CBX2 Induces Glioma Cell Proliferation and Invasion Through the Akt/PI3K Pathway

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Research Article

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Abstract

**Background** Glioma is the most common primary intracranial tumor. Abnormal expression of CBX2(ChromoBox2) is associated with tumorigenesis and tumor development.

**Methods** TCGA data in UALCAN showed that CBX2 was overexpressed in glioma tissue. To confirm the role of CBX2 in glioma, we regulated the level of CBX2 and conducted colony formation, Transwell, and CCK-8 assays to verify the effect of CBX2.

**Results** The results showed that CBX2 knockdown reduced glioma cell proliferation and invasion and that the cells were less tumorigenic. CBX2 overexpression induced glioma cell proliferation and invasion and glioma stem cell self-renewal. The animal experiments showed that CBX2 knockdown inhibited glioma growth and improved survival time. CBX2 knockdown inhibited activation of the Akt/PI3K pathway. EGF rescued the effects of CBX2.

**Conclusion** We hypothesized that CBX2 induced the growth and invasion of glioma cells via the Akt/PI3K pathway.

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**Background**

Glioma is the most common primary intracranial tumor and is characterized by aggressive growth, difficulty of complete resection and easy relapse[1]. Most gliomas are characterized by invasive growth. Surgical resection combined with regular radiotherapy and chemotherapy is still the main treatment, but the treatment efficacy is poor, and relapse easily occurs[2]. It has been reported that the occurrence of gliomas is closely related to the abnormal expression of various genes and is usually accompanied by abnormally high expression of oncogenes and the deletion or low expression of tumor suppressor genes[3]. Therefore, it has become a hot topic in the field of medical research to further explore the root causes of glioma occurrence and development at the genetic level.

ChromoBox (CBX) proteins include 8 members and are crucial proteins associated with gene expression and development[4, 5]. Five members make up the PRC1 complex: CBX2, CBX4, CBX6, CBX7 and CBX8[6]. Studies have shown that CBX2 can bind H3K9me3 or H3K27me3, and CBX4 is associated with H3K9me3[6]. CBX proteins play important roles in the progression of tumor development. CBX4 overexpression is associated with overall survival in colorectal carcinoma, CBX6 downregulation occurs in glioblastoma, and CBX7 is overexpressed in gastric and prostate cancers[7, 8, 9, 10].

In this study, we found that CBX2 was overexpressed in glioma compared to normal brain tissue. However, the mechanism was not clear. To confirm the role of CBX2 in the proliferation and invasion of glioma cells, we conducted an in vitro and in vivo study.

**Methods**
The cancer genome atlas (TCGA) dataset analysis

Glioma and normal brain tissue data were obtained from the TCGA database in UALCAN (http://ualcan.path.uab.edu/index.html).

Cell culture

The glioma cell lines U87 and U251 were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C with 5% CO₂.

Transfection

U87 cells were transfected with CBX2-shRNA to knock down the expression of CBX2, and the negative control group was transfected with Scramble-shRNA. The sequences are shown in Table S1. The overexpression plasmid, pcDNA3.1-C-(k)DKY encoding CBX2 protein.

Quantitative real-time PCR (qRT-PCR)

Total RNA isolation was followed by reverse transcription, and SYBR Green master mix (Promega, USA) was used for qRT-PCR. The CBX2 primer sequences were as follows: forward: 5’-GCCAGCAGTGGACAGAAC-3’ and reverse: 5’-CACTGTCACGGTGATGAGGTT-3’. GAPDH was used as the reference gene, and the primer sequences for GAPDH were as follows: forward: 5’-TGTGGGCATCAATGGATTTGG-3’ and reverse: 5’-ACACCATGTATTCCGGGTCAAT-3’. The 2^(-ΔΔCq) method was used to calculate the relative expression.

Western blotting

Total protein was extracted and mixed with 5× SDS loading buffer, separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, USA). The membranes were incubated with primary antibodies overnight and then incubated with HRP-conjugated secondary antibodies. Protein expression was visualized using an enhanced chemiluminescence kit (Millipore). The bands were analyzed with ImageJ software.

Transwell invasion assay

Matrigel and DMEM were diluted at a ratio of 1:2 and served as the extracellular matrix, and the Matrigel solidified after being incubated for 30 min at 37°C. After transfection for 48 h, the cells were cultured in the wells with Matrigel under general conditions for an additional 48 h. The invaded cells were detected by staining the cells with crystal violet, and the cells were counted and imaged with a microscope at ×200 magnification.

Cell counting kit-8 assay

The transfected cells were cultured in 96-well plates at 2000 cells per well. Twenty microliters of CCK-8 solution (Solarbio, Beijing, China) was added to each well and incubated at 37°C for 4 h after 24, 48, 72
and 96 h of culture. The results were measured via spectrophotometry at 450 nm (BioTek Instruments, Inc., Winooski, VT, USA).

**Colony formation assay**

After transection for 48 h, the cells were seeded in 6-well plates at a density of 1000 cells/well and cultured for 14 days. The colonies were fixed with paraformaldehyde (4% w/v) and stained with crystal violet (0.5% w/v). The number of colonies was counted with a microscope (Olympus Corporation).

**Extreme limiting dilution analysis**

Glioma cells were seeded into 96-well plates and cultured with 100 μl of serum-free DMEM/F12 medium containing B27, insulin, FGF and EGF. After 14 days, GSCs were detected by extreme limiting dilution analysis.

**Animal experiments**

To verify the effect of CBX2 knockdown, transfected U87 cells were injected into the brains of BALB/c-A nude mice by intracranial inoculation. The mice were imaged for Fluc activity using bioluminescence imaging. We recorded the weights of the mice and their survival.

**Immunohistochemical staining**

The paraffin-embedded sections were used for immunohistochemical staining detected with the avidin-biotin complex method. The sections were incubated with antibodies specific for CBX2 and Ki67 overnight at 4°C, followed by secondary antibody incubation at 37°C for 1 h. The expression of CBX2 and Ki67 was determined by coloration with DAB.

**Statistical analysis**

The significance of Kaplan-Meier analysis was determined by the log-rank test. Multivariate analysis was performed with a multivariate Cox regression model. The data in the study are presented as the mean ± SD. A value of P<0.05 was determined to be statistically significant. Significance was set at *P<0.05, **P<0.01, *** P<0.001. SPSS 17.0 (SPSS, Chicago, IL) was used in the present study.

**Results**

**The level of CBX2 was higher in glioma tissues than in normal brain tissue**

The data obtained from UALCAN demonstrated that CBX2 was overexpressed in glioma compared to normal brain tissues (Fig. 1A, p < 0.001). There was no association between the level of CBX2 and the stage of glioma.
Cbx2 Induced Glioma Cells Proliferation And Invasion

We measured the level of CBX2 in glioma cell lines by RT-PCR and western blotting (Fig. 1B, C). The expression of CBX2 in U87, U251 and LN229 cells was higher than that in the HEB cell line. The U87 cell line was used for CBX2 knockdown, and the LN229 cell line was used for CBX2 overexpression. The efficiencies were verified by RT-PCR and western blotting (Fig. 1D, E, P < 0.001). To analyze the effect of CBX2 on the proliferation, invasion and stem cell self-renewal of glioma cells, CCK-8, colony formation, Transwell and extreme limiting dilution assays were conducted. The outcomes showed that downregulation reduced the growth of glioma cells (Fig. 1F, G, P < 0.01). The number of colonies in the scramble group was 103.7 ± 18.4, and the average number of colonies in the CBX2-shRNA1 and CBX2-shRNA2 groups was 21.3 ± 3.7 and 25.6 ± 4.1, respectively. In the transwell assays, the numbers of cells that crossed the membrane in the three groups were 65.0 ± 9.9, 26.7 ± 6.0 and 31.6 ± 7.7, and the number of cells was decreased following transfection with CBX2-shRNA1 and CBX2-shRNA2 (Fig. 1H, P < 0.05). To verify the tumorigenic properties of glioma cells after CBX2 knockdown, the formation of tumor spheres was examined after 14 days. We found that the cells in the scramble group were more tumorigenic than the cells that were transfected with CBX2-shRNA (P < 0.05 and P < 0.05, respectively, Fig. 1I).

Furthermore, we conducted western blotting to measure the changes in EMT-related proteins after CBX2 knockdown. The expression of N-cadherin, Slug and Snail was decreased, and that of E-cadherin was increased. These results indicate that CBX2 knockdown repressed EMT.

To further confirm the role of CBX2, the level of CBX2 in glioma cells was upregulated by transfection with the CBX2 plasmid. To confirm the overexpression of CBX2 in LN229 cells, RT-PCR and western blotting were conducted after transfection with the CBX2 plasmid (Fig. 2A, B). The results of the CCK-8 and colony formation assays showed that the proliferation of glioma cells was improved after transfection with the CBX2 plasmid (Fig. 2C, D). The colony numbers in the Vector and CBX2 groups were 122.0 ± 11.6 and 197.7 ± 18.9, respectively. Then, we measured cell invasion. The number of cells that crossed the membrane in the vector group was 95.7 ± 10.5 and 194.0 ± 18.9 in the CBX2 group, and the number of cells was increased following transfection with the CBX2 plasmid (Fig. 2E, P < 0.01). The tumor spheres were cultured after transfection with the CBX2 plasmid. The cells that were transfected with CBX2 were more tumorigenic than those in the vector group (Fig. 2F, P < 0.01). We conducted western blotting to measure changes in EMT-related proteins after the upregulation of CBX2 (Fig. 2G). The levels of N-cadherin, Slug and Snail were increased, and the expression of E-cadherin was decreased. These results indicate that the upregulation of CBX2 induced EMT.

Cbx2 Increased Tumor Growth In The Animal Model

The orthotopic mouse model was established with U87 cells. The tumors were analyzed by Fluc bioluminescence imaging, and the tumors were suppressed in the CBX-shRNA1 group (Fig. 3A). CBX2 knockdown improved the survival time compared to that of the control group (Fig. 3B, P < 0.05). Mouse
body weight was measured, and we found that weight loss in the control group was obvious (Fig. 3C, P < 0.05). The expression of CBX2 and Ki67 in the tumor tissue was determined by immunohistochemical staining. The level of CBX2 was reduced in the CBX2-shRNA1 group. The level of Ki67 indicates the proliferation of cells, and Ki67 was decreased following CBX2 knockdown.

**CBX2 induced glioma cell proliferation and invasion by regulating the activity of the Akt/PI3K signaling pathway**

In this study, we treated CBX2-knockdown cells with EGF (an agonist of the Akt/PI3K pathway, 100 ng/ml) to rescue the effects of CBX2 inhibition. We found that EGF upregulated the levels of p-Akt and p-GSK-3β (Fig. 4A). CCK-8 and colony formation assays were also performed. The colony numbers in the three groups were 113.3 ± 11.3, 33.7 ± 5.3 and 104.3 ± 9.6 (Fig. 4C, P < 0.01). The Transwell assay results demonstrated that the numbers of cells that crossed the membrane in the three groups were 73.7 ± 7.9, 24.0 ± 4.4 and 72.6 ± 9.2 (Fig. 4D, P < 0.01). In addition, the cells were more tumorigenic than those in the CBX2-shRNA1 group (Fig. 4E, P < 0.01). These results showed that EGF increased cell growth, invasion and stem cell self-renewal in CBX2-shRNA1 cells. We concluded that CBX2 induced glioma cell proliferation and invasion via the Akt/PI3K pathway.

**Discussion**

Polycomb group complex (PcG) proteins, including PRC1 and PRC2, are widely distributed in animals and plants. These proteins have a unique chromatin modification and play an inhibitory role on transcription[11]. PcG proteins are transcriptional repressors that regulate the growth and development of animals and plants[11–12–13]. PcG proteins are widely involved in many physiological and pathological processes, such as embryonic stem cell self-renewal, cell cycle control, cell growth and apoptosis[12–13–14]. CBX mainly targets PRC1 to chromatin. CBX2 is an important member of the CBX protein family. CBX2 is located in the nucleus and consists of 532 amino acids, and it is a crucial component of PcG. The C-terminal polycomb receptor box of CBX2 can specifically recognize H3K27me3, thus enhancing the inhibition of gene expression[15]. Recently, studies have shown that the expression of CBX2 is closely related to tumorigenesis and tumor development. Some studies have shown that the expression of CBX2 in osteosarcoma tissue is significantly increased, and the degree of the increase is closely related to metastasis, postoperative recurrence, chemotherapy reaction and adverse prognosis[16]. In advanced serous ovarian cancer, the expression of CBX2 was significantly increased. Knockdown of CBX2 significantly inhibited nonanchored proliferation and promoted anoikis in tumor cells, increasing the sensitivity of tumor cells to pods[17]. Studies have shown that abnormally high expression of CBX2 in breast cancer, prostate cancer and liver cancer is closely related to the degree of malignancy, lymph node metastasis, TNM stage, poor patient prognosis and sensitivity to chemotherapy drugs[18–19–20]. The expression of CBX2 in glioma, its effect on glioma and its mechanism have not been reported. In our study, we found that the level of CBX2 was higher in glioma tissue than in normal brain tissue. Using CCK-8, colony formation, and Transwell assays, we found that CBX2 played an important role in glioma progression. Moreover, we found that CBX2 affected the biological function of glioma cells via the
Akt/PI3K pathway. The Akt/PI3K signaling pathway is an important signal transduction pathway in cells that participates in the regulation of many important biological processes, blocks cell apoptosis, and promotes the occurrence and development of various human tumors\[21\]. Many cytokines can activate the PI3K/Akt signaling pathway in many kinds of tumors, such as liver cancer and breast cancer\[22, 23\]. In our study, we found that CBX2 knockdown decreased the expression of p-Akt and p-GSK-3β, which are core proteins of the PI3K/Akt pathway.

**Conclusions**

In conclusion, compared with that of normal tissues, the level of CBX2 in gliomas was significantly increased. In vitro and in vivo experiments confirmed that CBX2 knockdown could significantly inhibit the formation of glioma. Further studies showed that CBX2 knockdown decreased the expression of p-Akt and p-GSK-3β, suggesting that CBX2 may play a role as an oncogene by activating the PI3K/Akt signaling pathway.

**Abbreviations**

CBX2: ChromoBox2; TCGA: The Cancer Genome Atlas; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; qRT-PCR: Quantitative real-time PCR; SDS-PAGE: sulfate polyacrylamide gel electrophoresis; CCK-8: Cell counting kit-8 assay; DAB: diaminobezidin; FGF: fibroblast growth factor; EGF: Epidermal Growth Factor; PcG: Polycomb group complex; TNM: tumor node metastasis; AKT: protein kinase B; GSK-3β: Glycogen synthase kinase-3beta.

**Declarations**

**Ethics approval and consent to participate**

The study protocol was approved by the Ethics Committee of the Tianjin Medical University General Hospital.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The analyzed data sets generated during the study are available from the corresponding author on reasonable request. Inquiries for data access may be sent to the following e-mail address: wltj912@163.com.

**Competing interests**
The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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**Authors’ contributions**

Le Wang, Yang Nan and Yue Zhong designed this study and provide guidance for research. Le Wang and Bingcheng Ren conducted the word and analysis the data. Le Wang wroted the manuscript. All authors have approved the manuscript.

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Figures
Figure 1

Downregulation of CBX2 inhibited glioma cell proliferation, invasion and self-renewal. A. The data (UALCAN, http://ualcan.path.uab.edu/index.html) showed that the expression of CBX2 was higher than that in normal brain tissue (P<0.001). B, C. The expression of CBX2 was analyzed in glioma cell lines by RT-PCR and western blotting. D, E. CBX2-shRNA transfection efficiencies were verified by RT-PCR and western blotting (P<0.001). F, G. CCK-8 and colony formation assays demonstrated that CBX2-shRNA-
mediated knockdown of CBX2 decreased the proliferation of glioma cells (P<0.01). H. Transwell assays showed that CBX2 downregulation reduced invasion compared to that in the scramble group. I. Sphere formation analysis showed that the downregulation of CBX2 decreased the glioma stem cell frequencies (P<0.05). J. Knockdown of CBX2 reduced the levels of N-cadherin, Slug, and Snail and increased the level of E-cadherin.

Figure 2
Upregulation of CBX2 induced glioma cell proliferation, invasion and self-renewal. A, B. CBX2 plasmid transfection efficiencies were verified by RT-PCR and western blotting (p<0.01). C, D. CCK-8 and colony formation assays demonstrated that CBX2 plasmid-mediated overexpression of CBX2 induced the proliferation of glioma cells (P<0.01, P<0.05). E. Transwell assays showed that CBX2 upregulation induced invasion compared to that of the scramble group (P<0.01). F. The glioma stem cell frequency was increased following transfection with the CBX2 plasmid, and the effects were analyzed by sphere formation assays. G. The upregulation of CBX2 reduced the level of E-cadherin and increased the expression of N-cadherin, Slug, and Snail.

**Figure 3**

Knockdown of CBX2 repressed tumor growth in vivo. A. Fluc bioluminescence images showing the tumors in the nude mouse xenograft model. B. Overall survival analysis demonstrated that CBX2 downregulation improved the survival time (p<0.05). C. The mouse body weights were evaluated. D. Images of HE-stained tumor tissue. E. The expression of CBX2 and Ki67 was analyzed by immunohistochemical staining.
Figure 4

Knockdown of CBX2 inhibited glioma cell proliferation and invasion and glioma stem cell self-renewal via the downregulation of the AKT/PI3K pathway. A. Knockdown of CBX2 inhibited the levels of p-AKT and p-GSK-3β, reducing the activity of the AKT/PI3K pathway. The use of EGF, which is an AKT/PI3K pathway agonist, increased the levels of p-AKT and p-GSK-3β. B, C. CCK-8 and colony formation assays showed...
that EGF rescued the proliferation of glioma cells. D. Sphere formation assays showed that the cells in the CBX2-shRNA1+EGF group were more tumorigenic than those in the CBX2-shRNA1 group (P<0.05).

**Supplementary Files**

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- TableS1.xlsx