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CBX8 Promotes Cell Proliferation and Metastasis and Leads to Radiotherapy Tolerance of Glioma Cells

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ABSTRACT

AIM: To illustrate the role of CBX8 -a protein involved in protein metabolism and chromatin regulation/acetylation- in glioma cells, especially in DNA damage repair pathways.

MATERIAL and METHODS: Detect CBX8 expression in glioma cells and clinical samples by qPCR and Western blot. Overexpression and knockdown CBX8 cell lines were constructed by lentivirus infection. CCK8, wound healing, and transwell assays were used to verify the effects of CBX8 on proliferation, migration, and invasion of glioma cells. After radiation treatment, CCK8 and colony formation assays were used to detect cell sensitivity of CBX8 expression levels to radiotherapy. Western blot detected expression levels of p-ATM, p-ATR, BRCA-1, RAD51, and P53 in various cells after radiation treatment, demonstrating CBX8's effect on DNA damage and repair proteins. Finally, the sensitivity of tumors with different CBX8 expression levels to radiotherapy was verified in vivo.

RESULTS: CBX8 expression is significantly increased in glioma. High CBX8 expression promotes proliferation, invasion, and migration of glioma cells. It also causes glioma cells to resist radiotherapy. CBX8 affects protein expression related to DNA damage repair. In vivo, tumors with low CBX8 expression are more sensitive to radiotherapy.

CONCLUSION: CBX8 promotes proliferation and metastasis of glioma cells and reduces cell sensitivity to radiotherapy by affecting DNA damage repair pathways.

KEYWORDS: Glioma, CBX8, DNA damage repair, Radiotherapy tolerance, Oncogene

ABBREVIATIONS: ANOVA: One-way analysis of variance, **CBX8:** Chromobox 8, **ESCC:** Esophageal squamous cell carcinoma, **HCC:** Hepatocellular carcinoma, **QPCR:** Quantitative real-time PCR, **WHO:** World Health Organization, **YBX1:** Y-box binding protein 1

INTRODUCTION

Glioma is the most common primary tumor of the brain and spinal cord (7,9,14). Histologically, they share characteristics with normal glial cells. Glioma diagnosis and classification is based on histopathology. In the World Health Organization (WHO) classification, there are seven molecular subtypes: astrocytoma, glioblastoma multiforme, oligodendroglioma, ependymoma, mixed glioma, medulloblastoma, and choroid plexus papilloma (7,9).

Standard glioma treatments include surgery, radiation therapy, chemotherapy, molecular, and immunotherapy (11). Postoperative adjuvant radiotherapy and chemotherapy are conventional treatment methods. Research on the combination of immune checkpoint inhibitors and radiotherapy in high-grade glioma population is ongoing (2,3).

CBX8 (Chromobox 8) is a protein involved in protein metabolism and chromatin regulation/acetylation. It has ubiquitin-protein transferase activity and can bind single-stranded

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RNA. CBX8 is an important part of the polycomb inhibitory complex, directly regulating expression of many target genes and influencing cell fate (4). CBX8 plays an important role in various malignant tumors. In esophageal cancer, CBX8 functions as an oncogene that promotes tumor cell proliferation and improves tumor chemotherapy resistance. It is one poor prognosis indicator for patients with esophageal squamous cell carcinoma (ESCC) (16). CBX8 is significantly increased in gastric cancer tissues compared to marginal tissues (5). In hepatocellular carcinoma (HCC), CBX8 acts as an oncogene, up-regulating EGR1 and miR-365-3p to stimulate the AKT/β-catenin pathway (18). CBX8 interacts with Y-Box binding protein 1 (YBX1) and promotes HCC cell proliferation (15). Additionally, CBX8 drives HCC stem cell-like and metastatic behavior and regulates BMP4 expression (13). Studies report that high CBX8 expression promotes cancer cell proliferation by inhibiting the p53 pathway. Also, it has an important carcinogenic effect on the invasiveness of bladder urothelial cancer cells (17). CBX8 plays an important role in transcriptional regulation of MLL-AF9 and leukemia occurrence (12). In breast cancer, ectopic CBX8 expression can promote breast cancer progression (8).

Studies show that CBX8 plays an important regulatory role in the DNA damage response (10). The role of CBX8 in glioma has not been clearly reported. Our study illustrates the role of CBX8 in glioma cells, especially in DNA damage repair pathways.

MATERIAL and METHODS

Tissue Preparation

Clinical tissue samples from 15 glioma patients were collected for mRNA and protein detection. Approval and consent for the use of human tissue were obtained from the Ethics Committee. All persons gave their informed consent prior to inclusion in the study.

Cell Lines and Culture

Glioma cells U87, T98G, U373, U251, and immortalized human normal glial cells SVGP12 were provided by the Chinese Academy of Sciences Stem Cell Bank. Cells were inoculated in DMEM containing 10% fetal bovine serum (HyClone, Logan, UT, USA), and incubated in a 5% CO_2, 37 °C constant temperature incubator.

Migration and Invasion Assays

Transwell (Millipore Corporation, USA) assay was used to detect the effect of CBX8 on cell migration and invasion. Cells in the logarithmic growth phase were diluted with serum-free DMEM so the final number inoculated was ~5 × 10^4 cells/200 µl/chamber. Chambers were previously coated with matrigel, and 500 µl medium containing 20% FBS was added to lower transwell chambers while 200 µl cell suspension was added to upper chambers. After 48 h, cells were fixed with 4% formaldehyde for 20 min and stained with crystal violet for 10–15 min. Results were recorded under a microscope (200×).

Quantitative Real-time PCR (QPCR)

Total cell RNA was extracted by Trizol reagent (Invitrogen, US), reverse transcribed into cDNA, and detected by quantitative real-time PCR with three replicates per sample. *GAPDH* was the internal reference gene, and the relative transcription amount was calculated by the $2^{-\Delta\Delta CT}$ method. Primers used are as follows:

CBX8 qPCR primers: 5'-AACATCCTGGATGCTCGCTTGC-3' (forward sequence); 5'-TTTGAGGAGGAAGGTTTTGGGCT-3' (reverse sequence). *GAPDH* qPCR primers: 5'-GTCTCCTCTGACTTCAACAGCG-3' (forward sequence); 5'-ACCACCCTGTTGCTGTAGCCAA-3' (reverse sequence).

Western Blot Analysis

Anti-CBX8 antibody (ab70796, Abcam, UK), Anti-p-ATM antibody (ab81292, Abcam, UK), Anti-p-ATR antibody (ab223258, Abcam, UK), Anti-RAD51 antibody (ab133534, Abcam, UK), Anti-BRCA-1 antibody (ab191042, Abcam, UK), Anti-P53 antibody (ab26, Abcam, UK), anti-GAPDH antibody (HRP) (ab9482, Abcam, UK) and Goat Anti-Rabbit IgG H&L antibody (HRP) (ab205718, Abcam, UK) were purchased from Abcam, and Western blot was performed according to conventional methods.

Overexpression and Knockdown of CBX8

The coding sequence of human *CBX8* (NM_020649.3) was constructed into pLV-EF1α-EGFP-N vector to achieve CBX8 overexpression. CBX8 shRNA was designed and constructed into pLKO.1 vector for knockdown experiments. shRNA sequence:5'-CCGGCACGGACGTGACCTCAAACTTCTCGAGAAGTTTGAGGTCACGTCCGTGTTTTTG-3'. The lentivirus was constructed separately and used to transfect cells to achieve overexpression and knockdown of *CBX8*.

Radiotherapy Sensitivity Assay

Glioma cells cultured for 48 hours (cell confluence 80%–90%) were treated with 2Gy radiation. Inhibition rate was calculated by colony formation and CCK8 assays.

Colony Formation Assay

Cells were enzymatically digested and diluted with complete medium to 1000 cells/mL, inoculated in a six-well plate and cultured for 10 days. They were fixed with formaldehyde and stained with crystal violet. Colonies were counted and analyzed.

Animal Experiment

All animal experiments were approved by the Ethics Committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Twenty 5-week-old BALB/c male nude mice were selected, randomly divided into 4 groups, and kept in an SPF animal laboratory. The control cells and CBX8 KD cells were resuspended in PBS and injected subcutaneously into nude mice, 4 × 10^6 cells/100 µL/mouse. One week after inoculation, the volume of subcutaneous transplanted tumor was measured every three days. Three weeks after the inoculation, two groups of tumor-bearing mice were given radiotherapy, 5 times a week, 2 Gy each time. After continuous treatment for two weeks, the nude mice were

euthanized, and tumor volume was measured to calculate the inhibition rate of radiotherapy.

Statistical Analyses

All experiments were repeated 3 times, and SPSS 17.0 software package was used for analysis. Data were expressed as mean ± standard deviation (Mean ± SD). One-way analysis of variance (ANOVA) was used between the data. $p<0.05$ was considered statistically significant.

RESULTS

CBX8 Expression Is Significantly Increased in Glioma

QPCR and Western blot detected the CBX8 expression level in glioma and adjacent tissues. Results showed that the expression level of CBX8 in glioma tissues was significantly higher than that in adjacent tissues (Figure 1A, B). Simultaneously, four types of glioma cells, U87, T98G, U373, and U251, and a normal glioma cell, SVGP12, were detected. Results showed that the expression level of CBX8 in glioma cells T98G, U373, and U251 was significantly higher than in normal glial cells (Figure 1C, D). These results show that up-regulation of CBX8 expression may be involved in the occurrence and development of glioma.

High Expression of CBX8 Promotes Proliferation, Migration, and Invasion of Glioma Cells

qPCR and Western blot results showed that CBX8 can be stably over-expressed and knocked-down in U87 and U251 cells after lentivirus infection (Figure 2A). The CCK8 results showed that CBX8 overexpression promoted proliferation of glioma cells, while knockdown inhibited cell proliferation (Figure 2B). The results of wound healing and transwell assays showed that knockdown of *CBX8* in U251 cells reduced invasiveness, while that of U87 cells overexpressing CBX8 increased. The difference was statistically significant ($p<0.05$) (Figure 2C, D). The experimental results suggested that high CBX8 expression promoted proliferation, migration, and invasion of glioma cells.

High Expression of CBX8 Causes Glioma Cell Insensitive to Radiotherapy

Standard treatment for malignant glioma is surgical resection, radiotherapy, and chemotherapy. CBX8 plays an important, but unclear, regulatory role in the DNA damage response (10). Glioma cells with different CBX8 expression levels were treated with radiation. The inhibition rate was determined by colony formation and CCK8 assays. Results showed that glioma cells with high CBX8 expression were not sensitive to

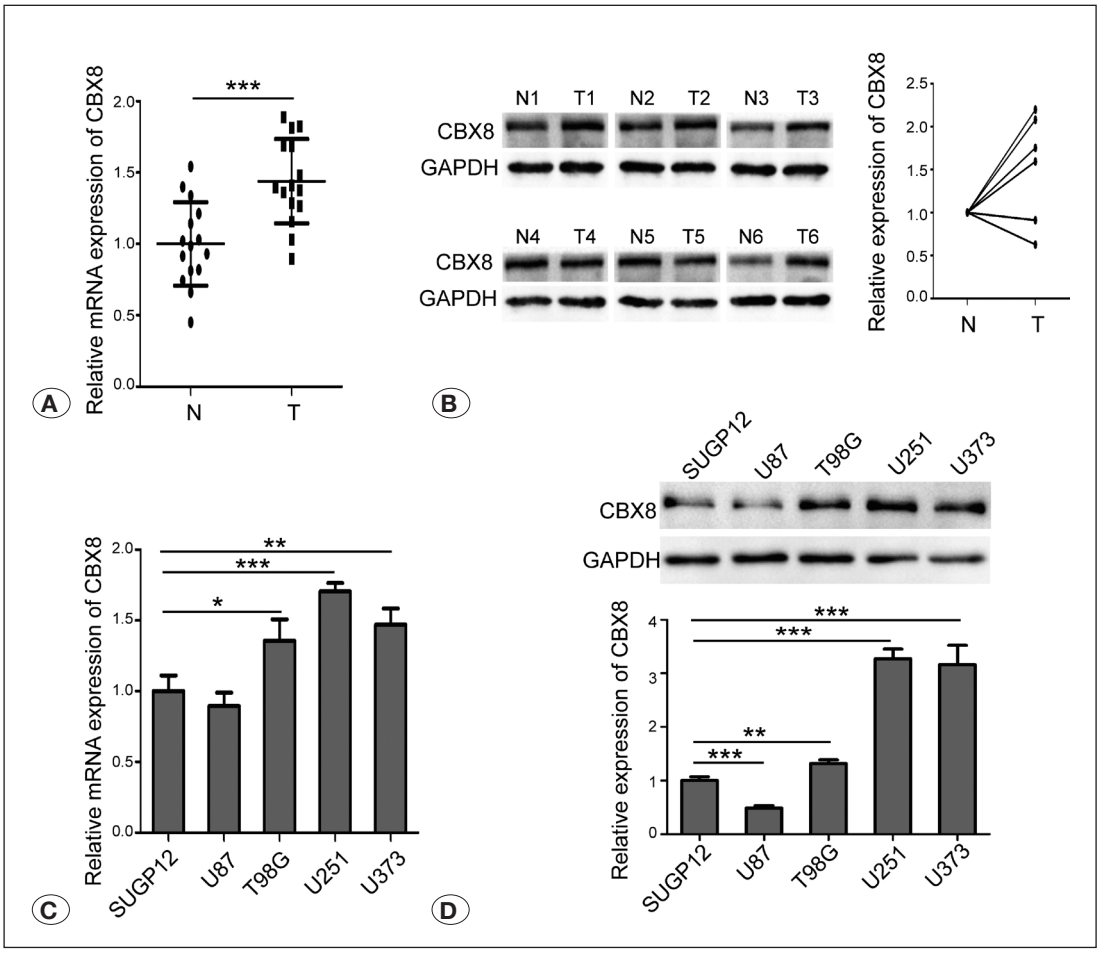

Figure 1: CBX8 expression is significantly increased in glioma. **A)** qPCR detection of *CBX8* transcription levels in glioma tissue samples. **B)** WB detection of CBX8 protein levels in glioma tissue samples. **N:** normal; **T:** tumor. **C)** qPCR detection of *CBX8* transcription levels in glioma cells. **D)** WB detection of CBX8 protein levels in glioma cells (***$p<0.001$, **$p<0.01$; *$p<0.05$).

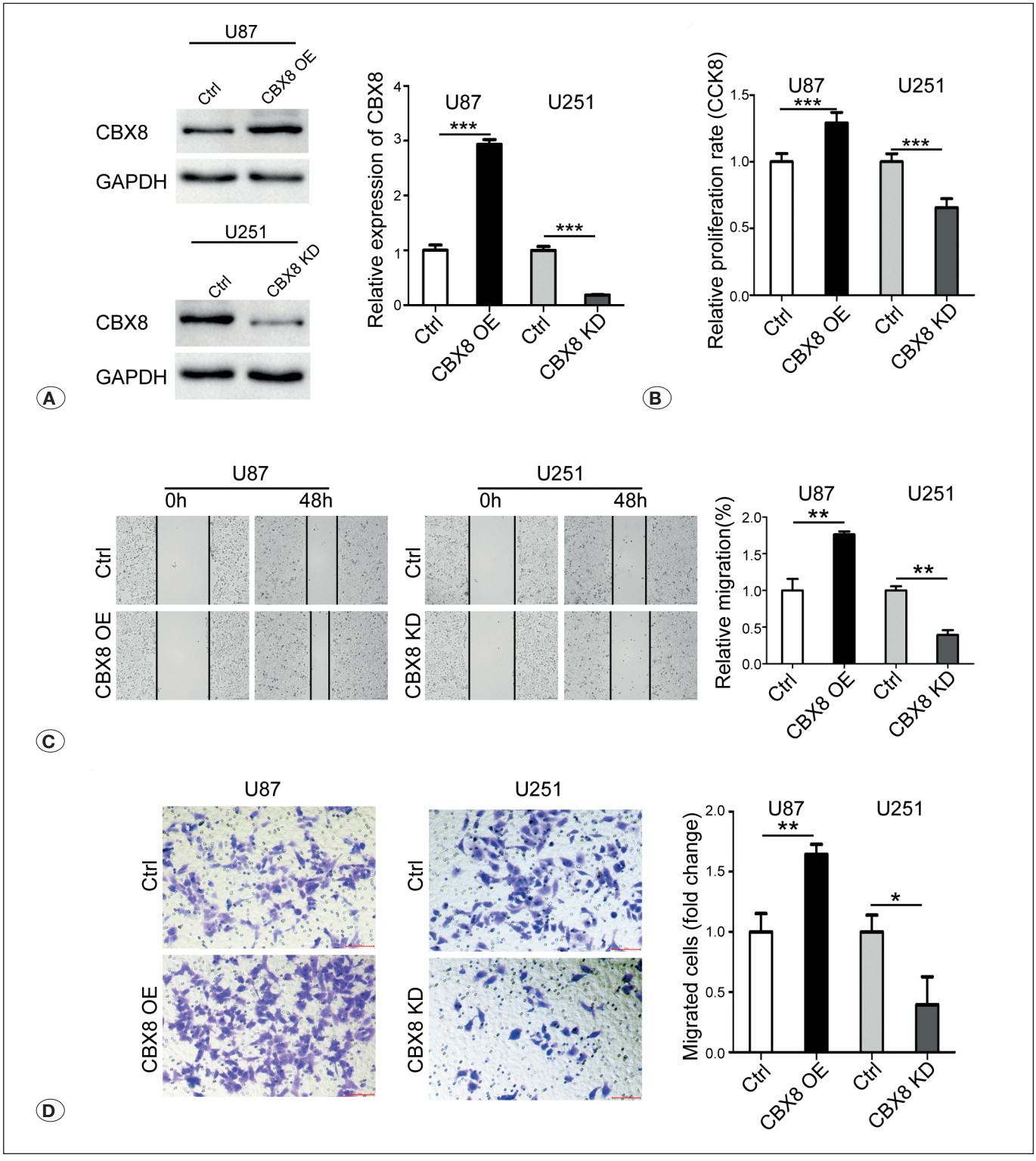

Figure 2: High CBX8 expression promotes proliferation and metastasis of glioma cells. **A)** Overexpression and knockdown of *CBX8* were verified by WB. **B)** CCK8 assay detected cell proliferation (***p<0.001). **C)** Wound healing assay and statistical results (**p<0.01). **D)** Transwell assay and statistical results (**p<0.01; *p<0.05).

radiation treatment. The inhibition rate of radiation treatment on CBX8 high-expressing cells was significantly lower than that of the control group. The knockdown of CBX8 showed the opposite result (Figure 3). We speculate that CBX8 might change cell sensitivity to radiotherapy by affecting DNA damage repair in glioma cells.

CBX8 Affects the Expression of Proteins Related to DNA Damage Repair

To determine whether CBX8 affects cell sensitivity to radiotherapy by affecting the DNA damage repair process, western blot was used to detect the expression level of p-ATM, p-ATR, BRCA-1, RAD51, and P53 in cells after radiation treatment. The results showed that p-ATM, p-ATR, and P53 significantly increased in CBX8 KD cells, and BRCA-1 and RAD51 were significantly decreased in CBX8 KD cells. The opposite result was shown in CBX8 OE cells (Figure 4). This result indicates that when high CBX8 expression cells are subjected to radiation treatment, low levels of p-ATM, p-ATR, and P53 cannot normally initiate apoptosis and cell cycle arrest procedures. Higher levels of BRCA-1 and RAD51 promote an efficient DNA damage repair process. This result is the direct cause of cell insensitivity to radiotherapy. We speculate that the abnormally high expression of CBX8 seriously affects protein expression levels of multiple important molecules involved in the DNA damage and repair process of glioma cells, which affects the normal response of this pathway to radiotherapy.

In Vivo, Tumors with Low CBX8 Expression Are More Sensitive to Radiotherapy

Xenograft tumor experiments performed in nude mice showed that the tumorigenicity and proliferation rate of CBX8 knockdown cells were significantly lower than those of the control group (Figure 5A). After formation of visible tumor masses, experimental mice were given radiotherapy. The results showed that CBX8 knockdown mice were significantly more sensitive to radiotherapy with a significantly improved inhibition rate (Figure 5B). The results demonstrated that CBX8 knockdown could increase glioma cell sensitivity to radiotherapy. CBX8 may be a therapeutic target for glioma.

DISCUSSION

Glioma resistance to radiotherapy and chemotherapy significantly contributes to the poor prognosis of glioma (1,6).

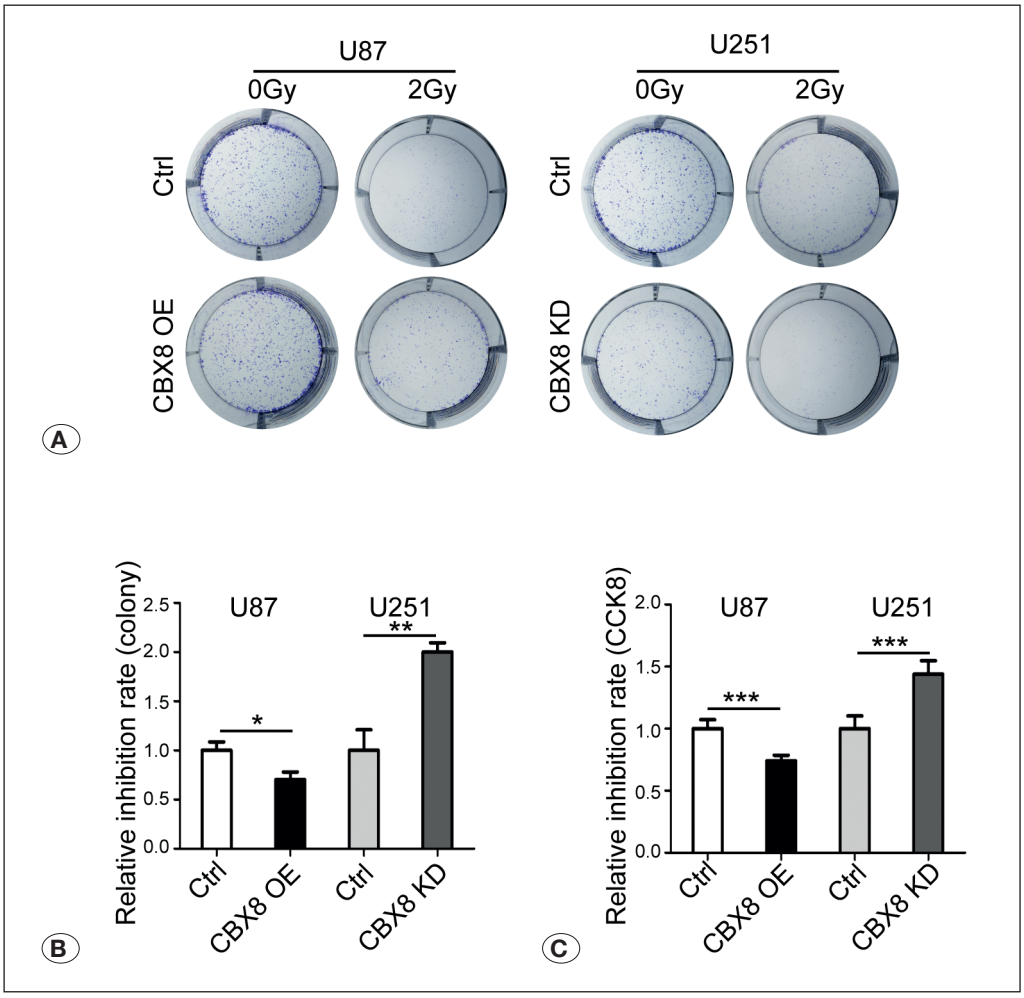

Figure 3: The relationship between CBX8 expression level and radiotherapy sensitivity. **A, B)** Colony formation assay to detect the inhibition rate of radiotherapy and statistical results (**p<0.01; *p<0.05). **C)** CCK8 assay to detect the inhibition rate of radiotherapy (***p<0.001).

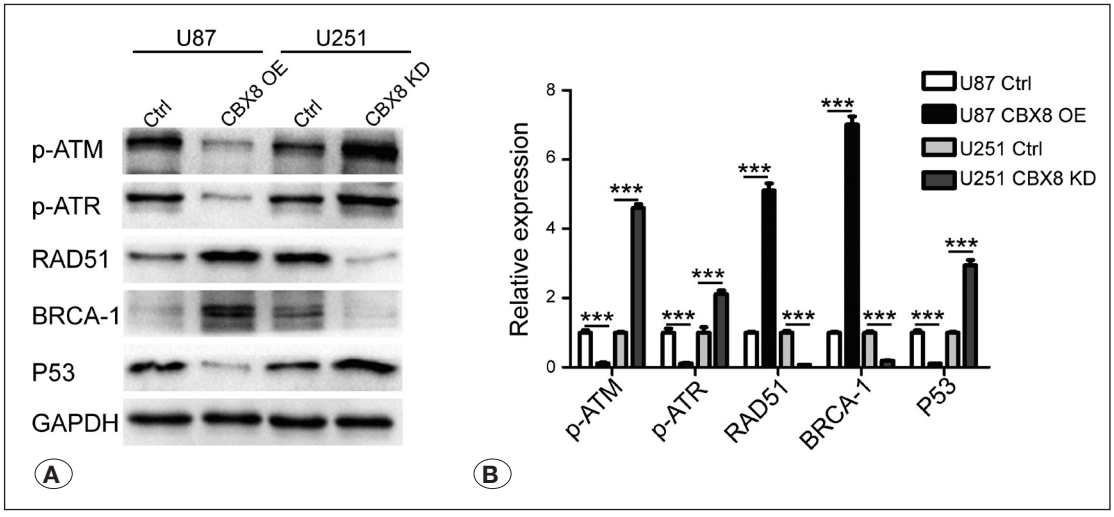

Figure 4: DNA damage repair process related proteins are affected by CBX8 expression. **A, B)** Western blot detected the expression and statistical results of DNA damage repair related proteins p-ATM, p-ATR, BRCA-1, RAD51, P53 after radiotherapy in glioma cells (***p<0.001).

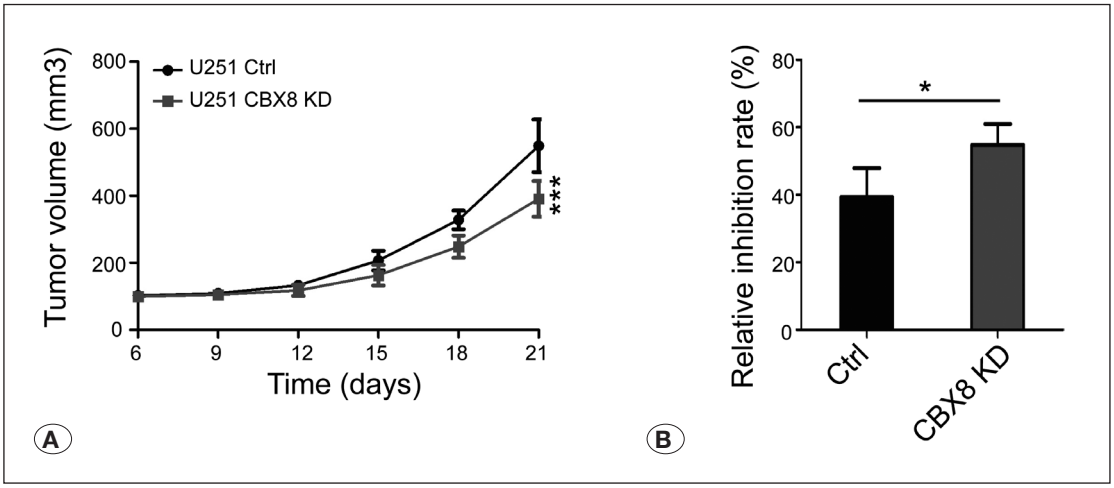

Figure 5: *In vivo*, tumors with low CBX8 expression are more sensitive to radiation therapy. **A)** Cells with different CBX8 expression levels have different tumorigenicity in nude mice (***p<0.001). **B)** Cells with different CBX8 expression levels have different sensitivity to radiotherapy (*p<0.05).

Its molecular mechanism is a complex network composed of multiple signaling pathways. Recent studies show that DNA damage checkpoints ATM, ATR, Chk1, Chk2, Rad17, Rad1, Rad9, P53, BRCA-1, and RAD51 and their regulatory pathways play an important role in cell proliferation, genome stability, tumor occurrence, and tumor radio- and chemotherapy resistance. Interfering with cell DNA damage checkpoints can increase the sensitivity of radio- and chemotherapy-resistant tumor cells and improve efficacy.

CBX8 up-regulation leads to significant decreases in p-ATM, p-ATR, and P53, which are the main factors involved in the G2/M DNA damage and repair signaling pathway of malignant glioma, while BRCA-1 and RAD51 increase significantly. When cellular DNA is damaged by radiation, changes in these key molecules can lead to the following results: low levels of p-ATM, p-ATR, and P53 cannot normally initiate cell apoptosis and cycle arrest programs, while higher levels of BRCA-1 and RAD51 began an efficient DNA damage repair process. Inhibiting cell apoptosis and promoting DNA damage repair drive radiotherapy insensitivity. It is speculated that abnormally high CBX8 expression seriously affects the protein expression levels of important molecules involved in the DNA damage and repair process of glioma cells, which affects the normal response of this pathway to radiotherapy.

Additionally, high CBX8 expression also promotes the proliferation and metastasis of malignant glioma cells. It may be one inducer leading to development of malignant glioma. Under environmental factors that induce DNA mutations, cells cannot respond correctly, that is, the cycle is stalled to repair DNA damage or induce cell apoptosis. Cells with harmful mutations continue to survive and even proliferate, leading to malignant tumors. For malignant glioma, one of the main treatments is radiation therapy. High CBX8 expression can lead to radiotherapy tolerance and poor prognosis.

CONCLUSION

CBX8 promotes the proliferation and metastasis of glioma cells and reduces the sensitivity of cells to radiotherapy by affecting DNA damage repair pathways. This result suggests that for patients with high CBX8 expression, radiotherapy is not the best option for postoperative adjuvant treatment. Cell therapy, gene therapy, or targeted therapy will gradually become alternative adjuvant therapies.

AUTHORSHIP CONTRIBUTION

Study conception and design: QL, XL
Data collection: QL, XL
Analysis and interpretation of results: QL, XL
Draft manuscript preparation: QL
Critical revision of the article and study supervision: WG
All authors (QL, XL, WG) reviewed the results and approved the final version of the manuscript.

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