Up-regulation of miR-1825 inhibits the progression of glioblastoma by suppressing CDK14 though Wnt/β-catenin signaling pathway

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Abstract
Mounting evidences displayed that miRNAs modulated glioblastoma (GBM) development. Here, our goal was to investigate whether miR-1825 could regulate GBM development and explore its potential mechanism. Our findings displayed that miR-1825 was decreased in GBM tissue specimens by qRT-PCR and it confirmed as a prognostic marker of GBM by Kaplan-Meier survival analysis. Moreover, we also found that miR-1825 up-regulation suppressed GBM cell viability, tumor growth, invasion and migration by MTT assay, Xenograft assay and Transwell assay. Furthermore, CDK14 was first identified as the target of miR-1825 by Luciferase reporter assay and CDK14 acted as an oncogene in GBM development by Immunohistochemistry. In addition, Western blot analysis demonstrated that Wnt/β-catenin signaling pathway took part in GBM development modulating by miR-1825. In conclusion, miR-1825 up-regulation suppressed GBM progression by targeting CDK14 through Wnt/β-catenin pathway.

Introduction
Gliomas are tumors derived from neuroepithelial tumors, accounting for 40% to 50% of brain tumors [1 2]. They are the most common intracranial malignant tumors, which are caused by the interaction of congenital genetic high-risk factors and environmental carcinogenic factors [3 4]. Although the current treatment of GBM has made great progress, the survival rate of GBM patients was still very poor. Moreover, the high recurrence of GBM makes it very difficult to cure. Thus, it's very urgent to understand the underlying mechanism of GBM and then search for the target therapies for GBM. Currently, miRNAs were determined as the potential targets in cancer treatment [5 6] and modulated tumors development and progression by regulating their target genes [7-9]. In GBM, many miRNAs have been proved to participate in cell progression and development. For instance, miR-365 displayed the suppression effect on GBM cell viability and migration via targeting PAX6 [10]. Besides, miR-210 was proved to inhibit GBM cell invasion and migration ability by targeting BNDF [11]. Moreover, the findings of Cui T et al displayed that miR-4516 targeted PTPN14 to promote GBM cell progression and this axis provided an insight for treating GBM [12]. However, the researches on miR-1825 in GBM development and progression are very little. Xing W and his colleagues showed that miR-1825 was
decreased in glioma and it could suppress cell proliferation, invasion and facilitate cell apoptosis [13]. The reports about the underlying mechanism of miR-1825 in GBM have not been found in the literature.

TargetScan predicts some target genes for miR-1825, including CDK14. Cyclin-Dependent Kinase 14 (CDK14) is well known to regulate cell cycle and play important roles in cellular activities [14]. As previous studies displayed, CDK14 took part in multiple cancers development as a target of miRNAs [15]. For example, it was the target of miR-542 and involved in ovarian cancer cell proliferation, invasion and tumorigenesis [16]. Also, in regulating pancreatic cancer development, CDK14 acted as the target of miR-431 [17]. Furthermore, CDK14 functioned as an oncogene in glioma and served as a target gene of miR-613 [18]. Based on the studies above, we went to verify whether CDK14 was the target of miR-1825 in regulating GBM progression, which has not been reported until now.

Epithelial-mesenchymal transition (EMT) is an important biological process for the migration and invasion of epithelial-derived malignant cells [19]. The activation of Wnt/β-catenin signaling pathway promotes tumors progression [20]. Therefore, we further investigated miR-1825 effect on EMT and Wnt/β-catenin axis.

Correctively, here, the goal of this study was to investigate miR-1825 role in GBM progression and explore whether miR-1825 impeded cell proliferation, invasion and migration by regulating CDK14 and Wnt/β-catenin axis.

Materials And Methods

**GBM tissues**

Fifty-five paired fresh tissue specimens were collected from GBM patients who recruited from Jinan Zhangqiu District Hospital of TCM. All GBM patients have not received any treatment before surgery. The fresh tissues were verified by pathologists and stored at −80°C refrigerator for further use. All patients should provide written consent to allow for research purposes prior to the collection of tissue samples. The research processes follow the guidelines approved by Jinan Zhangqiu District Hospital of TCM and the ethic committee of Jinan Zhangqiu District Hospital of TCM.

**Cell culture**
GBM cell lines U251, U87 and A172 were obtained from BeNa Culture Collection (Suzhou, China). All the cells were cultured as previously described [21]. Then the cells were maintained in a humidified incubator containing 5% CO2 at 37 °C.

**MiR-1825 mimic and miR-1825 inhibitor**

The mimic or inhibitor of miR-1825 was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). They were used for increasing or decreasing the expressional level of miR-1825. A172 cells were the selected cells for further analysis. The transfection was conducted for 48h following the instructions of Lipofectamine 2000 reagent.

**Real time PCR (RT-PCR)**

The mRNA expression of miR-1825 and CDK14 was measured by RT-PCR. The total RNAs were firstly isolated from GBM tissues and cells. The miScript Reverse Transcription kit was carried out for producing cDNA. Then, quantitative PCR was conducted by a miScript SYBR-Green PCR kit. U6 was applied for normalizing miR-1825 relative mRNA expression and GAPDH for normalizing CDK14.

$2^{-\Delta\Delta Cq}$ method was used for calculating the gene expression. The primers were shown in *supplemental table 1*.

**Proteins analysis**

The relative proteins level was tested by Western blot. In brief, the total proteins were exacted from GBM cells and then conducted the protein concentration by BCA kit. After the equal protein separated by SDS-PAGE, they were transferred to the NC membranes. Then, the membranes were blocked with 5% skimmed milk powder at 37°C for 1h, incubated with primary antibodies at 4°C overnight, and secondary antibodies at 37°C for 1h. Finally, an enhanced chemiluminescence (ECL) method and the Image J software were applied for detecting the immune complexes and quantified protein levels, respectively.

**Cell proliferation analysis**

Cell proliferation was evaluated by MTT assay. Briefly, A172 cells were placed in 96-well plates. When the cells were cultured for 1, 2, 3, 4 days, MTT solution was added. After incubation with MTT solution for another 4h, DMSO was added and MTT solution was removed. Finally, the optical density was
detected at a wavelength of 490 nm using a microplate reader.

**Cell migration and invasion analysis**

The invasiveness and metastasis of U87 cells were measured by Transwell assay. Detection of invasion and migration was similar, except for the upper chamber coated with Matrigel. Briefly, the cells were added to the upper chamber and DMEM medium containing 20% FBS was added to the lower chamber. After incubation for 24h, the cells traversed the membrane were fixed, stained and counted. These traversed cells were used to evaluate cell invasion and migration.

**CDK14 protein density analysis**

Immunohistochemistry was applied for detecting CDK14 protein density. Firstly, the paraffin sections were obtained from GBM tissues. Then they were incubated with 3% H$_2$O$_2$ for 15min. After blocking with goat serum for 2h at room temperature, the sections were incubated with primary antibodies at 4°C overnight, and secondary antibodies at 37°C for 1h. Next, the sections were performed staining using DAB solution, followed by alcohol dehydration, xylene decolorization and neutral resin sealing. Finally, the results were observed and photographed by a microscopic.

**Tumor growth analysis**

Xenograft tumor formation assays were applied for measuring the tumor growth in vivo. The Animal Research Committee should approve these animal experiments and the operation should meet the standards for laboratory animal care and use in XXX. A172 cells treated with miR-1825 mimic or NC were injected into right flank of nude mice subcutaneously, which were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Then, the tumor size and weight was measured every four days for 28days by a vernier caliper and electronic scale, respectively.

**Luciferase activity analysis**

Luciferase reporter assay was applied for detecting the relative luciferase activity. Firstly, the CDK14 3’UTR-pGL3-reporter vector was constructed. Then, A172 cells were con-transfected with the vector and miR-1825 mimic with. After transfection for 48h, the luciferase activity was tested by the Dual-Luciferase Reporter Assay System.

**Statistics analysis**
The values were represented as mean ± SD and each experiment was conducted three times independently. Data was analyzed by SPSS 22.0 statistical software and the statistics was performed by GraphPad Prism 6. Student’s t-test was applied for comparing the difference between two groups and Tukey’s post hoc test was carried out for more than two groups. Pearson test was applied for determining the relationship between miR-1825 and CDK14. Log-rank test was applied for analyzing the survival rate. A p value <0.05 was considered as significant.

Results

**MiR-1825 down-regulation was associated with poor prognosis**

A previous study had showed that miR-1825 acted as a tumor suppressor in GBM. Here, we also detected miR-1825 expression in GBM tissue specimens. The findings displayed that miR-1825 average expression was declined in GBM tissues (Figure 1A). Moreover, we investigated whether the differential expression of miR-1825 was related to patients’ survival rate. As Figure 1B results displayed that GBM with high expression of miR-1825 predicted good prognosis, while low expression predicted poor prognosis. Moreover, miR-1825 was significantly associated with GBM clinicopathological features, including WHO grade (Table1). These findings demonstrated that miR-1825 down-regulation served as an indicator for poor prognosis of GBM patients.

**MiR-1825 up-regulation impeded GBM progression**

MiR-1825 expression was declined in GBM tissues. We then tested miR-1825 expression in GBM cell lines. As results shown in Figure 2A, miR-1825 was reduced in all GBM cell lines. We then selected A172 cells for further study. To see miR-1825 role in GBM cell viability, invasiveness and metastasis, miR-1825 expression was increased or decreased by mimic or inhibitor. As we saw in Figure 2B, miR-1825 mimic or inhibitor was transfected into A172 cells successfully. Next, MTT assay was carried out for testing A172 cell viability. The findings displayed that increasing miR-1825 impeded, while decreasing miR-1825 enhanced cell viability (Figure 2C). Transwell assay found that A172 cell migration was declined by miR-1825 mimic, but increased by miR-1825 inhibitor (Figure 2C). MiR-1825 showed the same effect on A172 cell invasion (Figure 2D). The findings above indicated that miR-1825 exhibited hindrance effect on cell proliferation, invasion and migration.
**MiR-1825 up-regulation blocked tumor growth in vivo**

Then we tested miR-1825 effect on the size and weight of tumors extracted from GBM mice. As we saw in Figure 3A, the tumors size in miR-1825 group was smaller than that in normal control group. Also, miR-1825 mimic made the tumor growth rate more slowly than normal control (Figure 3B). Moreover, miR-1825 mimic reduced the tumors weight in comparison with control (Figure 3C). All the results demonstrated that miR-1825 up-regulation impeded tumor growth.

**MiR-1825 negatively modulated CDK14 expression**

As TargetScan prediction, CDK14 was the possible target of miR-1825 and Figure 4A displayed the binding sides of miR-1825 with CDK14. Furthermore, luciferase reporter assay was applied for further confirming CDK14 was the target of miR-1825. As Figure 4B displayed that luciferase activity was reduced by miR-1825 mimic in wide type, but not in mutant type. Next, CDK14 protein level and mRNA expression were measured after A172 cells treated with miR-1825 mimic or inhibitor. The findings exerted that miR-1825 mimic exhibited a reduced expression of CDK14, while miR-1825 inhibitor displayed a raised CDK14 expression (Figure 4C-4D). Moreover, Figure 4E showed that CDK14 and miR-1825 expression was inversely correlated. These results indicated that CDK14 was the target of miR-1825.

**CDK14 up-regulation was associated with poor prognosis**

We then checked CDK14 expression in GBM tissue samples. The findings displayed that CDK14 was located in cell membrane (Figure 5A) and its protein density was raised in GBM tissues (Figure 5B). Moreover, we investigated whether the differential expression of CDK14 was related to patients' survival rate. As Figure 5C results displayed that the low expression of CDK14 in GBM tissues predicted good prognosis, while high expression predicted good prognosis. The findings demonstrated that CDK14 up-regulation served as an indicator of poor prognosis in GBM patients.

**MiR-1825 up-regulation blocked EMT and Wnt/β-catenin signaling pathway**

We then inquire into the mechanism of miR-1825 in A172 cells. The findings showed that miR-1825 up-regulation facilitated E-cadherin expression, but inhibited N-cadherin and Vimentin expression. However, miR-1825 down-regulation exhibited the opposite effect (Figure 6A). Next, the downstream...
genes of Wnt/β-catenin axis were tested by Western blot, too. The results in Figure 6B displayed that increasing miR-1825 reduced β-catenin, c-myc, p-c-Jun expression, whereas decreasing miR-1825 induced their expression. The results demonstrated that miR-1825 upregulation blocked Wnt/β-catenin signaling pathway in U87 cells.

Discussion
Many studies have revealed that miRNAs affect GBM development, including miR-146b, miR-148a and miR-34a [22–24]. These outcomes have attracted in-depth research into miRNAs in GBM. Here, in our study, we revealed that miR-1825 expression was declined in GBM tissue specimens and its downregulation predicted poor prognosis. However, we also found that CDK14 expression was raised in GBM tissues and its upregulation predicted poor prognosis. Moreover, increasing miR-1825 impeded tumor growth, GBM cell proliferation, invasion and migration. MiR-1825 negatively regulated CDK14 expression and Wnt/β-catenin axis.

Researches on miR-1825 in tumors revealed that it served as a potential biomarker in multiple cancers. For example, miR-1825 was high expressed in prostate cancer and it acted as prostate cancer biomarker [25]. Also, miR-1825 was upregulated in larynx cancer and involved in tumor progression [26]. However, miR-1825 was downregulated in glioma and associated with tumorigenesis [13]. In this study, we revealed that miR-1825 expression was declined in GBM and used as a predictor for prognosis. Moreover, re-expression of miR-1825 impeded GBM cell viability, invasiveness and metastasis, which in line with the research that miR-1825 played an important role in glioma cell proliferation, apoptosis and invasion [13].

We also revealed that CDK14 high expression was associated with the poor survival time of GBM patients. CDK14 was used as an oncogene in several cancers. For instance, its expression was raised in hepatocellular carcinoma as a target of miR-1202 [27]. Besides, CDK14 expression was higher in glioma than normal and took part in glioma progression regulated by miR-613 [18]. All these results supported our above research. Moreover, we first time revealed that CDK14 was the direct target of miR-1825.

Wnt/β-catenin pathway played important roles in tumorigenesis, including GBM. Lots of studies
displayed that miRNAs regulated GBM development by modulating Wnt/β-catenin axis, such as miR-34a [28], miR-328 [29], miR-21 [30]. Here, we displayed that miR-1825 repressed GBM progression by inhibiting the activation of Wnt/β-catenin axis.

Correctively, we draw a conclusion that miR-1825 upregulation impeded GBM development by targeting CDK14 through suppressing Wnt/β-catenin axis.

Declarations

Ethics approval and consent to participate

Approval for the study was obtained from the Ethics Committee of Jinan Zhangqiu District Hospital of TCM (Jinan, China). Informed consent was obtained from all the patients whose tissues were used in this study.

Consent for publication

Not applicable.

Availability of data and materials

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

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**Competing interests**

The authors declare that they have no competing interests.
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**Authors' contributions**

Fengqin Lu and Chunhong Li contributed to the conception of the study. Yuping Sun and Ting Jia contributed significantly to the data analysis and study preparation. Na Li performed the data analyses and wrote the study. Haiyan Li helped perform the analysis with constructive discussions. All authors have read and approved the final study.

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Table
Table 1 The clinicopathological relevance analysis of miR-1825 expression in GBM patients

| Characteristics       | Cases | miR-1825 | P-value |
|-----------------------|-------|----------|---------|
|                       |       | High     | Low     |         |
| Age (years)           |       |          |         |         |
| ≥ 60                  | 25    | 9        | 16      | 0.761   |
| 60                    | 30    | 12       | 18      |         |
| Gender                |       |          |         |         |
| Male                  | 35    | 12       | 23      | 0.399   |
| Female                | 20    | 10       | 12      |         |
| WHO grade             |       |          |         |         |
| I+II                  | 20    | 12       | 8       | 0.022*  |
| III+IV                | 35    | 10       | 25      |         |
| Location              |       |          |         |         |
| Supratentorial        | 30    | 14       | 16      | 0.425   |
| Subtentorial          | 25    | 9        | 16      |         |

Statistical analyses were performed by the χ² test.
*P<0.05 was considered significant.

Figures
Figure 1

The association of miR-1825 differential expression with overall survival. (A) Decreased expression of miR-1825 in GBM tissue samples (n=55). (B) High expression of miR-1825 in GBM patients exhibited high survival rate of GBM patients. *P<0.05
Hindrance effect of miR-1825 on GBM progression. (A) Decreased expression of miR-1825 in GBM cell lines. (B) Decreased miR-1825 expression in miR-1825 mimic group and increased
miR-1825 expression in miR-1825 inhibitor group in A172 cells. (C) Cell viability was suppressed by miR-1825 mimic and promoted by miR-1825 inhibitor in A172 cells. (D) Cell migration was suppressed by miR-1825 mimic and promoted by miR-1825 inhibitor in A172 cells. (E) Cell invasion was repressed by miR-1825 mimic and facilitated by miR-1825 inhibitor in U87 cells. *P<0.05, **P<0.01.

Figure 3

Inhibition effect of miR-1825 on tumor growth. (A) The representative pictures of the size of the tumors in mice transfected with miR-1825 mimic or normal control (NC). (B) The slowly growth of tumors in miR-1825 mimic group than NC group. (C) The weight-loss tumors in mice treated with miR-1825 mimic compared to NC. *P<0.05, **P<0.01
Negatively regulation of CDK14 by miR-1825. (A) The binding sites of miR-1825 and CDK14. (B) Declined relative luciferase activity by miR-1825 mimic in wide type (WT). (C) Decreased CDK14 level by miR-1825 mimic and increased CDK14 level by miR-1825 inhibitor. (D) Decreased CDK14 mRNA expression by miR-1825 mimic and increased CDK14 mRNA expression by miR-1825 inhibitor. (E) Negatively relationship between CDK14 and miR-1825 ($r=-0.7550$, $p<0.0001$). **P<0.01
Figure 5

The association of CDK14 differential expression with overall survival. (A) Location of CDK14 in the cell of GBM tissues. (B) Increased protein density of CDK14 in GBM tissues. (C) Low expression of CDK14 in GBM patients exhibited high survival rate of GBM patients. *P<0.05
The activation of EMT and Wnt/β-catenin signaling pathway by miR-1825 downregulation.

(A) Decreased expression of E-cadherin expression, but increased N-cadherin and Vimentin expression by miR-1825 mimic. Increased E-cadherin expression, but decreased N-cadherin and Vimentin expression by miR-1825 inhibitor. (B) Reduced β-catenin, c-myc, p-c-Jun expression by miR-1825 mimic and increased expression of β-catenin, c-myc, p-c-Jun expression by miR-1825 inhibitor.
Supplementary Files

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