Received: 27 December 2018 | Revised: 9 March 2019 | Accepted: 11 March 2019

DOI: 10.1111/cas.14002

ORIGINAL ARTICLE

WILEY Cancer Science

AC016405.3, a novel long noncoding RNA, acts as a tumor suppressor through modulation of TET2 by microRNA-19a-5p sponging in glioblastoma

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Funding information
National Natural Science Foundation of China, Grant/Award Number: 81172180

Long non-coding RNAs (lncRNAs) are crucial regulators in various malignancies including glioblastoma multiforme (GBM). In the present study, we screened out a new lncRNA, AC016405.3, through a previous genome-wide lncRNA microarray analysis in GBM. It showed that AC016405.3 was downregulated in GBM tissue specimens and cell lines, and it also illustrated that the downregulated AC016405.3 was closely correlated with several aggressive features of patients with GBM. Functionally, we found that overexpression of AC016405.3 suppressed GBM cells' proliferation and metastasis using a gain of function experiment. We further showed that microRNA (miR)-19a-5p, a carcinogenic miRNA, was a downstream miRNA of AC016405.3. AC016405.3 was revealed as a target of miR-19a-5p, and overexpression of miR-19a-5p reversed the inhibitive effect of AC016405.3 on GBM cell proliferation and metastasis. Furthermore, a novel downstream gene of miR-19a-5p, *TET2*, was identified through a constructed microarray analysis. We showed that TET2 was downregulated in GBM and was involved in miR-19a-5p-mediated proliferation and metastasis by directly being targeted. Finally, through a western blot assay and a series of functional CCK-8 and metastatic assays, we showed that AC016405.3 suppressed proliferation and metastasis through modulation of TET2 by sponging of miR-19a-5p in GBM cells. In summary, the findings of the current study identified a novel lncRNA and illustrated that AC016405.3, acting as an anti-oncogene, suppressed GBM cell proliferation and metastasis by regulating TET through miR-19a-5p sponging. Our present study might provide a new axis in the molecular treatment of GBM.

KEYWORDS
ceRNA, glioblastoma, lncRNA AC016405.3, miR-19a-5p, proliferation/metastasis

1 | INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and mortal primary brain tumor in the adult central nervous system and is characterized by its highly aggressive and invasive behavior.[1] The total clinical outcomes of patients with GBM are not optimistic with an average median survival time of less than 15 months.[2] The aggressive and invasive characteristics lead to the high recurrence rate

and poor prognosis of GBM. Although combined treatments, including surgical resection, radiotherapy, chemotherapy, and immunotherapy, achieve a measure of progress, the late-stage outcomes of GBM are not encouraging due to high metastasis and invasiveness.[3] Therefore, seeking out new molecules correlated to GMB progression is of extreme urgency.

Long noncoding RNAs (lncRNAs), a group of nonprotein coding transcriptions with a length of more than 200 nt, are extensively involved in various diseases including cancer.[4] Long noncoding RNAs work as oncogenes or anti-oncogenes and participate in various aspects of cancer-related cell events including epithelial-mesenchymal transition, apoptosis, cancer stem cell differentiation, cell proliferation, and metastasis.[5-9] He et al reported that lncRNA urothelial carcinoma associated 1 interacted with microRNA (miR)-182 to modulate glioma proliferation and migration by targeting inhibitor of the apoptosis-stimulating protein of p53 (iASPP).[10] Kang et al found that lncRNA RP5-833A20.1 inhibited U251 cell proliferation, metastasis, and cell cycle progression by suppressing the expression of nuclear factor IA.[11] Based on a previous genome-wide analysis of lncRNA expression in GBM (GSE104267), we found that lncRNA AC016405.3, a novel lncRNA also named RP11-44N11.2, was significantly downregulated in GBM. Low expression of AC016405.3 was closely related to an aggressive phenotype and poor prognosis of patients with GBM. These findings also indicated that AC016405.3 might be an anti-oncogene in GBM. Up to the present, related research on the expression and function of AC016405.3 in GBM has been rare.

Long noncoding RNAs are well known to exert their functions through a mechanism of competing endogenous RNA (ceRNA). It is supposed that RNA transcripts communicate with others through "talking" with a new "language" mediated by miRNA response elements (MREs).[12,13] Qin et al reported that lncRNA MEG3 inhibited glioma cell proliferation, migration, and invasion by acting as a ceRNA of miR-19a.[14] Xiao et al reported that lncRNA TP73-AS1 suppressed glioma growth and metastasis by working as a ceRNA through miR-124-dependent inhibitor of iASPP regulation.[15] Until now, it has been unclear whether AC016405.3 can act as a ceRNA and cowork with any miRNAs in GBM.

In the present study, AC016405.3 was shown to work as an anti-oncogene in GBM. AC016405.3 suppressed GBM cell proliferation and metastasis by miR-19a sponging to modulate ten-eleven-translocation-2 (TET2).

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

The present research was granted approval by the Institute Research Medical Ethics Committee of Dalian Medical University (Dalian, China). Formal written informed consent was provided by the patients whose tissues were used in the present study. Three freshly frozen GBM tissues and paired paratumor tissue specimens as well as 64 formalin-fixed paraffin-embedded GBM specimens (from July 2012 to November 2016) were collected from the First Affiliated Hospital of Dalian Medical University. All GBM tissue samples were graded according to the 2007 WHO classification by two neuropathologists: grade I and II (n = 30), and grade III and IV (n = 34).

2.2 | Cell culture

A human GBM cell line U87MG was purchased from Shanghai Institutes for Biological Sciences Cell Resource Center (Shanghai, China). Another human GBM cell line U251MG and normal human astrocytes (NHA) were saved in the Central Laboratory of College of Pharmacy, Dalian Medical University. All cells lines were cultured in high glucose DMEM (Gibco, El Paso, TX, USA) with 10% FBS (TransGen Biotech, Shanghai, China), 100 IU/mL penicillin (Yeasen, Shanghai, China), and 100 mg/mL streptomycin (Yeasen). All cell lines were maintained in a humidified incubator at 37°C with 5% CO_2.

2.3 | Reverse transcription and quantitative real-time PCR

The procedure was carried out as previously reported.[16] Total RNAs were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Quantitative PCRs were carried out by using a SYBR Green PCR Kit (Takara, Dalian, China) on a CFX96 Touch sequence detection system (Bio-Rad, Hercules, CA, USA). GAPDH and U6 were applied to internal controls of lncRNAs and TET2 mRNA, and miRNAs, respectively. Expression levels of targeted genes were calculated by the $2^{-\Delta\Delta Ct}$ method.[17] Specific primers for each targeted gene were chemically synthesized by RiboBio (Guangzhou, China) and listed in Table S1.

2.4 | In situ hybridizations assay and immunohistochemistry assay

In situ hybridization (ISH) was undertaken by applying an ISH Kit (Boster Bio-Engineering Company, Wuhan, China) as previously described.[18] Formalin-fixed paraffin-embedded GBM tissue slices were deparaffinized and deproteinated. Slices were prehybridized in a prehybridization solution at 42°C for 2 hours and incubated with a DIG-labeled specific probe targeting AC016405.3 (RiboBio) at 42°C overnight. After strictly washing, the slides were then exposed to a streptavidin-peroxidase reaction system and stained with 3, 3′diaminobenzidine (ZSGB-BIO, Beijing, China). Hematoxylin was used to counterstain the slices. All slices were observed and photographed under a microscope (Leica, Wetzlar, Germany).

The immunohistochemistry procedure was carried out as previously described.[19]

2.5 | Oligonucleotide and plasmid transfection

AC016405.3 overexpression plasmids (AC016405.3), TET2 overexpression plasmids (oeTET2), corresponding blank vector, specific siRNA oligonucleotides that targeted AC016405.3 (siAC016405.3-01 and siAC016405.3-02) and scramble siRNA were purchased from

GenePharma (Shanghai, China). Wild AC016405.3 overexpression plasmids (AC016405.3-wt) and mutant AC016405.3 overexpression plasmids (AC016405.3-mut) were constructed by GenePharma as well. MicroRNA-19a-5p mimics, negative control (NC) mimic, miR-19a-5p inhibitor, and NC inhibitor were purchased from RiboBio. When GBM cells grew to 70% confluence, the plasmids or oligonucleotides were transfected into the GBM cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions to up- or downregulate targeted genes. Compared with siAC016405.3-02, siAC016405.3-01 presented a higher silencing efficacy (qualified by quantitative [q]RT-PCR assay) and was selected in the following AC016405.3 knockdown experiment (Figure S1). MicroRNA-19a-5p shRNAs (shmiR-19a-5p1# and shmiR-19a-5p 2#) and control shRNA (shCtrl) were chemically synthesized by RiboBio. In order to gain a miR-19a-5p stable knockdown cell line, U87MG and U251 MG cells after transfection were selected by the culture medium containing 0.4 mg/mL Geneticin (G418; Invitrogen). After 6 weeks, G418-resistant cell clones were established. Total RNAs from these cell lines were extracted for further microarray analysis.

2.6 | Microarray analysis

Human Gene Expression Microarray V4.0 (Agilent Technology, Palo Alto CA, USA) was used to screen downstream genes of miR-19a-5p. Total RNAs from six match-paired sets of miR-19a-5p stably downregulated U87MG cells were extracted as previously described. The double-stranded cDNA was synthesized, purified, and eluted. The complementary RNA was synthesized with T7 Enzyme Mix (Thermo Scientific, Waltham, MA, USA) and the eluted double-stranded DNA products. After amplification, cDNA was purified and labeled. CapitalBio BioMixer II Hybridization Station (Agilent, Santa Clara, CA, USA) was used to cross the chip overnight, then the chip was washed and scanned. Microarray images were transformed into point intensity value. The signal was directly output to GeneSpring software (Agilent, Santa Clara, CA, USA) after deducting the background value and carrying out the quantile standardization. The data were further analyzed. Differentially expressed genes were chosen based on the following criteria: fold change >2 and $P < .05$. The hierarchical clustering analysis was carried out on the differentially expressed genes.

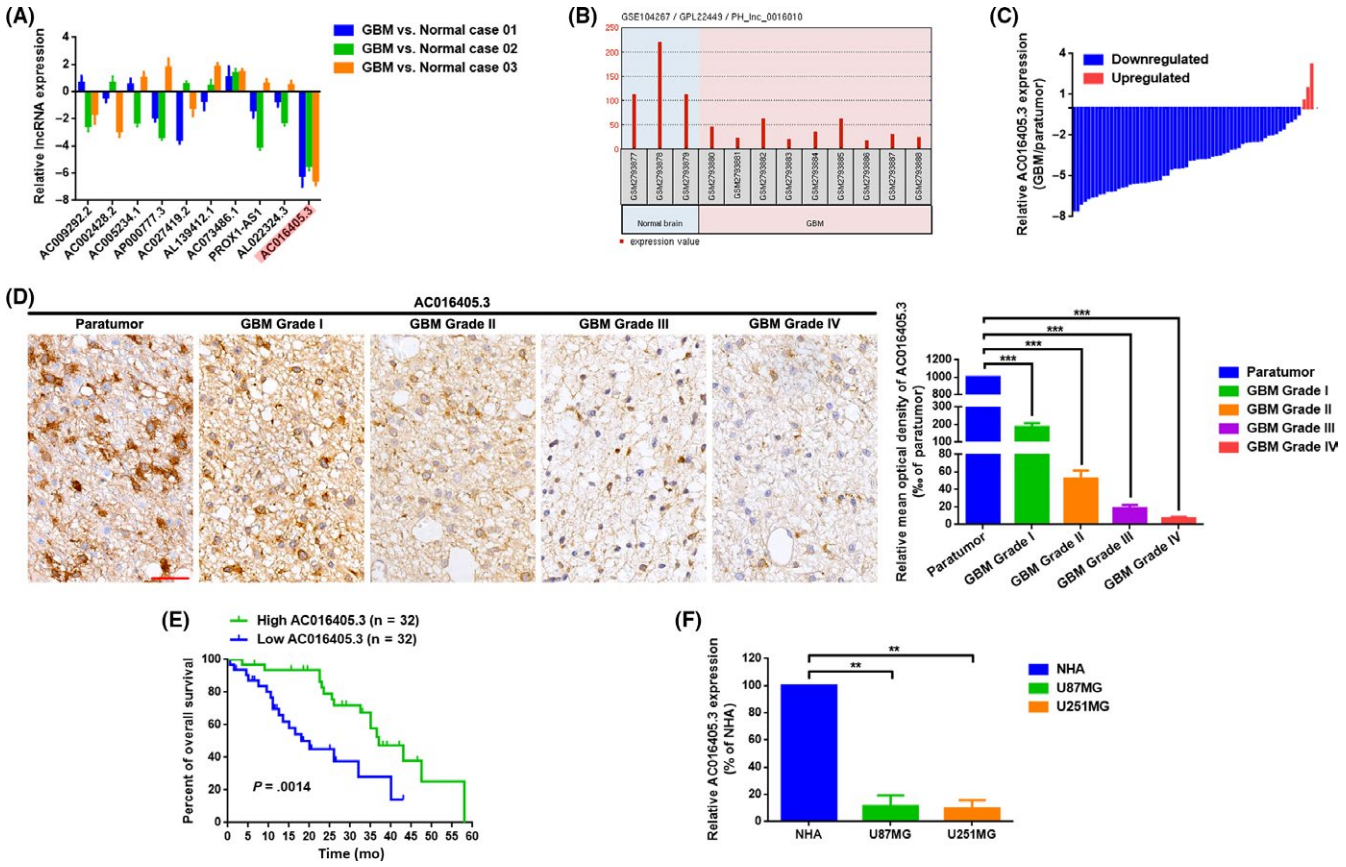


FIGURE 1 AC016405.3 was downregulated and correlated with poor prognosis in patients with glioblastoma multiforme (GBM). A, Expression of 10 long noncoding RNAs (lncRNAs) in 3 GBM tissue specimens and paired adjacent normal brain tissue specimens was measured by quantitative RT-PCR assay. B, AC016405.3 was downregulated in GBM tissue specimens according to an lncRNA expression profile of GSE104267. PH_lnc_0016010 represented for AC016405.3. C, Quantitative RT-PCR assay indicated that AC016405.3 was downregulated in 61 of 64 GBM tissue specimens. D, Mean optical density of AC016405.3 was gradually decreased with advanced staging as checked by an in situ hybridization assay. ***$P < .001$ vs paratumor group. E, Kaplan-Meier analyses showed that the overall survival of GBM patients with low AC016405.3 was shorter than that with high AC016405.3. F, AC016405.3 was also downregulated in GBM cell lines. Data were shown as mean ± SD from 3 independent experiments. **$P < .01$ vs normal human astrocyte (NHA) group

TABLE 1 Association of AC016405.3 expression with clinicopathological features of GBM

Clinical features	Cases (n)	AC016405.3		*P* value*
		High	Low	
Age				
<50	27	14	13	1.000
≥50	37	18	19	
Gender				
Female	29	14	15	1.000
Male	35	18	17	
Diameter				
<5 cm	29	22	9	0.002
≥5 cm	33	10	23	
Histological subtype				
IDH wild	55	28	27	0.839
IDH mutant	6	3	3	
Others	3	1	2	
Location				
Frontal temple	42	22	20	0.689
Cerebellum	4	2	2	
Paretio-occipita	7	2	5	
Periventricular	11	6	5	
Pathological grade				
I + II	30	23	7	0.000
III + IV	34	9	25	
Distant metastasis				
Without	21	22	11	0.012
With	29	10	21	

**P*-value obtained from Pearson's chi-square test or Fisher's exact test.

2.7 | Western blot analysis

Total proteins from GBM cells were extracted by a RIPA lysis buffer (Beyotime, Shanghai, China) and qualified by a BCA detecting kit (Beyotime). Protein samples were subjected to 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated with specific Abs targeting TET2 (Abcam, Cambridge, MA, UK; dilution rates of 1:250) and GAPDH (Abcam; dilution rates of 1:500) at 4°C overnight, separately. The next day, the membranes were incubated with a secondary Ab (Abcam; dilution rates of 1:2000) at room temperature for 1 hour. The protein expression images were obtained through a Bio-Spectrum Gel Imaging System (UVP, Upland, CA, USA).

2.8 | Cell counting assay

We applied the CCK-8 assay as previously reported to determine the proliferation ability changes of GBM cells.[20] U87MG and U251MG cells were incubated in 96-well plates (2×10^3) supplemented with complete growth medium and followed by specific transfections for 24 hours. At days 1, 2, 3, 4, and 5 after transfection, 10 μL CCK-8 solution was added into each well and incubated for 2 hours. The absorbance was measured at an optical density of 450 nm by a Microplate reader (Bio-Rad).

2.9 | Wound-healing assay and Transwell assay

For the wound-healing assay, U87MG and U251MG cells were seed onto 6-well plates and transfected with different plasmids or oligonucleotides for 48 hours; the subconfluent cell monolayers were formed into 3 parallel lines with a P-200 pipette tip. The detached cells were washed off twice gently, and the medium was then replaced with 1% FBS complete medium. Images were taken at 0 and 24 hours to

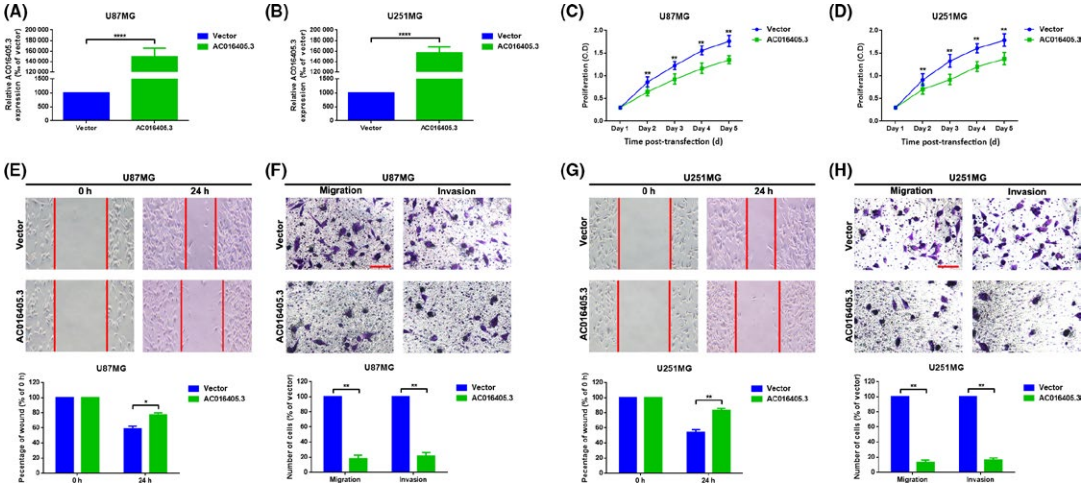


FIGURE 2 Upregulation of AC016405.3 suppressed proliferation and metastasis in U87MG and U251MG cells. A,B, AC016405.3 in U87MG (A) and U251MG (B) cells was remarkably upregulated by transfecting AC016405.3 overexpression plasmid as measured by quantitative RT-PCR assay. C,D, Upregulation of AC016405.3 suppressed U87MG (C) and U251MG (D) cell proliferation as determined by a CCK-8 assay. E-H, Upregulation of AC016405.3 suppressed U87MG (E,F) and U251MG (G,H) cell metastasis as checked by wound healing and Transwell assay, respectively. Data are shown as mean ± SD from 3 independent experiments. ***P* < .01 vs vector group

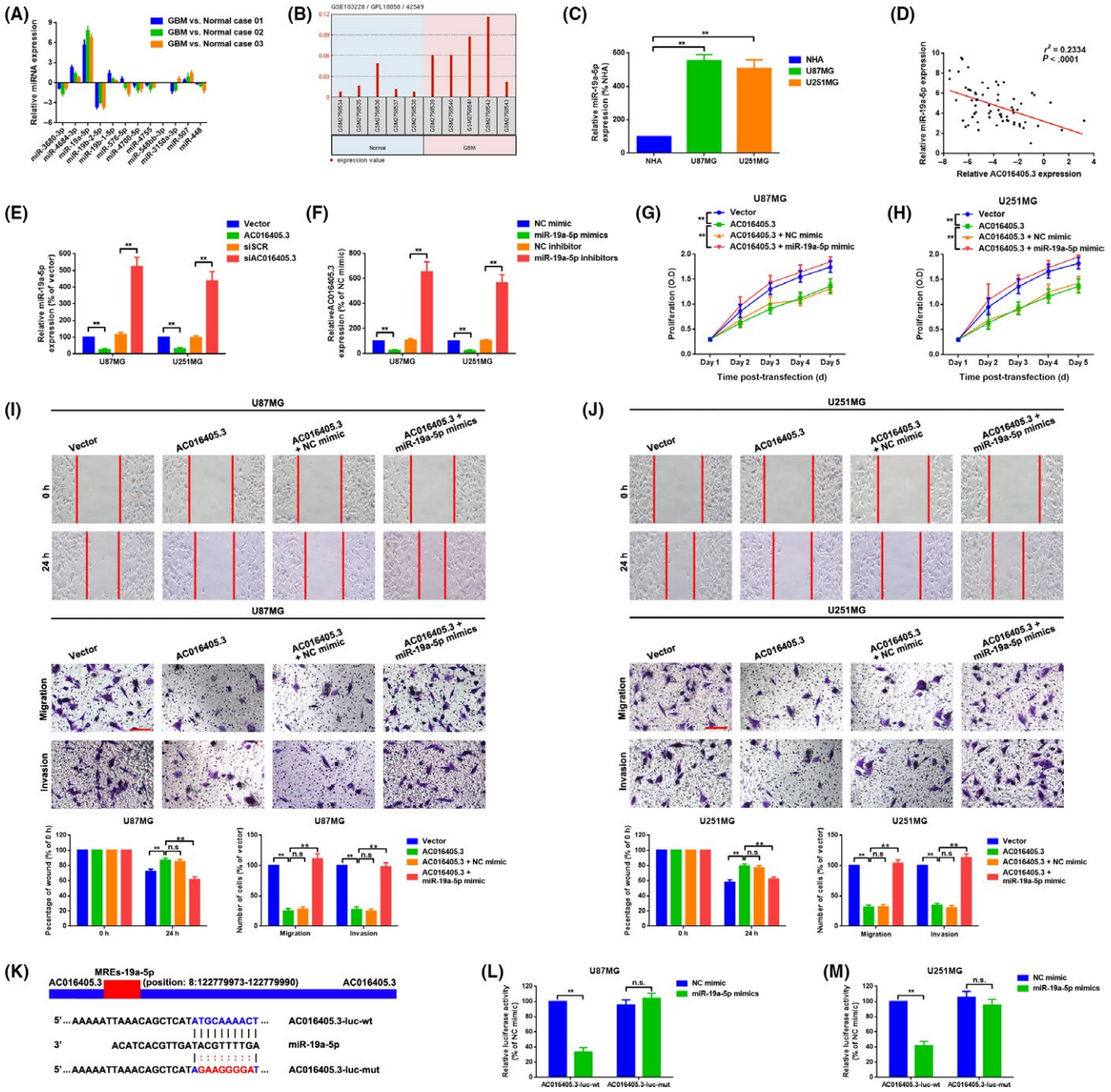


FIGURE 3 MicroRNA (miR)-19a-5p was involved in AC016405.3-mediated suppression of proliferation and metastasis in U87MG and U251MG cells. A, Expressions] of miRNAs in 3 glioblastoma multiforme (GBM) tissue specimens and paired adjacent normal brain tissue specimens were determined by quantitative RT-PCR assay. B, MiR-19a-5p was upregulated in GBM tissue specimens according to the miRNA expression profile of GEO dataset GSE103228. 42549 represented for miR-19a-5p. C, MiR-19a-5p was upregulated in GBM cell lines U87MG and U251MG compared to normal human astrocytes (NHA). $^{**}P < .01$ vs NHA group. D, Expression of miR-19a-5p was inversely correlated with AC016405.3. E, Upregulation and downregulation of AC016405.3 inversely regulated miR-19a-5p expression. $^{**}P < .01$ vs vector or scramble siRNA (siSCR) group, separately. F, Up- and downregulation of miR-19a-5p negatively regulated AC016405. $^{**}P < .01$ vs negative control (NC) mimic or NC inhibitor group, individually. G,H, Upregulation of AC016405 suppressed U87MG (G) and U251MG (H) cell proliferation, but the inhibitive effect was abrogated by enhancement of miR-19a-5p, determined by a - assay. I,J, Upregulation of AC016405 suppressed U87MG (G) and U251MG (H) cell metastasis, but the inhibitive effect was attenuated by an increase of miR-19a-5p, determined by wound healing and Transwell assays, respectively. $P > .05$ and $^{**}P < .01$ vs AC016405 group. n.s., not significant. K, Diagram of constructed luciferase reporter plasmids. L, Cotransfection of a WT reporter plasmid (AC016405-luc-wt) and miR-19a-5p mimics resulted in a significant weakening of luminescence as compared with cotransfection of AC016405-luc-wt and NC mimic. While the theoretical miRNA response elements (MREs)-19a-5p in AC016405 was mutated (cotransfection of AC016405-luc-mut and miR-19a-5p mimics), the luminescence was restrengthened. and Data are shown as mean ± SD from 3 independent experiments. $^{**}P < .01$ vs NC mimic group. n.s., $P > .05$

visualize wound healing. The percentage of wound closure (original width − width after cell migration) / original width was calculated.

For the Transwell assay, the procedure was carried out as previously described.[21] In short, GBM cells were incubated on uncoated (for migration assays) and Matrigel-coated (for invasion assays) upper chambers (BD Biosciences, Franklin Lakes, NJ, USA), respectively. Culture medium not containing or containing 10% FBS was supplemented into the upper and lower wells individually and cultured for a further 24 hours, after which the non-migrated and non-invaded cells were wiped out. Then, the filters were fixed in 90% alcohol, followed by crystal violet staining. Five random fields were counted per chamber with an inverted microscope (Olympus, Tokyo, Japan).

2.10 | Dual luciferase reporter assay

The procedure was carried out as previously described.[18] Wild and mutant reporter plasmids of AC016405.3 (AC016405.3-luc-wt and AC016405.3-luc-mut) and TET2 (TET2-luc-wt1 and TET2-luc-mut1, TET2-luc-wt2 and TET2-luc-mut2, TET2-luc-wt3 and TET2-luc-mut3, TET2-luc-wt4 and TET2-luc-mut4, TET2-luc-wt5 and TET2-luc-mut5) were purchased from GenePharma. When U87MG and U251MG cells grew to 70% confluence, 2 μg wild or mutant reporter plasmids were cotransfected with miR-19a-5p mimics or NC mimic using a Lipofectamine 2000 (Invitrogen), separately. Forty-eight hours later, the fluorescence changes in each group were measured by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol.

2.11 | Statistical analysis

All data were collected from 3 independent repeated experiments and expressed as mean ± SD. Statistical analysis was determined by GraphPad Prism V5.0 (GraphPad Software, La Jolla, CA, USA) software and SPSS 19.0 statistical software (IBM, Armonk, NY, USA). Correlations between AC016405.3 and clinicopathological features of patients with GBM were determined by Pearson's χ^2-test or Fisher's exact test. Survival analysis was undertaken using a log-rank test. Differences between 2 groups were analyzed by Student's *t* test. One-way ANOVA was used for analyzing differences among multiple sets of data. Differences were considered significant if $^{*}P < .05$, $^{**}P < .01$, or $^{***}P < .0001$, individually.

3 | RESULTS

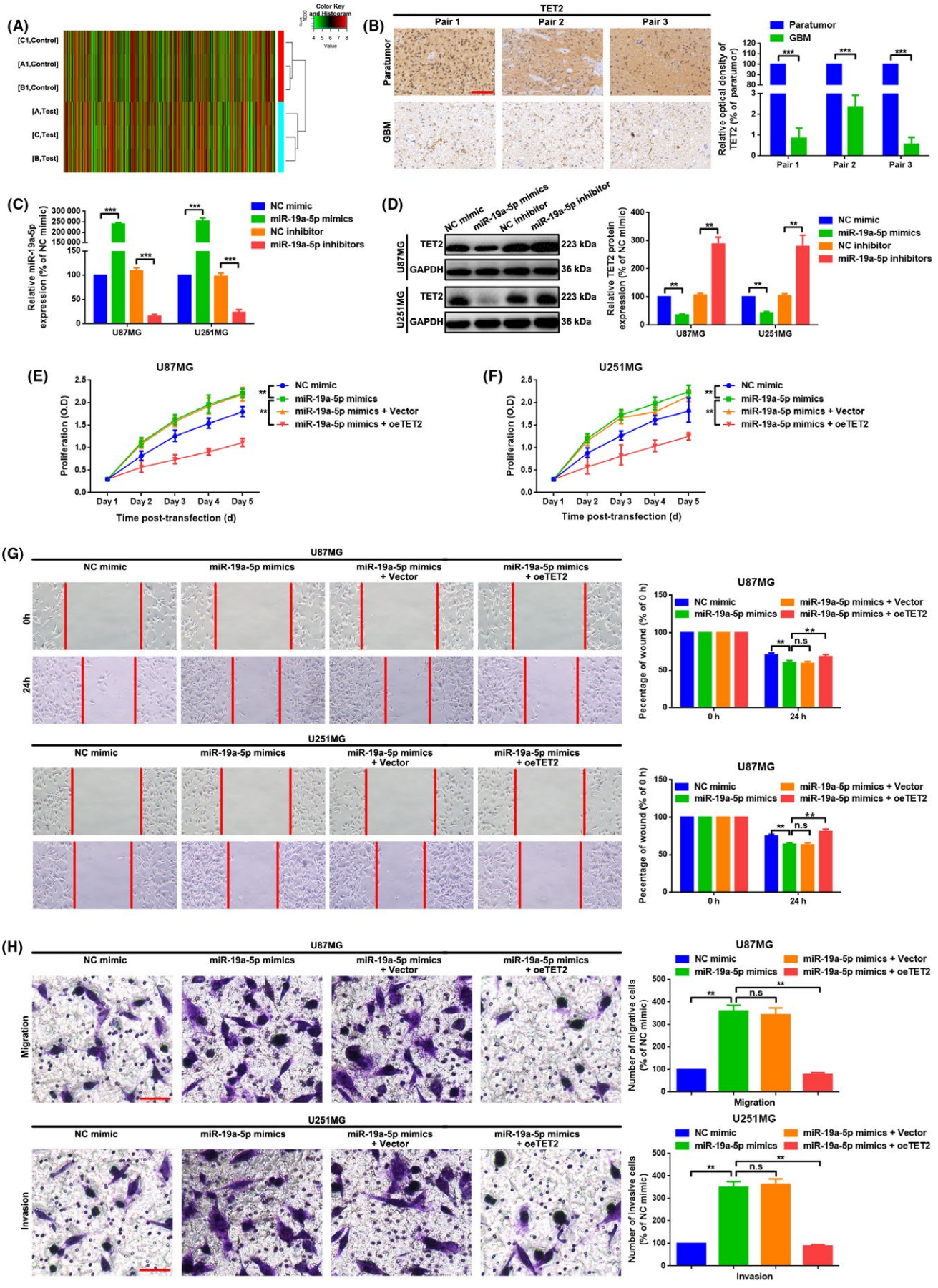
3.1 | AC016405.3 downregulated and correlated with poor prognosis in GBM patients

According to a previous genome-wide lncRNA microarray analysis in GBM (GSE104267), 13 downregulated lncRNAs with criteria of LogFC < −2 and $P < .001$ were primarily screened out (Table S2). Among them, we measured the expression of 10 lncRNAs whose lengths were <1000 bp in 3 collected freshly frozen GBM tissue specimens and 3 paired adjacent normal brain tissue specimens. As shown in the data presented in Figure 1A, AC016405.3 was stably downregulated in all 3 of the paired GBM tissue specimens, and was therefore selected for the following research. Furthermore, AC016405.3 also showed downregulation in 9 GBM samples compared to 3 normal brain samples according to an analysis of GEO dataset GSE104267 (Figure 1B). Additionally, the expression of AC016405.3 in 64 paraffin-embedded GBM tissues was determined. As the results in Figure 1C show, AC016405.3 was downregulated in most (95.31%, 61/64) GBM tissues. We also analyzed the expression of AC016405.3 in different grades of GBM tissue specimens by ISH analysis. As the representative photographs reveal in Figure 1D, the expression of AC016405.3 was gradually downregulated with an advanced grading ($P < .001$). Clinically, we analyzed the correlation between elevated AC016405.3 and the clinicopathological features of patients with GBM. As displayed in Figure 1E and Table 1, low expression of AC016405.3 was closely correlated with a shorter survival rate (determined by a Kaplan-Meier analysis, $P = .014$), a larger diameter ($P = .002$), a higher pathological grading ($P = .000$), and more frequent distant metastasis ($P = .012$). Finally, we detected the expression of AC016405.3 at a cellular level. As the findings of qRT-PCR show (Figure 1F), compared with NHA, AC016405.3 was downregulated in 2 GBM cell lines, U87MG and U251MG.

3.2 | Upregulation of AC016405.3 suppressed proliferation and metastasis in U87MG and U251MG cells

In this section, we undertook gain-of-function experiments to explore the role of AC016405.3 played in GBM. First, AC016405.3 was upregulated in U87MG and U251 cells by transfecting AC016405.3-overexpressing plasmid (Figure 2A,B). Then, we used a CCK-8 assay to detect the proliferative ability changes of U87MG and U251MG cells. As shown in Figure 2C,D, upregulation of AC016405.3

FIGURE 4 MicroRNA (miR)-19a-5p promoted proliferation and metastasis by targeting suppression of TET2 in U87MG and U251MG cells. A, Hierarchical clustering analysis of mRNAs that were differentially expressed between test (miR-19a-5p knocked down) and control (>2.0-fold; $P < .05$; filtered to show the upregulated or downregulated mRNAs). Expression values are represented in shades of red and green, indicating expression above and below the median expression value across all samples, respectively. B, TET2 was downregulated in glioblastoma multiforme (GBM) tissue specimens compared with that in paratumor tissue specimens, determined by immunohistochemistry assay. $^{***}P < .001$ vs paratumor group. C,D, Up- and downregulation of miR-19a-5p (C) inversely regulated TET2 protein expression (D). $^{***}P < .001$ vs negative control (NC) mimic or NC inhibitor group, individually. E,F, Upregulation of miR-19a-5p promoted U87MG (E) and U251MG (F) cell proliferation, but the facilitative effect was remarkably abolished by a TET2 overexpression plasmid (oeTET2), checked by a CCK-8 assay. G,H, Upregulation of miR-19a-5p promoted U87MG and U251MG cell metastasis, but the promotive effect was remarkably attenuated by an increase of TET2 as measured by a wound healing assay (G) and a Transwell assay (H). Data are shown as mean ± SD from 3 independent experiments. $^{**}P < .01$ vs miR-19a-5p mimics group. n.s., $P > .05$


(A)
[C1,Control]
[A1,Control]
[B1,Control]
[A,Test]
[C,Test]
[B,Test]
Color Key and Histogram
Value
(B)
TET2
Pair 1
Pair 2
Pair 3
Paratumor
GBM
Relative optical density of TET2 (% of paratumor)
Paratumor
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(C)
Relative miR-19a-5p expression (% of NC mimic)
NC mimic
miR-19a-5p mimics
NC inhibitor
miR-19a-5p inhibitors
U87MG
U251MG
(D)
NC mimic
miR-19a-5p mimics
NC inhibitor
miR-19a-5p inhibitor
U87MG
U251MG
TET2
GAPDH
223 kDa
36 kDa
Relative TET2 protein expression (% of NC mimic)
(E)
U87MG
Proliferation (O.D)
Time post-transfection (d)
Day 1
Day 2
Day 3
Day 4
Day 5
NC mimic
miR-19a-5p mimics
miR-19a-5p mimics + Vector
miR-19a-5p mimics + oeTET2
(F)
U251MG
(G)
U87MG
U251MG
0h
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Pecentage of wound (% of 0 h)
n.s
(H)
Migration
Invasion
Number of migrative cells (% of NC mimic)
Number of invasive cells (% of NC mimic)

suppressed U87MG and U251MG cell proliferation. Additionally, we found that upregulation of AC016405.3 inhibited the metastatic ability U87MG and U251MG cells (Figure 2E-H).

3.3 | MicroRNA-19a-5p involved in AC016405.3-mediated suppression of proliferation and metastasis by directly targeting U87MG and U251MG cells

Long noncoding RNAs are well known to work as ceRNAs by interacting with downstream miRNAs. According to the results of bioinformatics analysis by online software Diana-lncBase (http://carolina.imis.athena-innovation.gr), we first measured the expression of the top 12 high binding score (score >0.9) miRNAs that might bind to AC016405.3 (Figure S2) in the collected three freshly frozen GBM tissue specimens. As shown in Figure 3A, miR-19a-5p was stably highly expressed in the 3 collected freshly frozen GBM tissue specimens and thus selected for further study. According to another analysis of GEO dataset GSE103228, miR-19a-5p was upregulated in 5 GBM samples compared to 5 normal brain samples (Figure 3B). Additionally, we measured the expression of miR-19a-5p at a cellular level. As the data show in Figure 3C, miR-19a-5p was also upregulated in U87MG and U251MG cells compared to NHA. Furthermore, we found that miR-19a-5p was negatively correlated with AC016405.3 (Figure 3D). We illustrated that miR-19a-5p and AC016405.3 suppressed each other in a reciprocal manner (Figure 3E,F). We also found that an increase of miR-19a-5p partially reversed the suppressive effect that upregulation of AC016405.3 had on U87MG and U251MG cell proliferation and metastasis (Figure 3G-J). Finally, using a luciferase assay, we illustrated that miR-19a-5p could directly bind to AC016405.3 through the theoretical miRNA response element (Figure 3K-M).

3.4 | MicroRNA-19a-5p promoted proliferation and metastasis by targeting suppression of TET2 in U87MG and U251MG cells

According to the outcomes of our present research and previous studies, miR-19a-5p works as an oncogene in GBM.[20,22,23] Here, we tried to explore downstream genes of miR-19a-5p. We knocked down miR-19a-5p by transfecting miR-19a-5p shRNA (Figure S3) and then compared the differentially expressed gene profiles by microarray analysis. The results indicated 351 genes (fold change cut-off value 2.0) were upregulated in U87MG and U251MG cells with miR-19a-5p knockdown (Figure 4A and Table S3). Among the 351 genes, *TET2* was selected for its most significant changes (criteria of LogFC >6 and $P < .001$) for further study. First, *TET2* was found to be downregulated in GBM tissue specimens (Figure 4B). Second, upregulation and downregulation of miR-19a-5p were found to negatively regulate TET2 expression (Figure 4C,D). Third, miR-19a-5p was able to bind to different sites (positions 302-308, 820-826, 1671-1677, 2053-2059, and 3470-3476) of TET2 by direct targeting (Figure S4). Finally, a TET2 overexpression plasmid (oeTET2) was functionally displayed to remarkably attenuate the facilitative effect that upregulation of miR-19a-5p had on U87MG and U251MG cell proliferation and metastasis (Figure 4E-H).

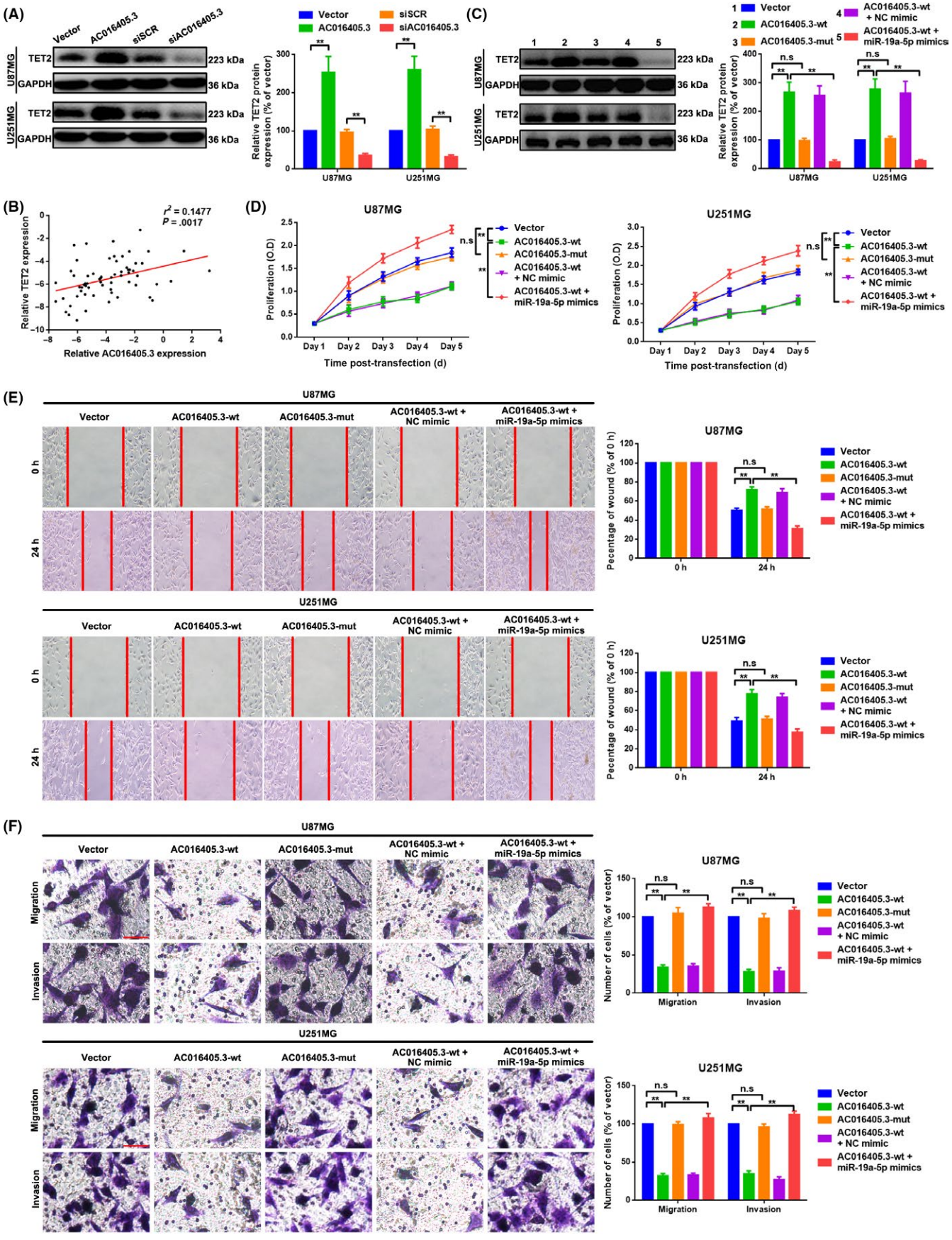
3.5 | AC016405.3 suppressed TET2-mediated proliferation and metastasis by miR-19a-5p sponging in U87MG and U251MG cells

In this section, we tried to determine the relationship among AC016405.3, miR-19a-5p, and TET2. First, we showed that upregulation and downregulation of AC016405.3 positively regulated TET2 protein expression (Figure 5A). Second, we found that the expression of TET2 was positively correlated with AC016405.3 (Figure 5B). In addition, we found that when the theoretical miR-19a-5p response elements (MRE-19a-5p) in AC016405.3 was mutated (AC016405.3-mut), the facilitative effect that AC016405.3 overexpression plasmids (AC016405.3-wt) had on TET2 was dismissed. Additionally, the promotive effect of AC016405.3-wt on TET2 was significantly attenuated by an upregulation of miR-19a-5p (cotransfection of AC016405.3-wt and miR-19a-5p mimics) (Figure 5C). Finally, we functionally identified that AC016405.3-wt but not AC016405.3-mut could suppress U87MG and U251MG cell proliferation and metastasis, and the suppressive effect was markedly reversed by an upregulation of miR-19a-5p (cotransfection of AC016405.3-wt and miR-19a-5p mimics) (5D-Ff). In summary, all the findings above indicated that AC016405.3 suppressed proliferation and metastasis through modulation of TET2 by sponging miR-19a-5p in U87MG and U251MG cells.

4 | DISCUSSION

Accumulative evidence has indicated that lncRNAs are ectopically expressed and are widely involved in multiple biological processes of GBM.[24-26] Long noncoding RNAs play crucial roles in proliferation and metastasis of GBM. Pastori et al reported that lncRNA HOX transcript antisense RNA (HOTAIR) is an essential driver of GBM cell proliferation; overexpression of HOTAIR in conjunction with I-BET151 treatment abrogates the antiproliferative activity of the BET bromodomain inhibitor.[27] Shi et al found that lncRNA

FIGURE 5 AC016405.3 suppressed TET2-mediated proliferation and metastasis through microRNA (miR)-19a-5p sponging in U87MG and U251MG cells. A, Expression of TET2 was positively correlated with AC016405.3. B, Up- and downregulation of AC016405.3 positively regulated TET2 protein expression. C, AC016405.3-wt, but not AC016405.3-mut, promoted TET2 protein expression, and the facilitative effect was abrogated by an increase of miR-19a-5p as measured by western blot assay. D, AC016405.3-wt, but not AC016405.3-mut, suppressed glioblastoma multiforme cell proliferation, but the inhibitive effect was reversed by an upregulation of miR-19a-5p as checked by a CCK-8 assay. E,F, AC016405.3-wt, but not AC016405.3-mut, suppressed glioblastoma multiforme cell metastasis, but the inhibitive effect was reversed by an upregulation of miR-19a-5p as determined by wound healing (E) and Transwell assays (F). $^{**}P < .01$ vs vector or AC016405.3-wt group, individually. n.s., $P > 0.05$


(A)
Vector
AC016405.3
siSCR
siAC016405.3
U87MG
TET2
GAPDH
223 kDa
36 kDa
U251MG
Relative TET2 protein expression (% of vector)
(B)
r² = 0.1477
P = .0017
Relative TET2 expression
Relative AC016405.3 expression
(C)
1 Vector
2 AC016405.3-wt
3 AC016405.3-mut
4 AC016405.3-wt + NC mimic
5 AC016405.3-wt + miR-19a-5p mimics
n.s
(D)
U87MG
U251MG
Proliferation (O.D)
Day 1
Day 2
Day 3
Day 4
Day 5
Time post-transfection (d)
(E)
U87MG
U251MG
Vector
AC016405.3-wt
AC016405.3-mut
AC016405.3-wt + NC mimic
AC016405.3-wt + miR-19a-5p mimics
0 h
24 h
Pecentage of wound (% of 0 h)
(F)
Migration
Invasion
Number of cells (% of vector)

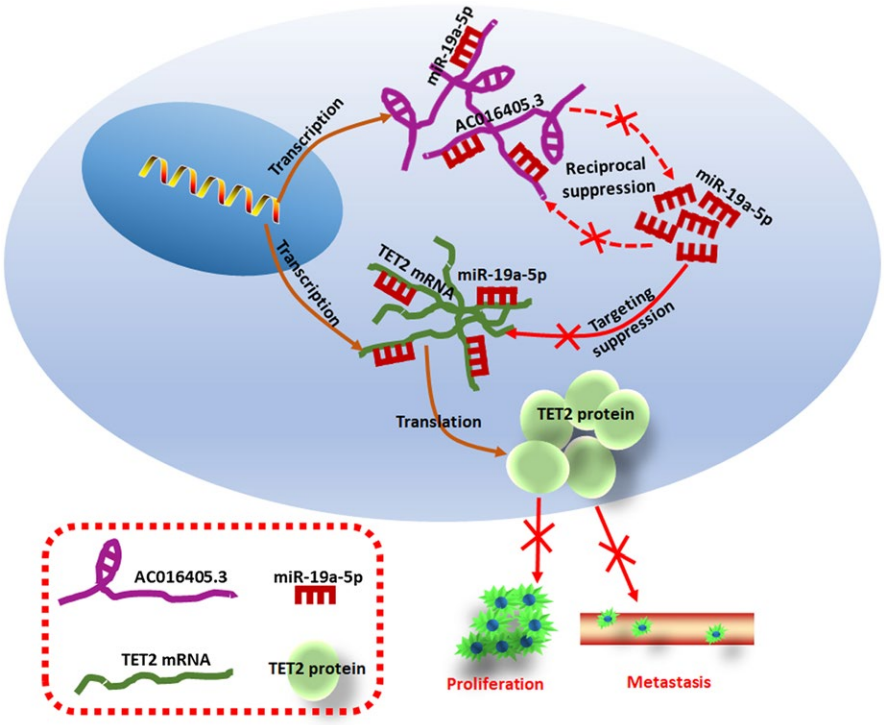


FIGURE 6 Schematic diagram of mechanism of this research. miR, microRNA

HERG promoted cell proliferation, migration, and invasion by acting as a ceRNA of miR-940 in GBM.[28] AC016405.3, also named RP11-44N11.2, is a novel lncRNA located at chromosome 8, from 122 779 971 to 122 780 830. AC016405.3 is 860 bp in length and contains 1 exon. According to the previous genome-wide lncRNA microarray analysis of GSE104267, AC016405.3 was downregulated in 9 GBM samples compared to 3 normal brain samples. Our present study also indicated that AC016405.3 was stably downregulated in GBM tissue specimens and GBM cell lines. Through a Pearson's χ^2 test and a Kaplan-Meier analysis, we found that AC016405.3 was closely correlated with several aggressive features, including a shorter survival rate, bigger tumor size, higher pathological grading, and more frequent distant metastasis in patients with GBM. Functionally, an upregulation of AC016405.3 was show to suppress GBM cell proliferation and metastasis, and these findings indicated that AC016405.3 might work as an anti-oncogene in GBM.

MicroRNAs are short (20-24 nt) noncoding RNAs that are involved in posttranscriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of mRNAs. MicroRNAs are also extensively involved in the complex pathogenesis of multiple cancers.[29] As a member of the miRNA family, miR-19a-5p is located at chromosome 13q31.3 and is reported as an oncogene in various malignancies. Chen et al found that miR-19a is upregulated in glioma; an elevation of miR-19a promoted glioma cell proliferation and invasion by targeting Ras homolog family member B.[30] In the current study, we also demonstrated that miR-19a-5p was upregulated in GBM tissue specimens and cell lines as previously reported. Additionally, miR-19a-5p was found to be negatively correlated with AC016405.3. We further showed a reciprocal inhibitive effect between miR-19a-5p and AC016405.3. Functionally, we identified that miR-19a-5p was a crucial downstream molecule in AC016405.3-mediated proliferation and metastasis. An upregulation of miR-19a-5p abrogated the suppressive effect that overexpression of AC016405.3 had on GBM cell proliferation and metastasis. Moreover, through microarray analysis, a series of functional experiments, and a constructed luciferase assay, we illustrated that TET2 was a downstream target of miR-19a-5p.

As a novel anti-oncogene, TET2 belongs to the TET family and is located at chromosome 4q24. It is most frequently mutated in several solid malignancies and is closely correlated with DNA methylation.[31] Zhu et al reported that TET2 inhibited tumorigenesis of MCF-7 cells by regulating caspase-4.[32] Chen et al found that TET2 was frequently downregulated in glioma and that ectopic expression of TET2 inhibited the invasion and proliferation of glioma cells.[33] In the present study, we found that the expression of TET2 was most significantly changed after miR-19a-5p knockdown in GBM cells. We further elucidated that TET2 was downregulated in GBM at the tissue level. As a downstream target of miR-19a-5p, ectopic overexpression of TET2 remarkably abrogated the facilitative effect of miR-19a-5p on GBM. These findings indicated that TET2 worked as an anti-oncogene in GBM. Also, the expression of TET2 and AC016405.3 was shown to present an obvious positive correlation. TET2 was positively regulated by AC016405.3. Finally, a wild AC016405.3 overexpression plasmid was shown to promote TET2 expression, and it mediated proliferation and metastasis; when the MREs-19a-5p in AC016405.3 was mutated, the promotive effect was reversed. Taken as a whole, as the diagram explains in Figure 6, all the outcomes presented in the current research indicated that

AC016405.3 was an anti-oncogene in GBM, and it suppressed TET2-mediated proliferation and metastasis by competitively sponging miR-19a-5p.

In conclusion, AC016405.3, a novel lncRNA, was identified as an anti-oncogenic lncRNA in GBM. We uncovered a novel pathway in which AC016405.3 sponges miR-19a-5p to modulate TET2-mediated GBM proliferation and metastasis. Our findings indicated a new molecular axis in the treatment of GBM.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (81172180).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Ren S, Xu Y. AC016405.3, a novel long noncoding RNA, acts as a tumor suppressor through modulation of TET2 by microRNA-19a-5p sponging in glioblastoma. *Cancer Sci*. 2019;110:1621–1632. https://doi.org/10.1111/cas.14002