

# Long non-coding RNA GHET1 promotes viability, migration and invasion of glioma cell line U251 by down-regulation of miR-216a

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**Abstract.** – **OBJECTIVE:** Glioma is among the most aggressive of all human malignancies. Long non-coding RNA (lncRNA) gastric carcinoma highly expressed transcript 1 (GHET1) was considered an important oncogene in tumors. However, the role of GHET1 in glioma was rarely studied. The present work aimed to explore the effect of GHET1 on glioma cell line U251.

**MATERIALS AND METHODS:** The viability, migration and invasion of U251 cells were analyzed by Cell Counting Kit-8 (CCK-8) assay, and transwell migration/invasion assay, respectively. The relative expression of GHET1 and miR-216a were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The expression of cell cycle-related proteins, cell metastasis-associated proteins and main factors in the JAK2/STAT3 and p53/survivin pathways was analyzed by Western blot. Cell transfection assay was conducted to alter expression of GHET1 and miR-216a in U251 cells.

**RESULTS:** The viability, migration and invasion of U251 cells were promoted by GHET1 overexpression. The pro-cell cycle genes including Cyclin D1, CDK4 and CDK6, and the pro-metastasis genes including MMP-9 and Vimentin were up-regulated after GHET1 was overexpressed. MiR-216a was found to be down-regulated by GHET1 overexpression, and it was involved in the effect of GHET1 on U251 cells. GHET1 might promote U251 cells by down-regulating miR-216a. Finally, we found that GHET1 overexpression activated the JAK2/STAT3 and p53/survivin signaling pathways by down-regulating miR-216a.

**CONCLUSIONS:** GHET1 overexpression increased viability, migration and invasion of U251 cells by down-regulating miR-216a. Mechanically, GHET1-miR-216a axis activated the JAK2/STAT3 and p53/survivin signaling pathways.

*Key Words:*

Glioma, GHET1, MiR-216a, JAK2/STAT3 pathway, p53/survivin pathway.

## Introduction

Glioma is considered the most aggressive brain tumor due to its high incidence and fatality rate<sup>1</sup>. Different therapeutic strategies have been explored to regress the tumor growth and progression; however, there is currently no effective drug therapy for glioma<sup>2</sup>. The major defects of drug treatment for glioma impede their application as the candidate drugs are not able to penetrate the blood-brain barrier and tumor cell specificity<sup>3,4</sup>. Therefore, targeting gene therapy which avoids the drug's shortcomings has attracted extensive attention.

Although research still has a long way to go to reach the goal of applying the targeting gene therapy in clinical practice, great progress in this field has been made. In recent decades, abundant long non-coding RNAs (lncRNAs) and microRNAs (miRNAs/miRs) were found to participate in molecular functions and pathological implications in cancers<sup>5</sup>. The prognostic and clinicopathological value of lncRNAs and miRNAs in cancers was also widely explored<sup>6-8</sup>. Abnormal lncRNA and miRNA expression profiles in clinical glioma specimens are related to malignant grades and tissue differentiation, which has significant clinical implications in glioma diagnosis of prognostication and sub-classification<sup>9-12</sup>.

lncRNA gastric carcinoma highly expressed transcript 1 (GHET1) has been identified as an oncogene in various types of cancers, including gastric cancer<sup>13</sup>, pancreatic cancer<sup>14</sup>, hepatocellular carcinoma<sup>15,16</sup> and esophageal squamous cell carcinoma<sup>17</sup>. Knockdown of GHET1 could suppress cancer cell proliferation and invasion<sup>18</sup>. Emerging evidence has confirmed the relevance of GHET1 to diverse types of human cancer, but the study analyzing the role of GHET1 in glioma is rare.

MiR-216a was found to be significantly decreased in glioma tissues and cell lines, and high level of miR-216a could suppress the proliferation, migration and invasion of glioma cells<sup>19</sup>, indicating the important role of miR-216a in glioma. Recent reports have indicated that lncRNAs could potentially interact with other classes of non-coding RNAs including miRNAs and also modulate their regulatory role by interacting with them. Jalali et al<sup>20</sup> hypothesized that lncRNAs could participate as a layer of regulatory interactions with miRNAs.

Herein, we analyzed the effect of GHET1 on glioma cells and explored the regulatory effect of GHET1 on miR-216a expression. Finally, the action mechanism of GHET1-miR-216a axis in glioma cells was studied by detecting the JAK2/STAT3 and p53/survivin signaling pathways.

## Materials and Methods

### Cell Culture

The human glioma cell line U251 was obtained from the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 2 mM L-glutamine was obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was added in RPMI-1640 medium with the percentage of 10%. U251 cells were cultured in RPMI-1640 medium at 37°C with saturated humidity and 5% CO<sub>2</sub>. The cells were passaged every 3 days with 0.25% trypsin digesting for 3 min followed by seeding of the cell suspension to the required concentration.

### Transfection Assay

GHET1 sequence with full length was ligated into the pcDNA3.1 vector, which was referred to as pc-GHET1. The pre-miR-216a, anti-miR-216a and their negative control (NC) were synthesized by GenePharma Co. (Shanghai, China). Cell transfection assay was conducted using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

### Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized using MultiScribe™ Re-

verse Transcriptase kit (Applied Biosystems, Foster City, CA, USA) and Real Time-Polymerase Chain Reaction (RT-PCR) was performed using TB Green™ Premix Ex Taq™ II (TaKaRa, Otsu, Shiga, Japan). The expression of GHET1 was normalized to GAPDH. For determining miR-216a expression, the TaqMan™ MicroRNA Reverse Transcription Kit and TaqMan™ Universal Master Mix II (both Applied Biosystems, Foster City, CA, USA) was used, and U6 acted as an internal control. The relative expression of GHET1 and miR-216a were calculated by relative quantification 2<sup>-ΔΔCt</sup> method<sup>21</sup>.

### Cell Counting Kit-8 (CCK-8) Assay

Cell viability was assessed by a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Dojindo, Kumamoto, Japan). Cells were inoculated in a 96-well plate with 5000 cells/well. After stimulation, 20 μL of CCK-8 solution was added to the culture medium, and the 96-well plate was incubated for 4 h at 37°C in humidified 95% air and 5% CO<sub>2</sub>. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

### Migration and Invasion Assay

The migratory and invasive abilities were determined by transwell assay using transwell chamber with the pore size of 8 μm. For evaluating cell migration, the transwell chamber was directly used, but for determining cell invasion, the insert was pre-coated with 80 μL of Matrigel solution (500 ng/μL; BD Biosciences, Franklin Lakes, NJ, USA). The upper chamber was added with 100 μL serum-free medium and cells were seeded in the medium with a concentration of 1000 cells/mL. Meanwhile, 600 μL of complete medium was added into the lower chamber. After incubation of 48 h, cells on the upper surface of the insert were removed. The migrated or invasive cells on the lower side of the membrane were stained with crystal violet and counted.

### Western Blot

Cells were collected and lysed in Triton X-100 lysis buffer containing 25 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 4°C. Equivalent amounts of proteins (20 μg) were denatured at 100°C in loading buffer for 10 min and resolved on 7.5-12% sodium do-

decyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) gels, followed by transfer to polyvinylidene difluoride (PVDF) membranes. The resulting blots were blocked overnight with 5% nonfat dry milk. The membrane was probed with primary antibodies and proper secondary antibodies. The primary antibodies including anti-CDK4 (ab137675), CDK6 (ab151247), anti-Cyclin D1 (ab226977), anti-MMP-9 (ab38898), anti-TIMP-1 (ab61224), anti-TIMP-2 (ab180630), anti-Vimentin (ab137321), anti-JAK2 (ab39636), anti-p-JAK2 (ab195055), anti-STAT3 (ab5073), anti-p-STAT3 (ab30647), anti-p53 (ab131442), and anti-survivin (ab469, all Abcam, Cambridge, MA, USA) were used at a dilution of 1:1000, which produced a signal of approximately 34 kDa, 37 kDa, 34 kDa, 92 kDa, 23 kDa, 24 kDa, 54 kDa, 131 kDa, 131 kDa, 85 kDa, 88 kDa, 53 kDa, and 16 kDa, respectively.  $\beta$ -actin (ab8227, Abcam, Cambridge, MA, USA) was used as a loading control. After washing, the bounding antibody was visualized using the enhanced chemiluminescence assay kit (Tiangen Biotechnology Corp., Beijing, China) according to the manufacturer's instructions.

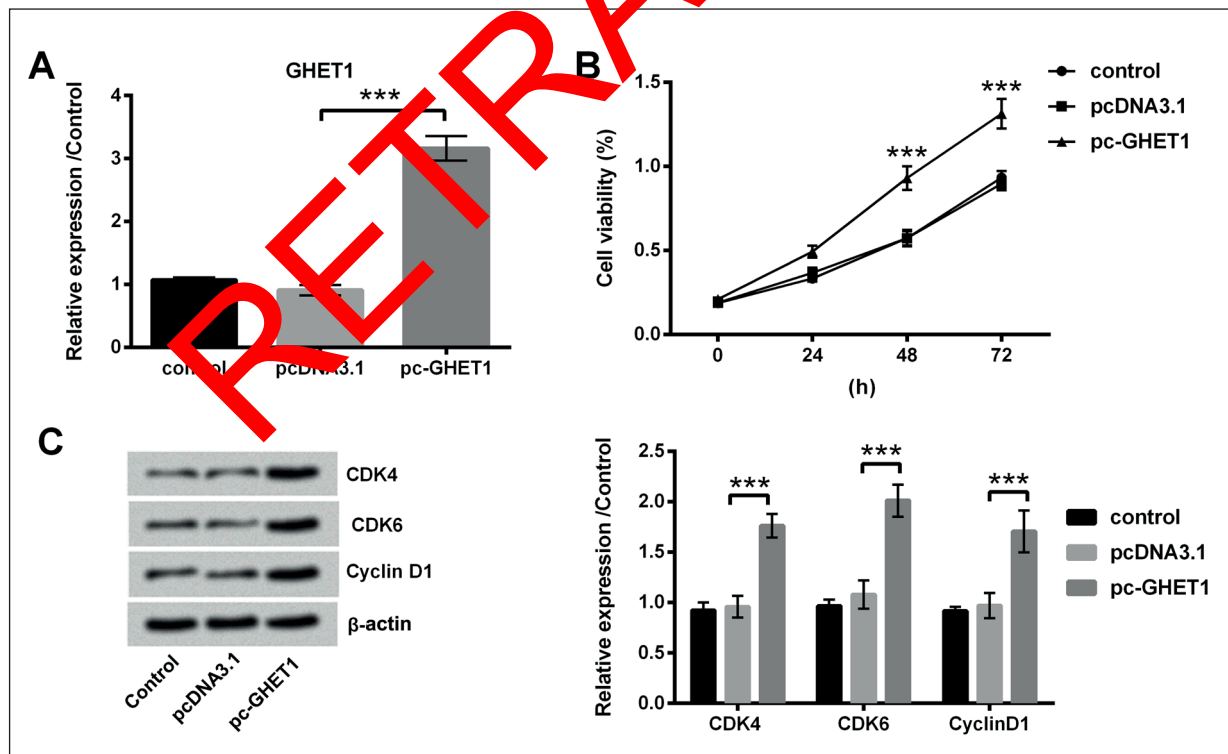
### Statistical Analysis

All data are shown as means  $\pm$  SD from three to six samples. Data analysis was performed using GraphPad Prism version 6.0 software (GraphPad Software, La Jolla, CA, USA). One- or two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used for statistical evaluation. A  $p$ -value below 0.05 ( $p < 0.05$ ) was considered statistically significant.

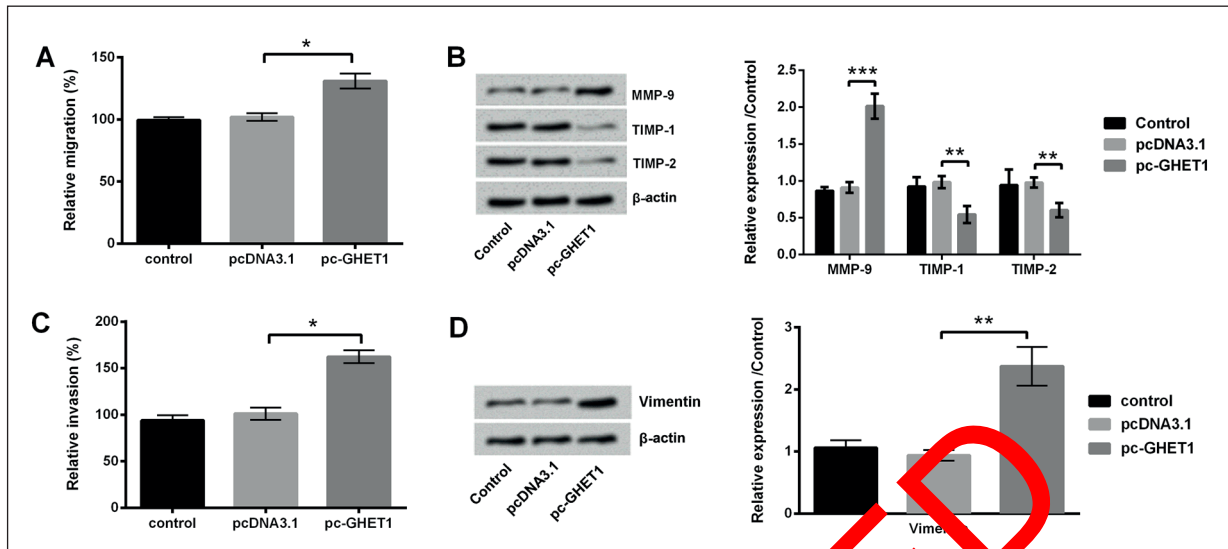
## Results

### GHET1 Promoted Growth of Glioma Cells

The expression of GHET1 was analyzed after transfection with pc-GHET1 and the result showed that it was up-regulated after transfection ( $p < 0.001$ , Figure 1A). Next, the effects of GHET1 overexpression on cell viability were evaluated after different incubation time. After incubation for 48 h and 72 h, cell viability was significantly enhanced by GHET1 overexpression (both  $p < 0.001$ , Figure 1B). Additionally, the expression



**Figure 1.** GHET1 overexpression promoted viability and growth of U251 cells. **A**, GHET1 overexpression after transfection with pc-GHET1. **B**, Viability of U251 cells in cells overexpressing GHET1. **C**, Expression of proliferation-associated proteins in cells expressing GHET1. \*\*\* $p < 0.001$ .



**Figure 2.** GHET1 overexpression increased migration and invasion of U251 cells. **A**, The increased cell migration in cells with GHET1 overexpression. **B**, The increased expression of the pro-migratory protein. **C**, The increased cell invasion in cells with GHET1 overexpression. **D**, The increased expression of the pro-invasive protein. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

of CDK4, CDK6, and Cyclin D1 was found to be increased in GHET1-overexpressed cells (all  $p < 0.001$ , Figure 1C).

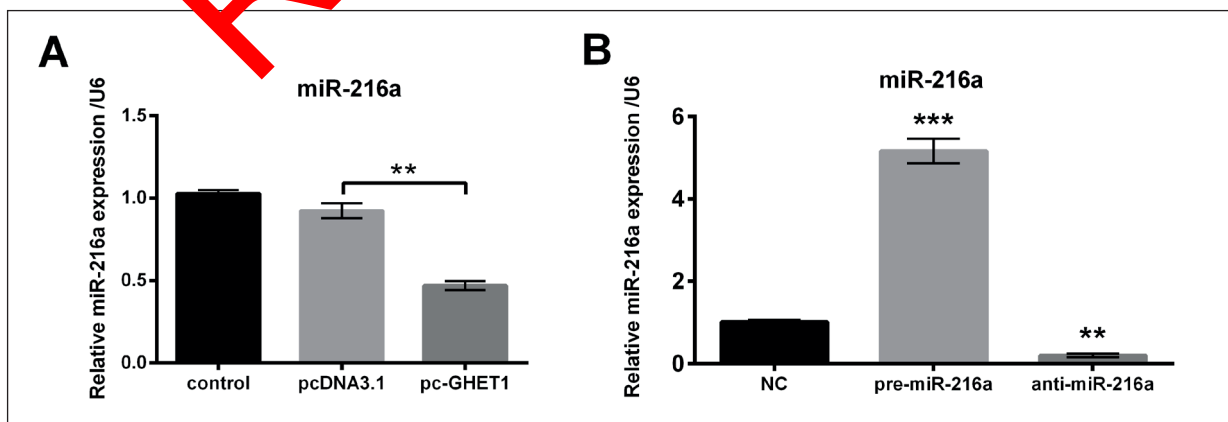
### GHET1 Promoted Migration and Invasion of Glioma Cells

The migratory and invasive capacities of U251 cells were analyzed using transwell assays after GHET1 was up-regulated. The data showed that migration of U251 cells was markedly increased ( $p < 0.05$ , Figure 2A), the level of pro-migration protein MMP-9 was elevated ( $p < 0.001$ ), and the levels of MMP-9 inhibitors, TIMP-1 and TIMP-2, were decreased (both  $p < 0.01$ , Figure 2B). The invasion of U251 cells was promoted ( $p < 0.05$ , Fig-

ure 2C) and Vimentin expression was enhanced after GHET1 was up-regulated ( $p < 0.01$ , Figure 2D).

### MiR-216a Was Downregulated by GHET1 Overexpression

MiR-216a expression was found to be down-regulated by GHET1 overexpression ( $p < 0.01$ , Figure 3A). However, whether miR-216a participated in the function of GHET1 in glioma cells was not clear. Therefore, the role of miR-216a in U251 cells was analyzed. MiR-216a expression was altered by transfection assay with significant increase in pre-miR-216a treatment ( $p < 0.001$ ) and significant decrease in anti-miR-216a treatment ( $p < 0.01$ , Figure 3B).



**Figure 3.** MiR-216a was silenced after GHET1 overexpression. **A**, The decreased expression of miR-216a after GHET1 overexpression. **B**, The transfection efficacy of miR-216a. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### ***GHET1 Promoted Growth, Migration, and Invasion of Glioma Cells by Downregulating miR-216a***

As described above, GHET1 down-regulated miR-216a expression. Therefore, we subsequently evaluated the role of miR-216a in U251 cells. The data demonstrated that miR-216 overexpression impaired the effects of GHET1 overexpression on U251 cells with inhibiting viability ( $p < 0.01$ , Figure 4A), decreasing levels of CDK4, CDK6, and Cyclin D1 ( $p < 0.01$  or  $p < 0.001$ , Figure 4B), suppressing migration and invasion (both  $p < 0.05$ , Figure 4C and 4E), as well as reducing level of MMP-9, increasing levels of TIMP-1 and TIMP-2 (all  $p < 0.01$ , Figure 4D), and decreasing level of Vimentin ( $p < 0.01$ , Figure 4F). Oppositely, miR-216a silence enhanced the effects of GHET1 on U251 cells. Compared with the pc-GHET1 + NC group, cell viability, migration and invasion were further increased in the pc-GHET1 + anti-miR-216a group (all  $p < 0.05$ , Figure 4A, 4C, 4E), and meanwhile, the levels of proteins promoting proliferation and metastasis were also increased ( $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$ , Figure 4B, 4D, 4F). These data suggested that GHET1 might promote viability, migration, and invasion of glioma cells partly due to the down-regulation of miR-216a.

### ***GHET1 Activated the JAK2/STAT3 and p53/Survivin Signaling Pathways in Glioma Cells***

The signaling pathways involved in the function of GHET1 were analyzed. Western blot analysis showed that phosphorylation levels of JAK2 and STAT3 were increased by GHET1 overexpression (both  $p < 0.001$ ), indicating that the JAK2/STAT3 pathway was activated in GHET1-overexpressed cells (Figure 5A). Similarly, we found that the level of survivin was increased after GHET1 was up-regulated ( $p < 0.001$ , Figure 5B). The phosphorylation levels of JAK2, STAT3 and expression levels of survivin were inhibited (all  $p < 0.001$ ). The expression levels of p53 was increased ( $p < 0.001$ ) after miR-216a was overexpressed, whereas miR-216a silence exhibited the contrary effects. Thus, we speculated that GHET1 might activate the JAK2/STAT3 and p53/survivin signaling pathways by down-regulation of miR-216a.

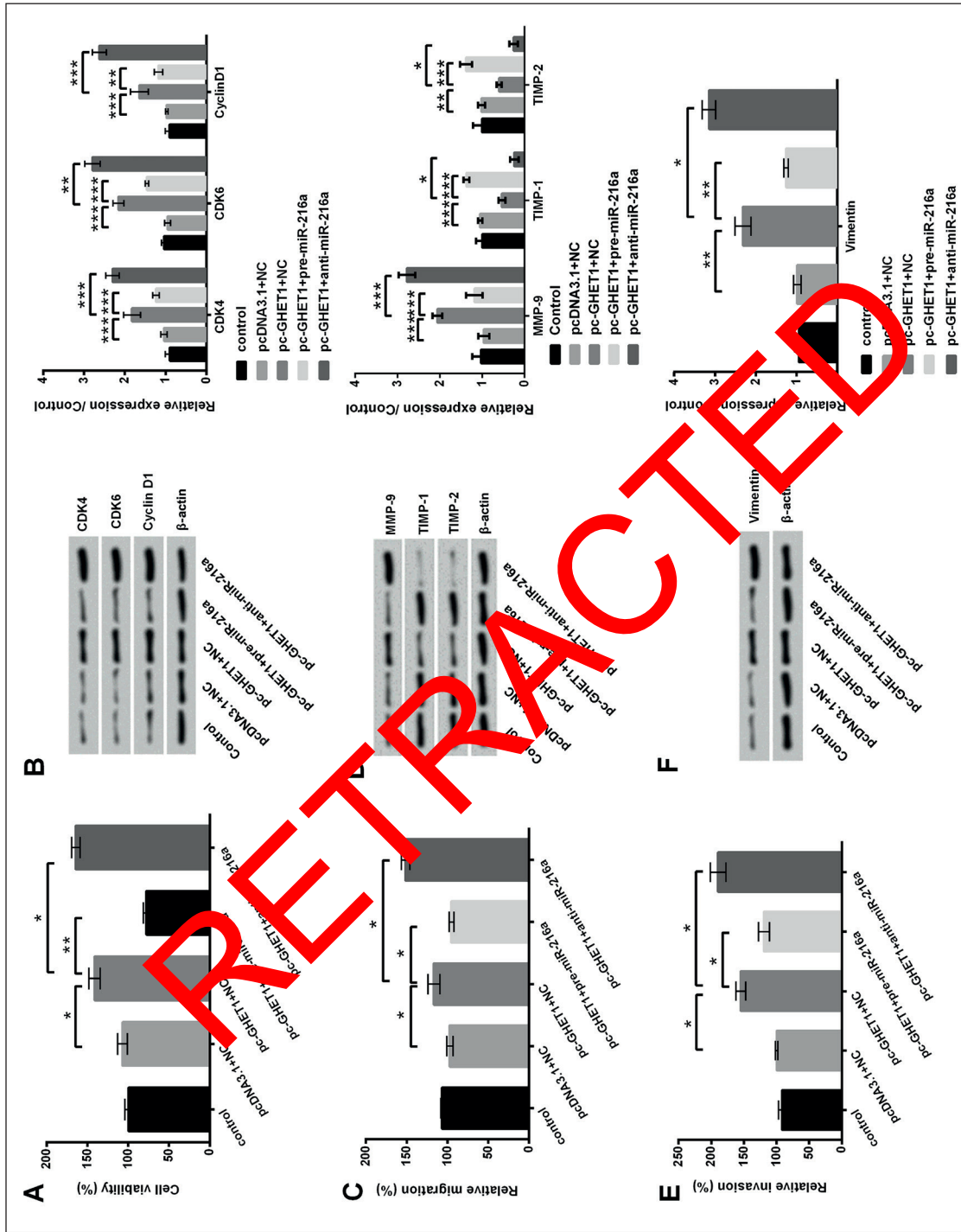
## **Discussion**

Glioma is one of the most prevalent forms of primary intracranial carcinoma. The increasing

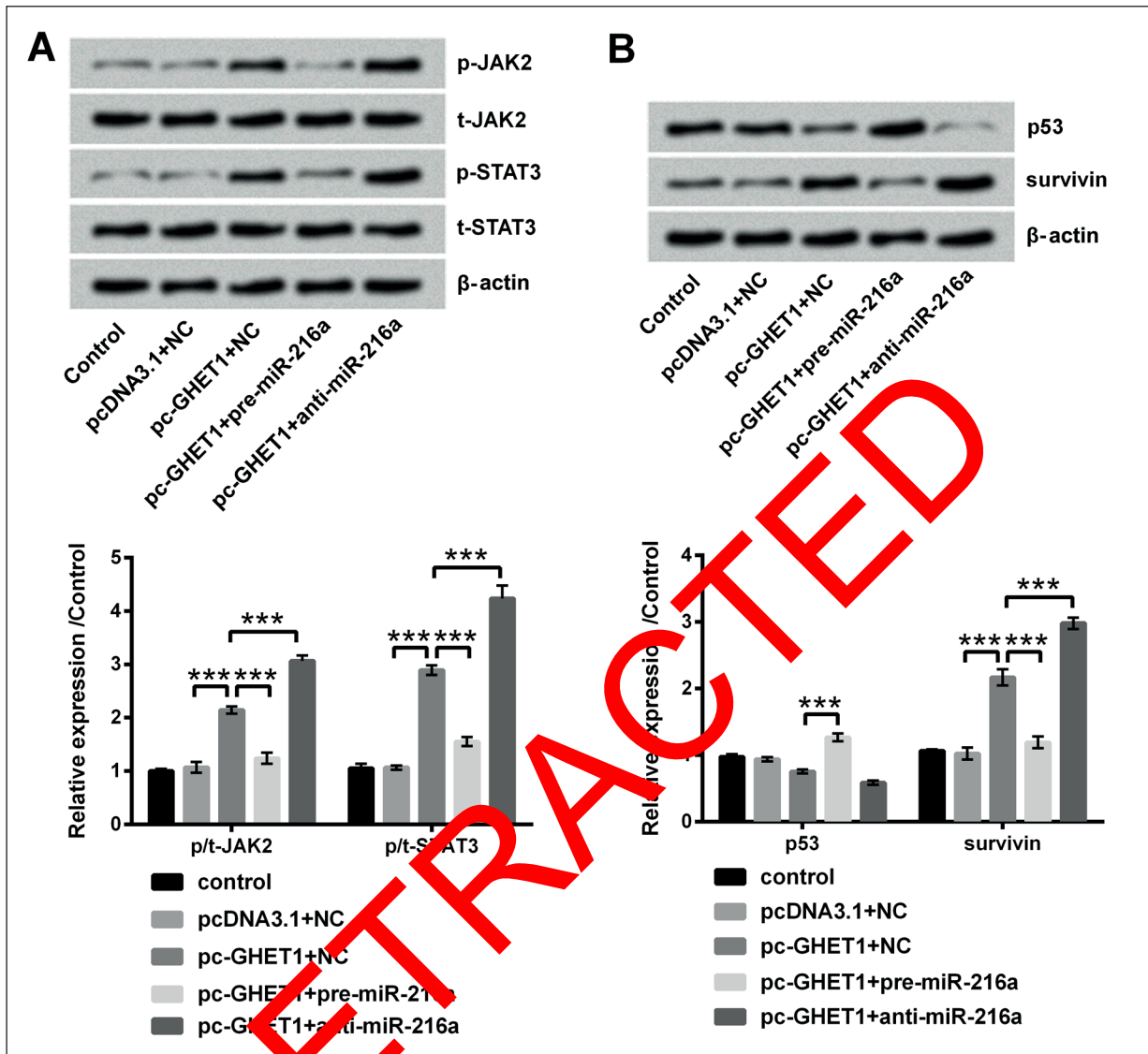
number of functional lncRNAs aberrantly expressed in glioma tissues and cell lines might be critical for glioma initiation, progression and other malignant phenotypes<sup>11</sup>. In our study, the effects of lncRNA GHET1 on viability, migration and invasion of glioma cell line U251 were analyzed. According to our data, GHET1 overexpression increased viability, migration and invasion of U251 cells. Subsequently, our work revealed that miR-216a had a key role in regulating growth and metastasis of U251 cells and miR-216a was negatively regulated by GHET1. We speculated that GHET1 might promote viability, migration and invasion of U251 cells partly by down-regulating miR-216a.

Increase evidence demonstrates that GHET1 is a tumor-promoter in many cancer types. The expression of GHET1 was relatively high in gastric cancer<sup>14</sup> and pancreatic cancer<sup>22</sup> and GHET1 expression was positively correlated to the tumor, node and metastasis staging of cancer<sup>14</sup>. GHET1 overexpression promoted viability, proliferation, migration and invasion of cancer cells, but GHET1 knockdown exerted the contrary results with increased apoptosis, inducing cell cycle arrest and inhibiting metastasis<sup>16,18,23</sup>, which were consistent with our work.

During the study of GHET1, we evaluated the expression of cell cycle-related protein (CDK4, CDK6, and Cyclin D1), cell migration/invasion-associated protein (MMP-9, TIMP-1, TIMP-2 and Vimentin). It is well-known that CDK4 and CDK6 are essential for the initiation of the cell cycle in response to mitogenic stimuli<sup>24</sup> and Cyclin D1 is a nuclear protein required for cell cycle progression in G1 phase<sup>25</sup>. The expression of these proteins was increased after GHET1 was overexpressed, indicating that the proliferation of U251 cells was promoted. MMP-9, an important member of the MMP gene family, is implicated in tissue destruction in various pathophysiologic conditions. Cell migration was in relation to the activation of MMP-9<sup>26</sup>. TIMP-1 and TIMP-2 are members of TIMP family capable of inhibiting the activities of all known MMPs<sup>27</sup>. TIMP-1 and TIMP-2 were shown to repress tumor growth, invasion and metastasis in experimental models<sup>28</sup>, and the expression of which was down-regulated in GHET1-overexpressed cells in our study. The intermediate filament protein Vimentin is a significant marker of epithelial-mesenchymal transition and a necessary regulator of mesenchymal cell migration. Excessive expression of Vimentin was found after GHET1 overexpression. All of



**Figure 4.** GHET1 promoted growth, migration, and invasion of U251 cells by down-regulating miR-216a. The role of miR-216a in the effects of GHET1 on *A*, viability, *B*, growth-associated gene expression, *C*, migration, *D*, migration-related gene expression, *E*, invasion, and *F*, invasion-related gene expression. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 5.** GHET1 activated the JAK2/STAT3 and p53/survivin signaling pathways by down-regulating miR-216a. **A**, The regulatory effect of GHET1-miR-216a on the JAK2/STAT3 pathway. **B**, The regulatory effect of GHET1-miR-216a on the p53/survivin pathway. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

these further confirmed that GHET1 was an oncogene in glioma.

To clarify the action of GHET1 in U251 cells, we analyzed the role of miR-216a because miR-216a expression could be negatively regulated by GHET1. The data demonstrated that miR-216a overexpression inhibited the increases of viability, migration and invasion of U251 cells induced by GHET1 overexpression; however, miR-216a silence aggravated the pro-cancer effect of GHET1. GHET1 might promote U251 cells in part by down-regulating miR-216a. This is not the first study indicating the anti-glioma property of

miR-216a, but we first found the regulatory role of miR-216a in pro-glioma function of GHET1. The previous study from Zhang et al<sup>19</sup> showed that augmented expression of miR-216a potently suppressed the proliferation, migration and invasion of glioma cells by targeting leucine-rich repeat-containing G protein-coupled receptor 5, which was consistent with our results.

The JAK2/STAT3 signaling pathway was involved in the formation mechanism of glioma<sup>29</sup>, and the inhibition of the JAK2/STAT3 signaling could enhance glioma cell killing<sup>30</sup>. Therefore, the activation of the JAK2/STAT3 pathway was

analyzed. Additionally, the p53/survivin pathway was also evaluated. The regulation of the p53/survivin signaling pathway is important for cell survival<sup>31</sup>, which plays an important role in regulating apoptosis of glioma cells<sup>32</sup>. According to our findings, GHET1 promoted glioma cell growth and metastasis by activating the JAK2/STAT3 and p53/survivin pathways. MiR-216a was involved in the modulation of GHET1 on the JAK2/STAT3 and p53/survivin pathways.

## Conclusions

The present work displayed that GHET1 promoted viability, migration and invasion of glioma cells partly by down-regulating miR-216a, during which process, the JAK2/STAT3 and p53/survivin signaling pathways were activated. We showed the pro-glioma role of GHET1, which might provide a new clue for the understanding of lncRNA-controlled cellular events in glioma.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

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