of glioblastoma patients is still only around 14 months, despite improvements in the standard of care, including resection, radiotherapy, and chemotherapy. This dismal clinical outcome makes glioblastoma an urgent subject of cancer research. The development of improved therapies rests on a greater understanding of the molecular mechanism of glioblastoma development.

With advances in microarray and sequencing technology, it has been well accepted that at least 90% of the human genome is actively transcribed into noncoding RNAs (ncRNAs), whereas less than 2% of the genome sequences encode proteins.[6,12] According to their size, the ncRNAs are divided into 2 groups: small ncRNAs (< 200 nt) and long ncRNAs (lncRNAs). It is increasingly recognized that lncRNAs constitute an important component in the regulation of gene expression and carcinogenesis.[9,24] Long noncoding RNAs are dysregulated in different kinds of cancer, demonstrating both oncogenic and tumor-suppressive roles, thus suggesting that their aberrant expression may contribute substantially to cancer development.[11] However, the biological functions of the vast majority of lncRNAs remain unknown.

H19, also known as *ASM*, *ASM1*, and *BWS*, is an imprinted maternally expressed gene (formal name "H19, imprinted maternally expressed transcript [non-protein coding]," www.genenames.org) that encodes an lncRNA and is associated with Beckwith-Wiedemann syndrome and Silver-Russell syndrome. Increased expression of *H19* has been observed in some cancers, such as gastric[25,30] and bladder cancer,[16] and decreased expression in others, such as hepatocellular carcinoma (HCC),[13] suggesting that *H19* may have either oncogenic or tumor suppressor properties, although the exact mechanism is still elusive.[11] A previous study showed that *c-Myc (Myc)* significantly induces the expression of *H19* in diverse cell types, including breast epithelial, glioblastoma, and fibroblast cells, suggesting that *H19* may play an important role in glioblastoma development.[4] In this study, we examined the expression of *H19* in glioblastoma and nontumor tissue obtained from patients undergoing tumor resection. Using commercial cell lines, we then investigated the role of *H19* in the invasion, angiogenesis, stemness, and tumorigenicity of glioblastoma cells. Our data suggest that *H19* plays an important role in tumorigenicity and stemness of glioblastoma cells and thus could be a therapeutic target for treatment of glioblastoma in the future.

Methods

Cell Lines and Tissue Samples

Tissue samples were obtained during glioblastoma resection in 30 patients treated at Yijishan Hospital. Use of the tissue samples was approved by the Wannan Medical College–Yijishan Hospital institutional review board. None of the patients from whom the samples were obtained had undergone preoperative chemotherapy or radiotherapy. Nontumor brain tissue obtained during the tumor resection procedures was used to determine the level of *H19* expression in nontumor tissue in the same patients. These tissue samples were examined by a pathologist and confirmed to be free of tumor cells.

Human glioblastoma cell lines (U87 and U373) were purchased from American Type Culture Collection and cultured in the recommended medium with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO). Two primary glioblastoma cell cultures (GBM1 and GBM2) were derived from glioma patient samples as described previously.[29] The primary glioma cells were cultured as follows: patient biopsies were immediately dissociated by trypsinization and subsequently were cultured in Neurobasal Medium (Invitrogen), with 1× B-27 and 1× GlutaMax I (Invitrogen). All medium was supplemented with 10% FBS (Invitrogen) and 100 U/ml penicillin/streptomycin (Sigma).

Survival Analysis

Survival data were collected for all 30 patients. Patients were grouped into 2 subgroups according to level of *H19* expression in the glioblastoma tissue samples (low vs high), with the average expression level of *H19* in all 30 glioblastoma tissue samples serving as the cut-off point. The Kaplan-Meier method was used to analyze patient survival.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA from glioblastoma tissues and cell lines was extracted using TRIzol RNA isolation reagent (Invitrogen). The concentration of isolated total RNA was measured by means of a NanoDrop ND-1000 spectrophotometer (Agilent). The total RNA was reverse transcribed by using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The quantitative polymerase chain reaction (PCR) was performed by using SsoFast EvaGreenH Supermix (Bio-Rad). The PCR primers of *H19* are as follows: *H19* (5′-TGCTGCACTTTACAACCACTG-3′) upstream and (5′-ATGGTGTCTTTGATGTTGGGC-3′) downstream.[19] Amplification was done on a Bio-Rad CFX96 system in a 20-μl reaction volume. GAPDH was used as the internal control.

In Vitro Matrigel Invasion Assay

An in vitro cell invasion assay was performed as previously described.[28] In brief, transwell inserts with 8-μm pores (BD Biosciences) were coated with Matrigel (280 μg/ml). The U87 and U373 cells were seeded in the 24-well plates and transfected with pcDNA3.1-H19 or pcDNA3.1 control plasmids using Lipofectamine LTX with Plus Reagent (Life Technologies). Equal numbers of transfected glioblastoma cells were seeded in the upper chambers of the wells in 500 μl serum-free medium, while 800 μl medium supplemented with 10% FBS as a chemoattractant was placed in the lower chambers. The plates were incubated at 37°C in a CO_2 incubator. After 24 hours, the chambers were pulled out, and the noninvading cells on the upper surface were removed with the

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cotton swab. The cells that invaded to the lower surface of the membrane were fixed in methanol, air dried, and stained with 0.1% crystal violet for 5 minutes. The stained cells were counted in 3 random fields (at a magnification of 40×) using a light microscope. Tumor cell invasion was assessed as the number of cells that had passed through the Matrigel-coated membranes. The results are reported as the means of the values obtained in 3 independent experiments.

Endothelial Tube Formation Assay (In Vitro Angiogenesis)

The endothelial tube formation assay was conducted using the Angiogenesis Starter Kit (Life Technologies) according to the manufacturer's protocol. The Matrigel was dissolved at 4°C overnight, and each well of the prechilled 24-well plates was coated with 100 μl Matrigel. The plates were then incubated at 37°C for 30 minutes to allow the matrix to solidify. Human umbilical vein endothelial cells (HUVECs, provided in the Angiogenesis Starter Kit) (5×10^4) were cocultured with transfected U87 and U373 cells, and then the collected cells were added to 500 μl endothelial cell growth medium (Gibco Medium 200 supplemented with Gibco LVES [large vessel endothelial supplement]). After 24 hours of incubation at 37°C, 5% CO_2, endothelial cell tube formation was assessed with an inverted photomicroscope at 40× magnification. Tubular structures and branch points were quantified by manual counting. The results are reported as the means of the values obtained in 3 independent experiments.

Establishment of Glioma Stable Cell Clones

One day before transfection, U87 and U373 cells were seeded onto 6-well plates at about 60%–80% confluence. Cells were transfected with pcDNA3.1-H19 or pcDNA3.1 control plasmids using Lipofectamine LTX with Plus Reagent (Life Technologies) in the absence of antibiotic. After 48 hours, cells were subcultured to 10% confluence in medium containing 500 μg/ml G418 (Sigma). When all cells in the nontransfected control culture were killed, antibiotic-resistant clones were picked and passaged in medium containing half the concentration of G418 as in the first round of selection. The established glioma stable cell clones were designated as the U87-pcDNA3.1-H19 or U87-pcDNA3.1 and U373-pcDNA3.1-H19 or U373-pcDNA3.1.

Isolation of $CD133^+$ and $CD133^-$ Cell Populations

The U87 and U373 cell lines and the GBM1 and GBM2 cell clones were dissociated and resuspended in PBS containing 0.5% bovine serum albumin and 2 mmol/L EDTA. For magnetic labeling, CD133/1 Micro Beads were used (Miltenyi Biotech). Positive magnetic-activated cell sorting (MACS) was performed using several MACS columns in series. The isolated cells were stained with CD133/2-PE (Miltenyi Biotech) or isotype control antibody and analyzed on a FACSCanto II (BD Biosciences).

Neurosphere Formation Assay

The neurosphere formation assay was performed as previously described.[28] Briefly, the transfected single cells (1×10^3) were plated onto a 24-well ultra-low attachment plate (Corning) in serum-free DMEM-F12, supplemented with 10 ng/ml basic fibroblast growth factor, 20 ng/ml epidermal growth factor, 0.4% bovine serum albumin, and B-27 supplement (1:50 dilution, Invitrogen). After 2–3 weeks of culture, the number of neurospheres (diameter > 40 μm) was manually counted in 3 randomly selected fields at a magnification of 40× using an inverted microscope. This assay was performed in triplicate (i.e., in 3 independent experiments).

Animal Studies

Following our previous protocol,[28,29] glioblastoma tumor xenografts were established in female BALB/c athymic mice by subcutaneous injection into the flanks of mice (6 mice per group) of stable U87-pcDNA3.1-H19 cells (injected into the right flank) or U87-pcDNA3.1 control cells (injected into the left flank) and U373-pcDNA3.1-H19 cells (injected into the right flank) or U373-pcDNA3.1 control cells (injected into the left flank) (1.5×10^6 cells per injection). Tumor volumes were determined by measuring the length and the width with calipers once a week for 6 weeks. The tumor volume (V) was calculated according to the formula $V = ab^2/2$, where a indicates length and b indicates width. Paraffin sections of tumors were subjected to standard hematoxylin and eosin staining. The statistical significance of differences between U87-pcDNA3.1-H19 or U87-pcDNA3.1 and U373-pcDNA3.1-H19 or U373-pcDNA3.1 control tumors was evaluated using the Student t-test.

Immunohistochemistry

Paraffin-embedded tissue samples from resected glioblastomas and nontumor brain tissue were cut in 5-μm sections and placed on poly-lysine–coated slides; then the samples were deparaffinized in xylene and rehydrated using a series of graded alcohol concentrations. Antigen retrieval was performed by heat mediation in citrate buffer (pH 6; Dako). Samples were blocked with 10% goat serum before incubation with primary antibody. The samples were incubated overnight using a primary antibody, anti-Ki 67, anti-CD31 (1:100), or an isotype-matched IgG (Abcam) as a negative control in a humidified container at 4°C. Immunohistochemical staining was performed with the Dako Envision Plus System according to the manufacturer's instructions.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). The data were analyzed using the SPSS 12.0 Windows version software. Statistical analyses were done by ANOVA or Student t-test. A p value < 0.05 was considered statistically significant.

Results

H19 Is Overexpressed in Glioblastoma Tissues and Associated With Patient Survival

To identify the role of *H19* in glioblastoma develop-

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ment, the expression of *H19* mRNA in 30 samples of glioblastoma and 30 samples of nontumor brain tissues was examined by real-time quantitative reverse transcriptionPCR (RT-qPCR). The relative expression level of *H19* was normalized by the expression of internal control GAPDH using relative quantification ($2^{-\Delta Ct}$).[15] The results showed that the average expression level of *H19* was significantly higher in glioblastoma tumor tissues compared with nontumor brain tissue from the same patient ($p < 0.0001$, paired Student t-test; Fig. 1 upper). Next, the average relative expression value of all 30 glioblastoma tissue samples was chosen as the cut-off point. The Fisher exact test and Kaplan-Meier analysis showed that a high level of *H19* expression was significantly associated with a poor progression-free survival rate (Fig. 1 lower). These data suggest that *H19* is overexpressed in glioblastoma tissues and associated with patient survival.

Overexpression of *H19* Promotes Glioblastoma Cell Migration and Angiogenesis In Vitro

Recent understanding of the fundamental processes governing glioblastoma invasion and angiogenesis provide a renewed hope for development of novel strategies for glioblastoma treatment.[26] We performed in vitro Matrigel invasion and endothelial tube formation assays to investigate the effects of *H19* overexpression on glioblastoma cell invasion and angiogenesis in vitro. Overexpression of *H19* was produced in both U87 and U373 cells by transfection of pcDNA3.1-H19. Cells from both lines were also transfected with pcDNA3.1 (without *H19*) as controls. The overexpression of *H19* was examined by RT-qPCR (Fig. 2A). The in vitro Matrigel invasion assay showed that overexpression of *H19* significantly increased the invasion capability of glioblastoma cells compared with pcDNA3.1-control vector–transfected U87 and U373 glioblastoma cells (Fig. 2B and C). Next, the transfected U87 and U373 cells were cocultured with endothelial cells (HUVECs). We observed that more tubes were formed in HUVECs cocultured with pcDNA3.1-H19–transfected glioblastoma cells compared with pcDNA3.1 control vector–transfected cells (Fig. 2D and E). These results indicate that overexpression of *H19* promotes glioblastoma cell invasion and angiogenesis in vitro.

H19 Is Overexpressed in CD133$^+$ Glioblastoma Cells and Promotes Neurosphere Formation

The cancer stem cell marker is widely expressed in glioblastoma, and we have previously isolated and analyzed CD133$^+$ glioblastoma cells from glioblastoma cell lines and primary glioblastoma tissues.[28] Therefore, we next examined the expression of *H19* in our isolated CD133$^+$ and CD133$^-$ cell populations by means of RT-qPCR. Interestingly, we found that the expression of *H19* was significantly higher in CD133$^+$ cells than CD133$^-$ cells, suggesting that the expression of *H19* may contribute to the stemness of glioblastoma cells (Fig. 3A). To test this hypothesis, we further assessed the self-renewal ability of glioblastoma cells by analysis of neurosphere formation, which is considered a hallmark of glioma stem-like cells. When the stable transfected cells were cultured in the presence of suitable factors for 2–3 weeks, U87 and U373 cells transfected with pcDNA3.1-H19 formed twice as many neurospheres as pcDNA3.1 control vector–transfected cells (Fig. 3B and C). These results suggest that *H19* is overexpressed in CD133$^+$ glioblastoma cells and promotes stemness of glioblastoma cells.

Stable Overexpression of *H19* Promotes Tumor Formation of Glioblastoma Cells in Mice

To further substantiate the role of *H19* in glioblastoma carcinogenesis, we assessed the effects of *H19* overexpression on tumorigenicity of glioblastoma cells in vivo. The U87 and U373 cells were stable when transfected with pcDNA3.1-H19 and pcDNA3.1 control vectors and then implanted into the right and left flanks (1.5×10^6 cells per flank) of BALB/c athymic mice by subcutaneous injection. Tumor volumes were determined every week by measuring tumor length (a) and width (b). The tumor volume (V) was calculated according to the formula $V = ab^2/2$. At 42 days after injection, the mean volumes of tumors generated from U87 or U373 cells transfected with pcDNA3.1-H19 were significantly larger than those generated from U87 or U373 cells transfected with pcDNA3.1 control (Fig. 4A). The tissue sections from the tumors generated from transfected U87 cells (cells transfected with pcDNA3.1 [controls] as well as cells transfected with pcDNA3.1-H19) were collected and assayed by H & E staining and immunohistochemical analysis to detect the proliferation marker Ki-67 and the angiogenesis marker CD31. The results showed that the expression of Ki-67 and CD31 was significantly increased in tumor tissues derived from U87 cells transfected with pcDNA3.1-H19 compared with tumor tissues from control group (Fig. 4B). These results indicate that stable overexpression of *H19* promotes tumor formation of glioblastoma cells and induced tumor cell proliferation and angiogenesis in vivo.

Discussion

H19 was the first human imprinted noncoding gene to be identified, showing expression on only the maternal allele.[31] It is also imprinted in mice, and *H19* was mapped on the short arm of chromosome 11, band 15.5, homologous to a region of murine chromosome 7.[18] Increased expression of *H19* has been observed in some cancers, such as gastric[25,30] and bladder cancer,[16] and decreased expression in others, such as HCC,[13] suggesting that *H19* involves either oncogenic or tumor suppressor properties. Hao et al.[10] introduced a construct expressing the *H19* gene into the kidney tumor cells and observed that overexpression of *H19* induced a slower growth rate, a much lower anchorage-independent growth rate in soft agar, and no tumor formation in mice, which indicate the tumor suppressor properties of *H19* in kidney tumors. However, accumulating data do not support the idea of *H19* being a tumor suppressor gene. Moreover, studies of various tumors have demonstrated a re-expression or an overexpression of the *H19* gene in comparisons with healthy tissue.[18] Therefore, *H19* possesses diagnostic, prognostic, and therapeutic values in many cancers, and its essential role in tumorigenesis has been demonstrated. Modulation of the imprinting

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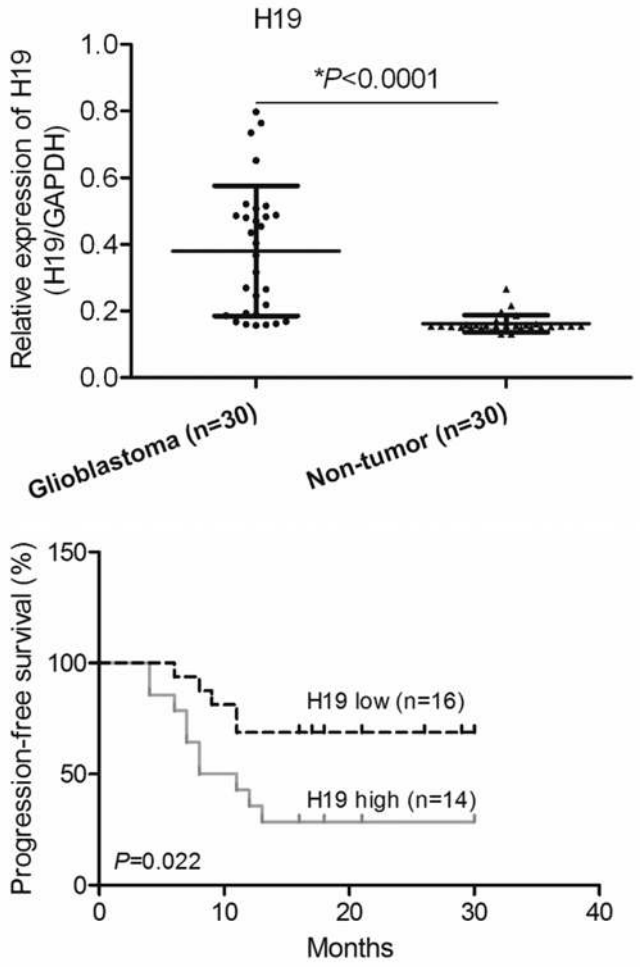

FIG. 1. Overexpression of *H19* in glioblastoma tissues and association with patient survival. **Upper:** The expression of *H19* in 30 glioblastoma specimens and 30 specimens of nontumor brain tissue obtained from the same patients was analyzed by RT-qPCR. **Lower:** The average expression level of all 30 glioblastoma tissue samples was chosen as the cut-off point, and the Kaplan-Meier method was used to analyze progression-free survival for patients in the high-expression and low-expression groups. There was a significant difference in the probability of patient survival (p = 0.022, Kaplan-Meier [log-rank] test).

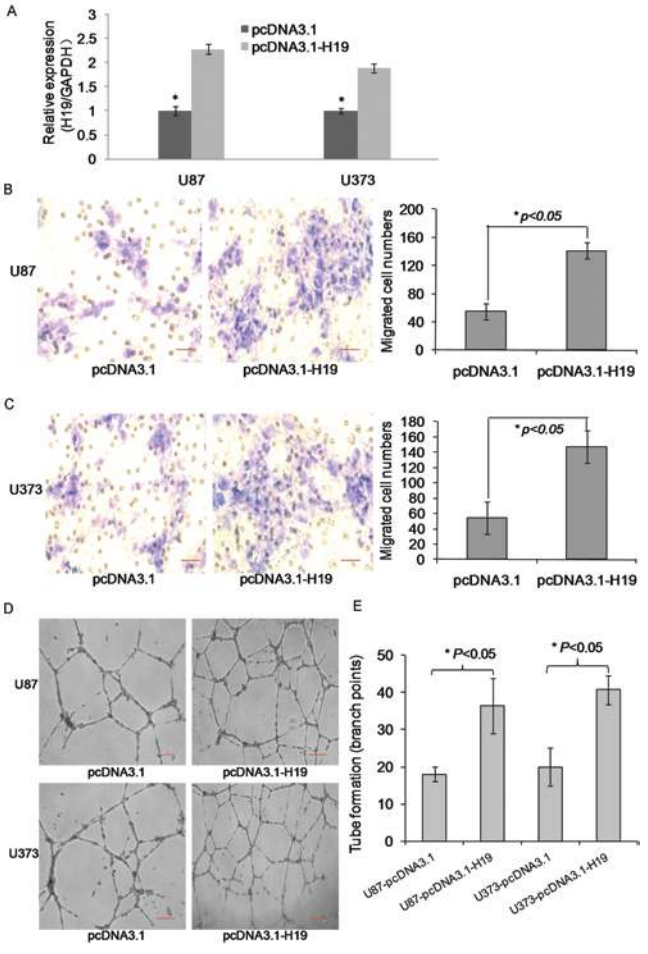

FIG. 2. Overexpression of *H19* and promotion of glioblastoma cell migration and angiogenesis in vitro. **A:** Expression of *H19* in glioblastoma cells transfected with pcDNA3.1-H19 and pcDNA3.1 control as determined by RT-qPCR analysis. **B and C:** Representative images showing invasiveness of U87 (B) and U373 (C) glioblastoma cells transfected with pcDNA3.1-H19 and pcDNA3.1 control as assessed by transwell Matrigel invasion assay, and graphs showing the average number of invasive cells counted in 3 independent repeated experiments. *p < 0.05. **D:** Representative photomicrographs showing HUVEC tube formation for HUVECs cocultured with U87 and U373 glioblastoma cells transfected with pcDNA3.1-H19 or pcDNA3.1 control. Original magnification ×100. **E:** Bar graph showing the difference in tube formation (number of branch points). Results are expressed as mean ± SD (n = 3). *p < 0.05. Figure is available in color online only.

status of *H19* may play an important role in the development of tumors including glioblastoma.[7]

Recent advances have identified a myriad of molecular functions related to tumorigenesis for several lncRNAs, including metastasis-associated lung adenocarcinoma transcript 1 *(MALAT1)*, prostate cancer associated noncoding RNA 1 *(PRNCR1)*, prostate cancer gene expression marker 1 (PCGEM1), homeobox transcript antisense intergenic RNA *(HOTAIR)*, and *H19* lncRNAs.[22] Long noncoding RNAs are emerging as key biomarkers in cancer diagnostics and therapeutics within the clinical setting.[3] Here, our results show that the average expression level of *H19* was significantly higher in glioblastoma tumor tissues than in their nontumor counterparts. Moreover, the Fisher exact test and Kaplan-Meier analysis showed that high-level *H19* expression was significantly associated with a poor progression-free survival rate. Consistently, a recent report also showed that the *H19* transcript was highly expressed in high-grade glioblastoma, and significant *H19* expression in other types of primary brain tumors as well as in brain metastases was also detected.[2] The different expression of *H19* RNA has also been associated with low-grade (WHO Grade II) diffuse glioblastoma (low expression) and high-grade (WHO Grades III and IV) diffuse glioblastoma (high expression).[2] These data suggest that the potential oncogenic properties of *H19* in glioblastoma development. Therefore, investigation of the important role of *H19* in glioblastoma may lead to its development as a potential prognostic tumor biomarker and therapeutic target for glioblastoma.

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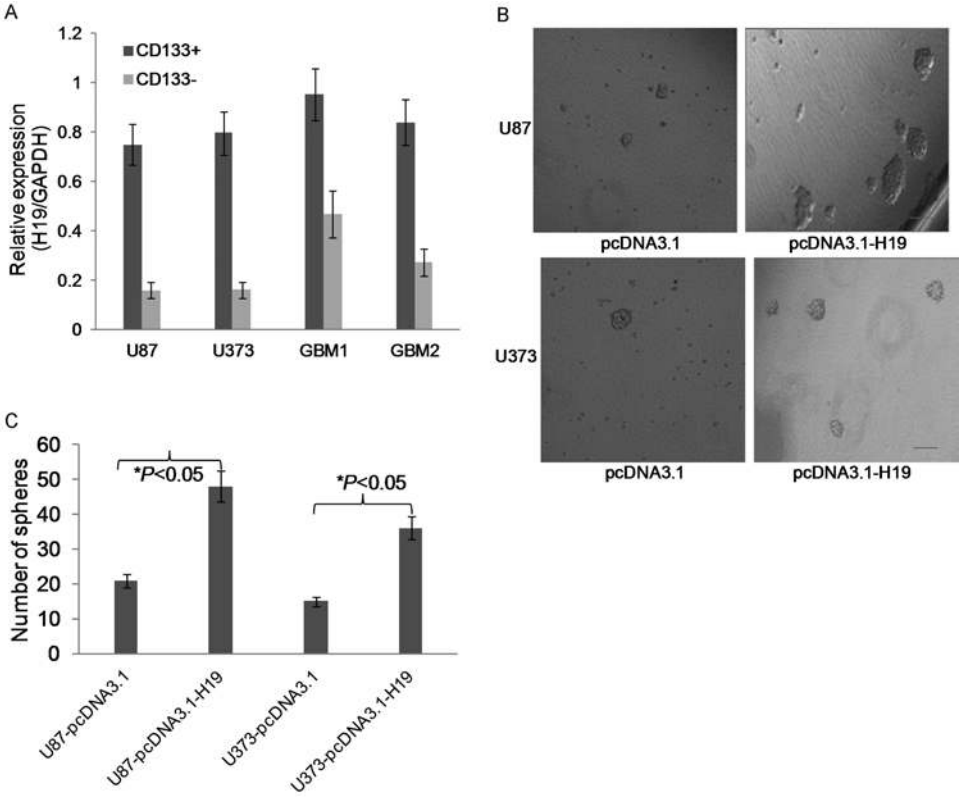

FIG. 3. *H19* is overexpressed in CD133⁺ glioblastoma cells and promotes sphere formation. **A:** The expression of *H19* in isolated CD133⁺ and CD133⁻ cell populations from commercial glioblastoma cell lines (U87 and U373) or our own glioblastoma cell clones (GBM1 and GBM2) as assessed by RT-qPCR. **B and C:** Representative images and numbers of neurospheres generated after 2–3 weeks of single-cell culture using the established stable glioblastoma cells transfected with pcDNA3.1-H19 or pcDNA3.1 control. Results are expressed as mean ± SD (n = 3). *p < 0.05.

Previous study showed that c-Myc significantly induces the expression of the *H19* in diverse cell types, including breast epithelial, glioblastoma, and fibroblast cells, suggesting *H19* may play important role in glioblastoma development.[4] Elevated *H19* expression has been reported in both primary and metastatic tumors, in morphogenesis and epithelial mesenchymal transition, in migration and angiogenesis, in inflammatory diseases and wound healing, and in multidrug resistance.[2] Moreover, Amit et al. have developed targeted therapy for a broad spectrum of cancers (pancreatic cancer, ovarian cancer, glioblastoma, and HCC) mediated by a double promoter plasmid expressing diphtheria toxin under the control of *H19* and *IGF2-P4* regulatory sequences.[1] Therefore, we further investigated the role of *H19* in glioblastoma cell invasion and angiogenesis. Our result showed that overexpression of *H19* promotes glioblastoma cell migration and angiogenesis in vitro. High levels of *H19* expression have been shown by utilizing gene array in glioblastoma-derived cancer stem cell lines, which are related to CD133⁺ cells. This might be related to the modulation of *H19* by hypoxia through upregulation of its ncRNA.[17] Therefore, we next examined the expression of *H19* in our isolated CD133⁺ and CD133⁻ cell populations by RT-qPCR. Interestingly, we found that the expression of *H19* was significantly higher in CD133⁺ cells than in CD133⁻ cells. Moreover, U87 and U373 cells transfected with pcDNA3.1-H19 formed twice as many neurospheres as pcDNA3.1 control vector–transfected cells. These results suggest that the expression of *H19* may contribute to the stemness of glioblastoma cells. Preclinical, clinical, and comparison studies in humans using a DNA plasmid containing *H19* regulatory sequences that drive the expression of an intracellular toxin (diphtheria toxin A-fragment) have demonstrated promising results in several types of carcinoma.[27] Further, our preclinical study in a murine model also indicated that stable overexpression of *H19* promotes tumor formation of glioblastoma cells and induces tumor cell proliferation and angiogenesis in vivo. Therefore, we believe that *H19* plays an important role in tumorigenicity and stemness of glioblastoma cells and thus may represent a therapeutic target for treatment of glioblastoma in the future.

Conclusions

Our results suggest that the expression of *H19* lncRNA was significantly increased in glioblastoma tissues and CD133⁺ cell populations. The increased expression of *H19* promotes migration, angiogenesis, stemness, and tumorigenicity of glioblastoma cells. Therefore, *H19* plays an important role in tumorigenicity and stemness of glioblastoma and thus can be a therapeutic target for treatment of glioblastoma in the future.

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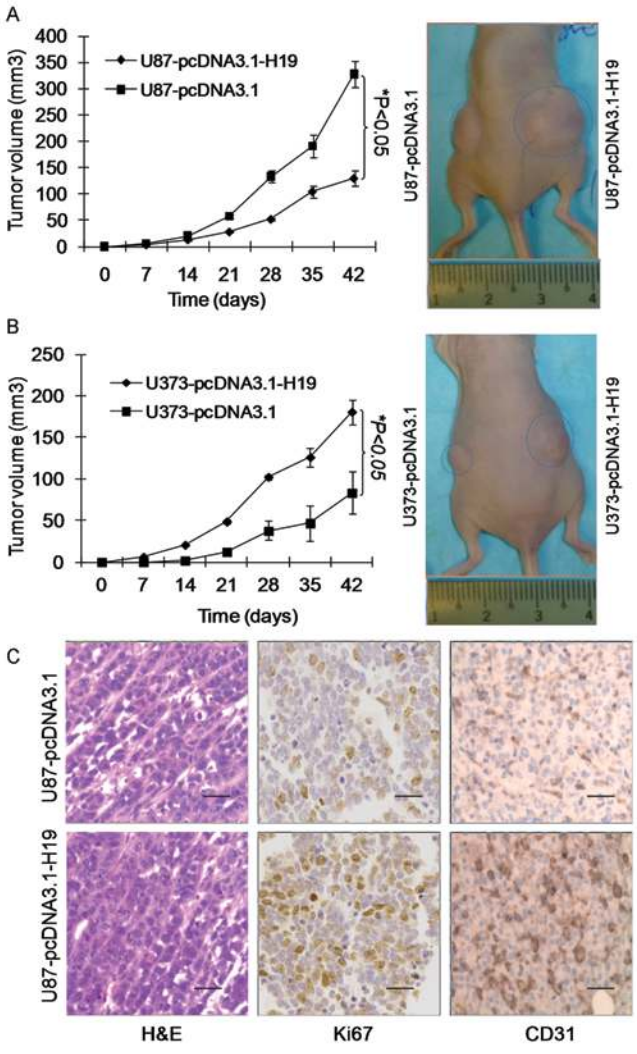

FIG. 4. Stable overexpression of *H19* promotes tumor growth and induces proliferation and angiogenesis of glioblastoma cells in mice. **A and B:** Determination of tumor growth in stable transfected U87 (A) and U373 (B) cells. Tumor volume was calculated once a week, starting 7 days after injection. Data represent mean ± SD of 3 independent experiments. *$p < 0.05$. **C:** Results of H & E staining and immunohistochemical analysis for detection of the proliferation marker Ki-67 and the angiogenesis marker CD31. Photomicrographs of representative fields from sections of tumors generated by injection of pcDNA3.1-H19 or pcDNA3.1 control stable transfected U87 cells. Figure is available in color online only.

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