Downregulation of KIF20A induces cell cycle arrest and apoptosis by suppressing PI3K/AKT in human glioblastoma

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Abstract: Background/Aim: Aberrant function of the kinesin family member 20A (KIF20A) has been reported to be vital in tumor genesis and development. However, the role of KIF20A in cell proliferation and invasion, as well as the mechanism underlying cell cycle arrest, remains unclear. Materials and Methods: Here, we first measured the expression of KIF20A in malignant astrocytoma and glioblastoma cell lines by immunohistochemistry and western blotting. Next, we knocked down KIF20A expression using siRNA to study its effect on cell proliferation and cycle. Results: We found that KIF20A knock down significantly suppressed cell proliferation, migration and invasion of glioblastoma cells. Importantly, KIF20A knock down induced cell cycle arrest in G0/G1 phase and promoted apoptosis by inactivating the PI3K/Akt pathway via c-Myc and activating the intrinsic apoptosis pathway. Conclusion: Taken together, our results suggest that KIF20A down-regulation can inhibit glioma tumorigenesis, which may provide a therapeutic target for treating glioma.

Keywords: KIF20A, glioblastoma, cell proliferation, cell migration, cell cycle, PI3K/Akt pathway

Introduction

Glioma represents approximately 30% of primary brain tumors of glial cell origin [1]. In humans, astrocytoma is the most common form of glioma, accounting for about 80% of malignancies of the central nervous system [2]. According to the World Health Organization (WHO) for Tumors in the Central Nervous System in 2007, the diffuse infiltrative astrocytic tumors can be graded on a three-tiered system: diffuse astrocytoma (grade II), anaplastic astrocytoma (grade III), and glioblastoma (grade IV) [3]. Despite advances in surgical and non-surgical treatments of gliomas, patients with glioblastoma still have a poor prognosis, with a median overall survival of 12-15 months [4]. However, the molecular mechanisms underlying glioma development and progression are still not well understood. In light of this, we sought to identify novel therapeutic targets to improve prognoses for glioma patients.

The kinesin superfamily proteins (KIFs) are responsible for transporting organelles, protein complexes and mRNAs [5, 6]. Research thus far indicates that abnormal expression and function of kinesins play a vital role in the genesis and development of various tumors [7]. KIF20A is overexpressed in pancreatic ductal adenocarcinoma cells (PDACs), and knocking down endogenous KIF20A expression reduced growth of PDACs [8]. In addition, overexpression of KIF20A has been reported in a few malignancies including hepatocellular carcinoma [9], breast cancer [10], bladder cancer [11], melanoma [12], gastric cancer [13], and cervical squamous cell carcinoma [14], among others [15]. It has been reported that KIF20A mRNA and protein levels are increased during hepatocyte proliferation and hepatocarcinogenesis [16]. Together, this evidence indicates that KIF20A may play a role in cell cycle arrest to regulate tumorigenesis. A proteomics study demonstrated that the anti-cancer agent, genistein, induced differential expression of 86 pro-
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teins in treated cells. Among these, 4 proteins from the kinesin family-KIF11, KIF20A, KIF22, and KIF23-were downregulated. Interestingly, all of these proteins are involved in the regulation of cell cycle, cell growth, and proliferation [17]. However, little is known about the role KIF20A plays in cell cycle regulation in gliomas. The intracellular phosphoinositide 3 kinase/Akt (PI3K/Akt) signaling pathway is important in the regulation of cell cycle, which is directly related to cellular quiescence, proliferation, cancer, and longevity [18]. Activation of BCL2, BCL-2-associated X protein (BAX) and caspases, and inhibition of KIF20A, mitogen-activated protein kinase (MAPK), and PI3K/Akt signaling pathways contribute to cell apoptosis during cancer. Thus, targeting these factors may affect the molecular mechanisms mediating the anti-cancer effects of genistein [19].

In this study, we investigated if KIF20A is associated with malignant tumor progression and cell cycle regulation in glioma. Our results demonstrate for the first time that knock down of KIF20A induces cell cycle arrest in G0/G1 phase and promotes apoptosis by inactivating the PI3K/Akt pathway via its downstream effector, c-Myc, and activating the intrinsic apoptosis pathway. Our study indicates that KIF20A plays a critical role in astrocytoma tumorigenesis, providing a promising therapeutic strategy for glioma.

Materials and methods

Assays for Cell Proliferation, Colony Formation, and Cell Migration and Invasion are described in the Supplementary Materials and Methods.

Tissue samples

Ninety-four human primary astrocytoma tissues were obtained from Qi Lu Hospital of Shandong University from 2010-2013. Tissues were formalin fixed followed by paraffin-embedding. Malignant astrocytomas included 11 diffuse astrocytomas (WHO grade II), 22 anaplastic astrocytomas (WHO grade III), and 33 glioblastomas (WHO grade IV). None of the patients received radiotherapy or chemotherapy before surgery. Ten cases of normal human brain tissues were obtained from patients who had received decompressive craniectomy at The Second People’s Hospital of Liaocheng City. All samples were examined by two pathologists to confirm histological diagnoses.

Ethics statement

The study was approved by both the Ethics Committee of the School of Medicine, Shandong University and the Ethics Committee of The Second People’s Hospital of Liaocheng, Shandong, China. Written informed consent was obtained from all participants involved in our study.

Immunohistochemistry

Paraffin embedded samples were heated for 50 min in an oven at 60°C. The slides were then deparaffinized twice in xylol for 10 min, rehydrated through graded ethanols, and incubated in 3% hydrogen peroxide solution for 10 min at room temperature. Antigen retrieval was performed by boiling the sections at 100°C for 4 min in 0.01 M citrate buffer (pH 6.0). Slides were incubated with KIF20A antibody (1:100, Abcam, Cambridge, UK) and Ki-67 (1:100, Abcam, Cambridge, UK) overnight at 4°C, and then incubated with biotinylated anti-mouse secondary antibody (PV-9000 2-step plus® reagent kit, Zhongshan Biotechnology, Beijing, China) at 37°C for 30 min. The slides were visualized with 3,3’-diaminobenzidin (DAB) substrate liquid and washed with deionized water before hematoxylin counterstaining.

Staining intensity was scored as follows: 0, absent; 1, weak; 2, moderate; 3, strong. The percentage of positive cells was scored as follows: 1 ≤ 25%; 2 ≤ 50%; 3 ≤ 75%; 4 > 75%. The final score was calculated by multiplying the intensity score by the percentage score. A composite score ≤ 6 was considered low KIF20A expression; a score > 6 was considered high KIF20A expression [20]. The Ki-67 labeling index was calculated as the mean percentage of immune-positive cells. One thousand neoplastic cells were counted in the five most-labeled areas [21].

Cell culture and transfection

The glioblastoma cell lines U251 and U87 were provided by Professor Li Gang, at the Brain Science Research Institute, Shandong University, T98 and LN229 cells were purchased from American Type Culture Collection (Man-
Small interfering RNA (siRNA) was synthesized by Gene-Pharma (Shanghai, China). Cells were transiently transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s protocol. Transfection efficiency was verified by real-time quantitative PCR (RT-qPCR) and western blot analyses.

**RT-qPCR**

Total RNA was extracted from glioblastoma cells by RNAiso Plus (TaKaRa, Dalian, China) and cDNA was synthesized with the ReverTra Ace qPCR RT Kit (Toyoto, Osaka, Japan) according to the manufacturer's protocol. RT-qPCR reaction was performed using the SYBR® Green Realtime PCR Master Mix assay kit (Toyoto, Osaka, Japan) under the following conditions of 95°C for 60 s, 40 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 45 s. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an endogenous control. The primer sequences were as follows: KIF20A: 5’-GCCAA-CTTCATCCAACACCT-3’ (forward); 5’-GTGGACAG-CTGCCCTCCTGGT-3’ (reverse); GAPDH: 5’-GAG-TCAACGGGATTTCGTCGT-3’ (forward); 5’-GACAA-GCTCCCGTCTCAG-3’ (reverse).

**Cell cycle assay**

Glioblastoma cells were harvested, washed, fixed overnight in 75% ethanol at 4°C following transfection. Before analysis, RNase A was added in the water bath for 30 min at 37°C. The cell suspension was stained with propidium iodide in the dark. Cell cycle distribution was determined by flow cytometry (FACSCalibur, BD Biosciences). The results were quantitated with ModFit analytic software.

**Apoptosis assay**

After transfection, glioblastoma cells were harvested and stained with the Annexin V-FITC/PI Apoptosis Detection Kit (BestBio, Shanghai, China) according to the manufacturer’s instructions. Stained cells were immediately analyzed by flow cytometry (FACSCalibur, BD Biosciences). The total apoptotic rate was the sum of the early apoptotic rate and the late apoptotic rate.

**Western blot**

Total protein was extracted from transfected glioblastoma cells after 48 h transfection. Lysates were isolated by electrophoresis and transferred to nitrocellulose membranes. The membranes were then incubated with antibodies against KIF20A (1:1,000, Abcam), phosphorylated (p)Akt (Ser473, 1:5,000, Abcam), Akt (1:5,000, Abcam), Cyclin D1 (1:10,000, Abcam), Bcl-2 (1:1,000, Abcam), Bax (1:5,000), c-Myc (1:5,000, CST, USA), and β-actin (1:1,000, Beyotime, Beijing, China). The bands were analyzed using ImageJ software.

**Statistical analysis**

Analysis was performed using the statistical software package for Social Sciences, version 20.0 (SPSS). Fisher’s exact test or chi-square test analysis was applied for the correlation between KIF20A expression and clinical-pathologic parameters. The correlation between KIF20A and Ki-67 was determined by Spearman’s correlation coefficient. Survival curves were assessed by the Kaplan-Meier method, and the differences between the survival curves were analyzed by the log-rank test. Univariate and multivariate survival data were analyzed using the Cox proportional hazard regression model. Student’s t-test was used to analyze the differences between two groups. p < 0.05 was considered statistically significant.

**Results**

**KIF20A is a prognostic marker for malignant astrocytoma**

We stained brain tissues with a KIF20A antibody to confirm that KIF20A is expressed in malignant astrocytomas. Smooth muscle tissue was used as a negative control. We found positive KIF20A staining in 65 of the 94 cases (69.1%) of malignant astrocytomas and in 1 out of the 10 (10%) normal brain tissues. The intensity and percentage of KIF20A positive staining was enhanced as the pathological grade increased (Figure 1A-E). Consistently, the Kaplan-Meier survival curves demonstrated significant association between KIF20A expression and overall survival of malignant as-
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In vitro knockdown of KIF20A suppresses glioblastoma cell proliferation

RT-qPCR and western blot analyses demonstrated that siRNA KIF20A transfection reduced KIF20A expression in the knock down group compared to the control group in both U251 and U87 cell lines (Figure S2A, S2B). The cell proliferation rate, as assessed by the CCK-8 assay, decreased in the KIF20A-siRNA transfected U251 and U87 cells compared to the control group (Figure S2C, S2D; *p < 0.05, **p < 0.01, respectively). Furthermore, knock down of KIF20A in U251 and U87 cells decreased the number of colonies as evidenced by colony formation assays (Figure 2A-D; p = 0.0009 and 0.0020, respectively).

Downregulation of KIF20A arrests cell cycle in G0/G1 phase

Flow cytometry revealed that the percentage of cells in G0/G1 phase was increased from 48.74 ± 1.33% to 60.26 ± 1.27% in U251 cells (Figure 3A-D; p = 0.0004) and from 48.33 ± 0.70% to 61.49 ± 3.55% in U87 cells (Figure 3A-D; p = 0.0032), while the percentage of cells in S phase was reduced from 35.53 ± 1.85% to 24.40 ± 0.41% in U251 cells (Figure 3A, 3B; p = 0.0005) and 38.08 ± 0.33% to 24.76 ± 2.16% in U87 cells (Figure 3C, 3D; p = 0.0005). These results suggest that knock down of KIF20A induces cell cycle arrest in G0/G1 phase, which may in part explain the growth inhibition of glioblastoma cells.

KIF20A knock down in glioblastoma cells induces apoptosis and inhibits migration and invasion

Next, we measured apoptosis following KIF20A knock down. We found that the apoptosis rate
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Table 1. Association of KIF20A expression with clinico-pathological parameters

<table>
<thead>
<tr>
<th>Clinical-pathological variable</th>
<th>n</th>
<th>KIF20A expression</th>
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<tr>
<td></td>
<td></td>
<td>Low (n, %)</td>
<td>High (n, %)**</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 40</td>
<td>32</td>
<td>24 (75.0)</td>
<td>8 (25.0)</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>62</td>
<td>44 (71.0)</td>
<td>18 (29.0)</td>
</tr>
<tr>
<td>Gender</td>
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</tr>
<tr>
<td>Male</td>
<td>55</td>
<td>42 (76.4)</td>
<td>13 (23.6)</td>
</tr>
<tr>
<td>Female</td>
<td>39</td>
<td>26 (66.7)</td>
<td>13 (33.3)</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
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<tr>
<td>&lt; 5</td>
<td>42</td>
<td>29 (69.0)</td>
<td>13 (31.0)</td>
</tr>
<tr>
<td>≥ 5</td>
<td>52</td>
<td>39 (75.0)</td>
<td>13 (25.0)</td>
</tr>
<tr>
<td>WHO grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>27</td>
<td>24 (88.9)</td>
<td>3 (11.1)</td>
</tr>
<tr>
<td>III</td>
<td>32</td>
<td>25 (78.1)</td>
<td>7 (21.9)</td>
</tr>
<tr>
<td>IV</td>
<td>35</td>
<td>18 (51.4)</td>
<td>17 (48.6)</td>
</tr>
<tr>
<td>Ki-67 (%)</td>
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<tr>
<td>&lt; 12</td>
<td>46</td>
<td>40 (87.0)</td>
<td>6 (13.0)</td>
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<tr>
<td>≥ 12</td>
<td>48</td>
<td>28 (58.3)</td>
<td>20 (41.7)</td>
</tr>
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</table>

*indicates p < 0.05, significant difference. **A composite score ≤ 6 was considered low KIF20A expression; a score > 6 was considered high KIF20A expression.

in U251 (Figure 4A, 4B; p = 0.0017) and U87 (Figure 4C, 4D; p = 0.0054) cells transfected with KIF20A siRNA was increased compared to control. The Transwell assay further showed that KIF20A knock down significantly decreased migration and invasion in U251 (Figure 4E, 4F; p = 0.0061 and p = 0.0083, respectively) and U87 (Figure 4G, 4H; p = 0.0010 and p = 0.0075, respectively) cells compared to the control group.

The effects of KIF20A knock down on critical cell cycle protein expression in cancer cells

To explore the downstream targets of KIF20A, we measured expression of several cell cycle regulated genes including phosphorylated AKT (PI3K/AKT signaling pathways), c-Myc, and Cyclin D1 (important for cancer formation, progression), BCL2, and BAX (critical factors for cell apoptosis). siRNA mediated knock down of KIF20A decreased pAKT (Ser473) protein expression in U251 (Figure 5A, 5B) and U87 (Figure 5A, 5C) cells compared to the control group. We further investigated the function of KIF20A in different glioblastoma cell lines and studied the downstream signaling of KIF20A.

Discussion

Glioma is a highly heterogeneous tumor, and its carcinomatous process is limited compared to other cancers. Many studies have focused on gene alternation in glioma to understand the molecular mechanisms of tumorigenesis and progression [22-24]. KIF20A is a member of the kinesin superfamily-6 and has two microtubule-binding sites, including the N-terminal kinesin motor domain and the C-terminus Rab6-binding domain [25, 26]. KIF20A is involved in key cellular functions such as intracellular movement of organelles and vesicles, spindle formation, and cytokinesis [27, 28]. Accumulating evidence has shown that ectopic overexpression of KIF20A exists in various tumors, which indicates that KIF20A contributes to tumorigenesis [8-13]. In the current study, KIF20A was highly expressed in malignant astrocytomas, and this elevated expression significantly correlated with advanced pathological grade and high Ki-67 index. These findings are consistent with recent studies that have also demonstrated that high KIF20A expression is an independent prognostic factor for overall survival in glioma patients, which suggests that KIF20A is a target for treating glioma [29, 30]. However, the previous research only explored the effects of KIF20A on glioblastoma cells without providing any new insight into the underlying molecular mechanism. In the present study, we are the first to report variation in cell cycle distribution in response to KIF20A knock down. We further investigated the function of KIF20A in different glioblastoma cell lines and studied the downstream signaling of KIF20A.
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A recent study demonstrated that silencing FOXM1 (a member of the Forkhead box family) and KIF20A similarly caused abnormal spindle morphology and chromosome alignment, which induces mitotic catastrophe in breast carcinoma paclitaxel-resistant MCF-7 TaxR cells [10]. However, up-regulated KIF20A expression led to impaired mitosis and cytokinesis, aberrant ploidy, and genomic instability in hepatoma cells [16]. Thus, we speculate that KIF20A’s mechanism of action in glioblastoma cells is analogous with the above points.

KIF20A accumulates in mitotic cells where it localizes to the midzone of the spindle during anaphase. Cell cycle regulation is believed to be the foundation of tumor cell proliferation and cell division. For the first time, we demonstrate that KIF20A knock down arrests glioblastoma cells in G0/G1 phase, which indicates that reducing KIF20A expression can suppress cell cycle transition from G0/G1 to S phase, causing inhibition of cell proliferation and colony formation. This result is consistent with some studies that showed that KIF20A downregulation caused accumulation of MCF7 in breast carcinoma cells [31]. In contrast, silencing KIF20A inhibited gastric cancer cell viability and induced cell cycle arrest in G2/M phase [17]. Thus, cell cycle arrest induced by KIF20A differs among cancers cells, which explains how silencing KIF20A can promote proliferation of glioblastoma cells by influencing cell cycle phase distribution.

Figure 2. Knockdown KIF20A suppresses proliferation of glioblastoma cells. A-D. Colony formation assay showed that knock down of KIF20A decreased colony formation in U251 and U87 cells \((p = 0.0009\) and \(0.0020\), respectively). \(N = 6\) in each group. Data are expressed as mean ± SEM.
Figure 3. Downregulation of KIF20A arrested cell cycle progression in G0/G1 phase. A-D. Flow cytometry was used to determine cell cycle distribution. The percentage of cells in G0/G1 phase was increased, while the percentage of cells in S phase decreased in U251 and U87 cells following KIF20 siRNA transfection. Knock down of KIF20A induced cell cycle arrest in G0/G1 phase. N = 6 in each group. Data are expressed as mean ± SEM.
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Figure 4. Downregulation of KIF20A induced cell apoptosis and inhibited migration and invasion in glioblastoma cells. (A-D) KIF20A siRNA induced cell apoptosis in U251 (A, B; \( p = 0.0017 \)) and U87 (C, D; \( p = 0.0054 \)) cells. (E-H) Migration and invasion of U251 (E, F; \( p = 0.0061 \) and \( p = 0.0083 \)) and U87 (G, H; \( p = 0.0010 \) and \( p = 0.0075 \)) cells decreased in the KIF20A siRNA group compared to the control group. \( N = 6 \) in each group. Data are expressed as mean ± SEM.
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The molecular mechanisms of kinesin superfamily members have been described in various types of tumors [32-34]. PI3K is a lipid kinase that generates second messengers involved in the regulation of cell functions including proliferation, apoptosis and survival, all of which play key roles in cell cycle progression, prevention of apoptosis, and malignant transformation [35]. One of PI3K’s major effectors is protein kinase B (Akt). Our results indicate that downregulation of KIF20A not only decreased pAKT (Ser473), but also suppressed the levels of cell cycle correlated genes, including c-Myc and Cyclin D1. This suggests that KIF20A is an upstream regulator of the PI3K/Akt pathway, and a decrease in KIF20A induces cell cycle arrest in G0/G1 phase by inactivating the Akt pathway via its downstream effector, c-Myc. The literature shows that Cyclin D1 is a G0/G1 related cell cycle regulatory factor [36, 37]; thus, a decrease in KIF20A expression arrests glioblastoma cells in G0/G1 phase rather than G2/M phase. Moreover, we also found that BCL2 levels were reduced while BAX levels were increased in glioblastoma cells transfected with KIF20A siRNA. These data suggest that KIF20A downregulation induces apoptosis through inactivating Akt and activating the downstream intrinsic apoptosis pathway via regulating the BCL-2/BAX ratio.

In conclusion, our results suggest that downregulating KIF20A inhibits cell proliferation and invasion, induces cell cycle arrest in G0/G1 phase, and promotes apoptosis. The KIF20A mediated effects on these cell processes is likely occurring by inactivation of the PI3K/Akt pathway and its downstream effectors. Our findings confirm that KIF20A is an attractive therapeutic target for glioma.

Disclosure of conflict of interest

None.

Figure 5. The effects of KIF20A downregulation on pAKT (Ser473), c-Myc, Cyclin D1, Bcl-2, and Bax protein expression in glioblastoma cell lines. (A-C) Western blot analysis of pAKT (Ser473), total AKT, c-Myc, Cyclin D1, BCL2, and BAX in U251 (A, B) and U87 (A, C) siRNA KIF20A transfected and control cells. Knock down of KIF20A decreased expression of pAKT (Ser473), c-Myc, Cyclin D1, and BCL2, and increased expression of BAX. *p < 0.05; **p < 0.01. N = 6 in each group. Data are expressed as mean ± SEM.
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References


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Supplementary materials and methods

Cell proliferation assay

U251 and U87 cells were seeded in 96-well plates at a density of $2 \times 10^3$ cells/well after transfection. The Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) was used to determine glioblastoma cell proliferation. Cells were incubated with 10 μL CCK-8 per well for 1 h at daily intervals (24, 48, 72, and 96 h), and absorbance was measured at 450 nm with a microplate reader.

Colony formation assay

U251 and U87 glioblastoma cells transfected with siRNA for 48 h were seeded in 6-well plates at a density of 1,000 cells per well. Cells were incubated for 10 days and then fixed and stained with crystal violet. The visible colonies were counted.

Cell migration and invasion assays

Migration assay was performed with a Transwell that included an 8.0 μm pore size polycarbonate membrane insert (Corning, New York, USA). The invasion assay was performed with the Transwell inserts pre-coated with Matrigel matrix (BD Science, Sparks, MD, USA). The glioblastoma cells ($4 \times 10^4$) were suspended in serum-free medium added to the upper chamber, while the lower chambers contained complete culture medium containing 10% FBS. The cells on the upper surface were removed with a cotton swab after several hours of incubation (18 h for U251 and U87 migration assays; 24 h for U251 and U87 invasion assays), and the lower surface was fixed, stained and counted under the microscope (Olympus, Japan).

Figure S1. Increased expression of KIF20A in glioblastoma. A. Kaplan-Meier analysis of total survival showed a significant difference between the low expression and high expression groups; high KIF20A expression was associated with poorer overall survival ($p = 0.000$). B. Western blot analysis of KIF20A expression in various glioblastoma cell lines.
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Table S1. Univariate and multivariate Cox proportional hazards regression analyses of overall survival after surgery

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<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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<tr>
<td></td>
<td>HR</td>
<td>CI (95%)</td>
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<tr>
<td>Overall survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &gt; 40 y</td>
<td>2.622</td>
<td>1.413-4.868</td>
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<tr>
<td>Ki-67 ≥ 12%</td>
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<tr>
<td>High expression of KIF20A</td>
<td>2.725</td>
<td>1.592-4.662</td>
</tr>
<tr>
<td>Advanced WHO pathological grade</td>
<td>4.274</td>
<td>2.854-6.399</td>
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HR-hazard ratio, CI-confidence interval. *indicates significant difference; p < 0.05.

Figure S2. Verification of KIF20A expression and cell proliferation rate in siRNA mediated KIF20A knock down. A, B. After transfection for 48 h, RT-qPCR and western blot were performed to analyze the expression of KIF20A between KIF20A siRNA and control groups in U251 and U87 cells. C, D. CCK-8 assay was used to measure proliferation. Compared to the control group, the proliferation rate of KIF20A-siRNA transfected U251 and U87 cells was significantly decreased (*p < 0.05, **p < 0.01). N = 6 in each group. Data are expressed as mean ± SEM.