CNN3 knockdown inhibits the proliferation, invasion and migration of glioma

Xu Xiao
Harbin Medical University Cancer Hospital
Yu Wang
Harbin Medical University Cancer Hospital
Han Xiao
The Forth Hospital Of Daqing
Chenlong Li
Harbin Medical University Cancer Hospital
Peng Liang (289404257@qq.com)
Harbin Medical University Cancer Hospital

Research Article

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Abstract

Background

Glioma is a severe, malignant tumor in the central nervous system. The present study detected the expression level and biological functions of calponin 3 (CNN3) in glioma to investigate the mechanism of glioma.

Results

The results revealed that CNN3 was significantly overexpressed in glioma cells and tissues, and was associated with poor prognosis. Knockdown of CNN3 reduced the proliferation, invasion and migration of glioma *in vitro*, and inhibited the growth of glioma *in vivo,* indicating that CNN3 was significantly associated with MEK/ERK signaling pathway.

Conclusions

CNN3 is a tumor-promoting gene in glioma, and a promising novel tumor marker and therapeutic target for glioma diagnosis.

Background

Glioma is one of the most aggressive and common malignant tumors in the central nervous system, which seriously threatens human health worldwide(1). According to the 2021 World Health Organization classification criteria (2), gliomas are classified into grades I-IV. Although low-grade glioma cells proliferate slowly and patients have a better prognosis, grade IV glioma [also known as glioblastoma multiforme (GBM)] is characterized by rapid recurrence and poor prognosis due to the high proliferative and invasive nature of its cancer cells (3). Despite developments in GBM treatment, the prognosis of patients with GBM has not improved significantly(4, 5). For patients with GBM, the median overall and progression-free survival time was only 14 and 7 months, respectively. For elderly glioma patients (age > 65 years), the 5-year survival rate is only ~ 2% (6). Currently, the standard treatment for GBM is surgery followed by chemoradiotherapy (7, 8). However, this treatment modality cannot effectively prevent recurrence or improve patient prognosis, which is associated with the current lack of clarity on the mechanism of GBM(8, 9).

Calponin (CNN) is an actin filament-associated regulatory protein expressed in smooth muscle and various non-muscle cells(10). Calponin family includes three isoforms: CNN1, CNN2 and CNN3. CNN3 was found to be expressed in neurons(11), astrocytes(12) and glial cells(13), where CNN3 functions include regulation of the actin cytoskeleton and neural plasticity (14). Various studies have demonstrated that CNN3 plays roles in a variety of malignant tumors. In cervical cancer, CNN3 can promote the progression of cervical cancer cells by affecting the expression of ribosomal protein lateral stalk subunit P1 (RPLP1) at the mRNA level (15). CNN3 promotes invasion and induction of drug resistance in colon

cancer cells (16). However, in non-small cell lung carcinoma (NSCLC), CNN3 acts as a tumor suppressor gene, and inhibits the progression of NSCLC by inhibiting the proliferation, invasion and migration of NSCLC cells (17). In summary, CNN3 plays different roles in a variety of cancer types, promoting or inhibiting the proliferation, invasion, metastasis and drug resistance of malignant tumors.

However, the association between CNN3 and glioma remains unclear. The present study aimed to investigate the effect and mechanism of CNN3 on glioma cell lines, and to clarify its potential as a therapeutic target and tumor marker for glioma.

Materials And Methods

Patients and specimens. Tumor and adjacent tissues were collected from 30 patients with glioma who underwent surgical resection in Harbin Medical University Cancer Hospital from 2020 to 2021 (15 females and 15 males; age, 20–58 years). None of the patients received chemotherapy or radiotherapy before surgery. All patients' pathology samples were verified by two senior pathologists. Tissue samples were stored for further experiments in liquid nitrogen at the Department of Pathology, Harbin Medical University Cancer Hospital. All glioma patients signed an informed consent form for specimen use in research, and all surgeries were conducted in line with the principles of surgical treatment for patients with glioma (18). The present study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital (Ethical code KY2021-22). The clinical information of the patients enrolled in the present study is presented in Table SI.

Cell culture. Normal human astrocyte (NHA) cells and human glioma cell lines (U251, T98G, LN229 and A172) were purchased from The American Type Culture Collection. All cell lines were cultured in 89% high-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.), 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. Cells were cultured in an incubator at 37˚C in the presence of 5% CO_2.

Transfection. Short hairpin (sh) RNA targeting CNN3 in plasma and its negative control (NC) were constructed by Shanghai GeneChem Co., Ltd. U251 and T98G were cultured in 6-well plates. When cells reached ~ 70% confluence, the plasmids with sh-RNA were transfected into cells by using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Transfection efficiency was examined by reverse transcription-quantitative PCR (RT-qPCR) and western blotting at 24 and 48 h after transfection. The sequences of sh-RNAs are presented in Table SII.

Bioinformatics data. RNA-seq data of glioma were downloaded from the Gene Expression Omnibus (GEO) (GSE16011 and GSE111260) and Gene Expression Profiling Interactive Analysis (GEPIA) databases. The expression of CNN3 in glioblastoma multiforme (GBM) and low grade glioma (LGG) were visualized by GEPIA online tool. The prognosis information were obtained from The Cancer Genome Atlas (TCGA) and Chinese Glioma Genome Atlas (CGGA) databases. The R package 'limma' was used to analyze differentially expressed genes at the mRNA level between glioma and normal tissues according

to the following thresholds: log2 fold-change (FC) > 2 and false-discovery rate < 0.01. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed using the Database for Annotation, Visualization and Integrated Discovery online tool. The prediction of correlations between CNN3 and other genes was performed with the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) online tool.

RT-qPCR. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total mRNA from cell lines and tissues. HiFiScript gDNA Removal cDNA Synthesis kit (CoWin Biosciences; Beijing) was used for RT. SYBR Green qPCR Mix (Biosharp Life Science; Beijing) was used to conduct RT-qPCR (95℃, 5 min, cycle 1; 95℃, 10 sec, cycle 40; 60℃, 30 sec, cycle 40). β-actin was used as an internal control in RT-qPCR. All the steps were performed according to the manufacturers' protocols. The sequences of primers of CNN3 and β-actin (Table SIII) were designed and constructed by Beijing Ruibiotech Biotechnology Co., LTD. CNN3 mRNA expression levels were normalized to that of the internal control, and the results were calculated according to the $2^{-\Delta\Delta Cq}$ method (19).

Western blotting. RIPA and PMSF were used to extract total proteins from different cell lines. A BCA protein assay kit (Beyotime Institute of Biotechnology; Shanghai) was used to calculate the concentration of the extracted proteins. 10% SDS-PAGE was used to separate the proteins (40 μg/ lane), which were then transferred onto PVDF membranes. Next, primary antibodies against CNN3 (Catalog number: 11509-1-AP) and GAPDH (Catalog number: 10494-1-AP) (rabbit polyclonal; 1:1,000; ProteinTech Group, Inc.) were incubated at 4˚C overnight, followed by 1-h incubation with the secondary antibody. Protein bands were visualized using ECL detection with a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc.).

Cell Counting Kit-8 (CCK-8) assay. Transfected U251 and T98G glioma cells were suspended and plated in 96-well plates (2500 cells/ plate). After 24 h, 10-μl CCK-8 solution (Beyotime Institute of Biotechnology; Shanghai) was added to each well of the 96-well plate. After incubation for 4 hours at 37℃, the optical density (OD) values were detected at 450 nm with a Tecan microplate reader (Infinite F50; Tecan Group, Ltd.).

Transwell assay. The invasion and migration abilities of glioma cells were detected by Transwell assay. The Matrigel (Beyotime Institute of Biotechnology; Shanghai) was diluted following the manufacturer's protocol and then added in the Transwell upper chamber (8-μm pore size; Corning, Inc.). A high-serum medium containing 20% FBS was placed in the lower Transwell chamber. Transfected U251 and T98G cells were suspended in non-FBS medium. The cells were placed in the upper chamber (3000 cells per chamber) and incubated at 37℃ for 24 h. The remaining cells and the Matrigel in the upper chamber were swabbed. The cells on the other side of the chamber were fixed with methanol for 5 min and then stained with crystal violet (Beijing Solarbio Science & Technology Co., Ltd.) for 15 min. Cell images were recorded with a light microscope. Transwell migration assays were performed as Transwell invasion assays with the exception of the Matrigel.

Immunohistochemistry (IHC). The specimens were fixed in formalin and embedded in paraffin. The slices were then heated at 66˚C for 1 h, dewaxed with xylene and placed in a gradient ranging from ethanol to

water. Upon washing at room temperature three times with PBS for 5 min each. Incubate with 3% hydrogen peroxide for 10 minutes. Upon washing at room temperature three times with PBS for 5 min each, the slides were incubated at 4℃ with the primary antibody (CNN3; Cat.no. 10494-1-AP; rabbit polyclonal; 1:300; ProteinTech Group, Inc.) overnight, and washed again with PBS for 5 min (three times). Next, the secondary antibody was added and incubated at room temperature for 30 min.

Glioma xenograft mouse model. 8 Female BALB/c nude mice (4 weeks of age, 15–20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., (Charles River Laboratories). All mice were housed in a specific pathogen-free animal house and provided free access to food and water. CNN3 was knocked down in U251 glioma cells with shRNA and sh-NC, and the cells were inoculated into the right armpit region of mice (n = 4/group, $5x10^5$ cells/mouse, 5 µl). Tumor volumes were measured every 7 days after inoculation (at days 7, 14, 21, 28 and 35) according to the following formula: Tumor volume = long diameter x short diameter^2 x 0.5. All mice were euthanized by intraperitoneal injection with sodium pentobarbital (130 mg/kg). All animal procedures performed in the present study met the requirements of the National Institutes of Health (CAB International, 2011) and were approved by the Ethics Committee of Harbin Medical University Cancer Hospital (Ethical code KY2021-22).

Statistical analysis. Statistical analyses were conducted with SPSS 22.0 statistical software (IBM Corp.). Differences between the means of two groups were compared using an independent samples Student's ttest, and comparisons between multiple groups were performed using a one-way ANOVA with a post hoc Tukey's test. Pearson’s correlation analysis was used to calculate the correlations between CNN3 and MEK1, MEK2, ERK1, ERK2. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

CNN3 expression is negatively correlated with prognosis in glioma. Firstly, GBM-related expression data were downloaded from the GEO (GSE16011 and GSE111260) and GEPIA databases. To identify differentially expressed genes in the aforementioned databases, the threshold was set to $FC > 2$, $P < 0.01$, and a Venn diagram was used for visualization (Fig. 1A). The results revealed the existence of 8 differentially expressed genes (namely, tetraspanin 6, butyrophilin subfamily 3 member A2, LIM domain and actin binding 1, xenotropic and polytropic retrovirus receptor 1, CNN3, RP2, dynein light chain Tctex-type 1 and nucleoporin 37). In order to investigate the associations between these 8 genes and glioma, KEGG analysis was performed on them, and their related genes were predicted by using the STRING database. The results suggested that CNN3 was significantly associated with glioma (Fig. 1C).

The expression level of CNN3 was compared between glioma (GBM and LGG) and normal brain tissues based on data from GEO and GEPIA databases by using the online tool GEPIA. The results verified that CNN3 was significantly increased in GBM and LGG tissues ($P < 0.001$; Fig. 1B).

To clarify the association between CNN3 and prognosis of patients with glioma, Kaplan-Meier survival curve analysis was performed using the clinical information of patients with glioma from the TCGA and

CGGA databases (Fig. 1D and E). The results showed that the expression of CNN3 was significantly negatively correlated with the survival of patients with glioma ($P < 0.001$). In conclusion, bioinformatics analysis suggested that CNN3 was overexpressed in glioma tissue and was associated with poor prognosis of patients with glioma. Overall, these results suggested that CNN3 may play a role as an oncogene in glioma.

CNN3 is overexpressed in glioma cell lines and tissue samples. To verify the aforementioned hypothesis, RT-qPCR and western blotting were used to detect the expression of CNN3 in glioma cell lines at the mRNA and protein level, respectively. The results showed that the expression of CNN3 at both the mRNA and protein level was significantly increased in glioma cell lines (U251, LN229, T98G and A172) compared with that in NHAs ($P < 0.05$; Fig. 2A and B).

Next, 30 GBM and adjacent normal tissues from patients with glioma were selected for RT-qPCR experiments. The results showed that the mRNA expression of CNN3 was significantly upregulated in GBM tissues ($P < 0.01$; Fig. 2C). In addition, immunohistochemical detection of GBM and adjacent normal tissues was performed, and the results were consistent with the above experimental findings (Fig. 2D). In conclusion, CNN3 was significantly upregulated in glioma cell lines and tissues.

Construction of a glioma cell line with low expression of CNN3. Since the U251 and T98G cell lines expressed higher levels of CNN3 than other glioma cell lines (Fig. 2A), these cell lines were selected for subsequent experiments. Three shRNAs (sh-CNN3-1, sh-CNN3-2 and shCNN3-3) and a sh-NC plasmid were transfected into U251 and T98G cells. After 24 h, RT-qPCR assays were used to detect the CNN3 mRNA expression level. The results showed that the knockdown efficiency of sh-CNN3-2 was the most remarkable (Fig. 3A). Therefore, sh-CNN-2 was selected for subsequent experiments. Western blotting was also performed on sh-CNN3-2 and sh-NC-transfected cells at 48 h after transfection. The results showed that CNN3 expression was also effectively suppressed by sh-CNN3-2 at the protein level (Fig. 3B).

Knockdown of CNN3 expression inhibits the proliferation, invasion and migration of glioma cells. To determine the effect of CNN3 on glioma *in vitro*, CCK-8 and Transwell assays were used to investigate the effect of CNN3 on cell proliferation, invasion and migration. As shown in Fig. 4A, compared with that of the sh-NC group, the increase in OD of the sh-CNN3 group was significantly slower. Furthermore, the numbers of invasive and migrating cells in the sh-CNN3 group were also significantly decreased compared with those in the sh-NC group (Fig. 4B and C). The above results suggested that knockdown of CNN3 could inhibit the proliferation, invasion and migration abilities of glioma cells.

Knockdown of CNN3 inhibits glioma growth in vivo. To explore the role of CNN3 *in vivo*, U251 cells of the knockdown CNN3 expression group (sh-CNN3) and the NC group (sh-NC) were inoculated into BALB/c nude mice ($n = 4$ mice/group). The volume of subcutaneous tumors in mice was measured every 7 days. Mice were sacrificed on day 35, and subcutaneous tumor weights were measured. In the NC group, 3 mice were successfully inoculated and 1 mouse was inoculated unsuccessfully, while 4 mice in the CNN3 knockdown group were successfully inoculated. The results showed that the tumor weight and growth

rate of mice in the CNN3 low-expression group were lower than those of mice in the NC group (Fig. 5A and B).

Bioinformatics analysis suggests that CNN3 can affect the MEK/ERK signaling pathway in glioma. To investigate the specific mechanism by which CNN3 affects glioma growth, the correlation of CNN3 with other genes was predicted using the online tool STRING. The results suggested that CNN3 may interact with ERK (Fig. 6A). Therefore, Pearson's correlation analysis was used to determine the correlation between CNN3 and the expression of known markers (MEK1/2 and ERK1/2) in the MAPK signaling pathway. The results showed that CNN3 was significantly correlated with the mRNA transcription levels of MEK1/2 and ERK1/2 (Fig. 6B-E, $P < 0.05$). These results suggested that CNN3 may affect glioma growth through the MEK/ERK signaling pathway.

Discussion

Glioma is one of the most malignant and aggressive tumors among the primary central nervous system tumors(20). Although great effort has been made in the treatment of glioma, the prognosis of patients with glioma has not effectively improved, likely due to the lack of understanding of the mechanism of glioma(4).

The present study performed bioinformatics analysis using expression data from the GEO, TCGA and GTEx databases. The results indicated that the mRNA transcription level of CNN3 in LGG and GBM was significantly increased compared with that in human normal brain tissues, which suggested that dysregulation of CNN3 expression may be involved in the pathogenesis and development of glioma. KEGG enrichment analysis of CNN3 showed the same trend. In addition, according to the survival curves generated using the data from TCGA and CGGA databases, the prognosis of patients in the CNN3 high-expression group was significantly worse than that of patients in the CNN3 low-expression group ($P < 0.05$).

Subsequently, to verify whether the mRNA transcription level of CNN3 was higher in glioma, as obtained by bioinformatics analysis, four commonly used glioma cell lines (U251, LN229, T98G and A172) and NHAs were selected for RT-qPCR assays. The results showed that CNN3 was significantly overexpressed in the above glioma cell lines compared with NHAs. In addition, the expression levels of CNN3 were highest in U251 and T98G cells. Thus, the U251 and T98G cell lines were selected for subsequent experiments. Western blotting was also employed to detect the protein level of CNN3 in the four aforementioned glioma cell lines and NHAs. The results also showed that CNN3 was highly expressed at the protein level in all the glioma cell lines. Furthermore, 30 pairs of glioma and adjacent normal tissues were selected from patients with glioma for RT-qPCR and IHC assays, and the experimental results were consistent with those obtained in cell lines. Thus, it was concluded that CNN3 was highly expressed in glioma at both the mRNA and protein level.

In different malignant tumors, the expression level and functional role of CNN3 are different. For example, in NSCLC, CNN3 exhibits low expression and acts as a tumor suppressor gene, which inhibits the

proliferation, migration and invasion of NSCLC cells by affecting the PI3K/AKT signaling pathway (17). By contrast, in cervical cancer, CNN3 is highly expressed, and by affecting the mRNA transcription level of RPLP1, it increases the proliferation and invasion abilities of cervical cancer cells, thereby shortening the survival time of patients with cancer (15). In osteosarcoma, CNN3 was reported to play an oncogenic role in tumor development by activating the ERK1/2 and p38 signaling pathways, and was associated with poor prognosis in patients with osteosarcoma (21). CNN3 is also associated with drug resistance. For example, (16)found that, in colorectal cancer, CNN3 can promote colon cancer lymph node metastasis and enhance colon cancer chemotherapy resistance by affecting β-catenin and p53. Therefore, the current study explored which biological processes of glioma cells were specifically affected by CNN3.

To compare the effects of different expression levels of CNN3 on the biological function of glioma cell lines, glioma cells with knocked down CNN3 expression were constructed. Cell transfection using shRNA is a commonly used and effective gene knockdown method. Three shRNAs with different knockdown sites (sh-CNN3-1, sh-CNN3-2 and sh-CNN3-3) were employed, and their CNN3 knockdown efficiency was detected at the mRNA and protein level by using RT-qPCR and western blotting, respectively. The results showed that the knockout efficiency and stability of sh-CNN3-1 and sh-CNN3-3 were lower than those of sh-CNN3-2; thus, sh-CNN3-2 was selected for subsequent functional experiments.

As aforementioned, CNN3 plays different biological functions in different types of tumor cells. Therefore, to explore the specific biological activities that CNN3 affects in glioma cells, CCK-8 assays were performed in the current study to explore the effect of CNN3 on the proliferation of glioma cells. The results showed that, compared with that of the NC group, the cell proliferation rate of the CNN3-knockdown group was significantly decreased in both the U251 and T98G cell lines. Subsequently, Transwell invasion and migration assays were performed on U251 and T98G cells in both the NC and CNN3 knockdown groups. The results revealed that, after knocking down CNN3 expression, the invasion and migration abilities of cancer cells were significantly decreased in both glioma cell lines. Thus, it was concluded that CNN3 could affect the proliferation, invasion and migration abilities of glioma cells.

To explore the effect of CNN3 on glioma growth *in vivo*, U251 transfected with sh-CNN3 and sh-NC were subcutaneously inoculated into mice (4 mice in each group) to construct a glioma xenograft model. One mouse in the NC group was inoculated unsuccessfully, which meant the glioma cells did not grow in the mouse subcutaneous tissue. The volume of subcutaneous tumors in mice was estimated every 7 days after inoculation (on days 7, 14, 21, 28 and 35) according to the formula ‘tumor volume = long diameter x short diameter^2 x 0.5’(22). The mice were sacrificed on day 35 after inoculation, and the subcutaneous tumors of each mouse were removed and their weights recorded. The results showed that the growth rate and quality of tumors of subcutaneous tumors in the CNN3 knockdown group were significantly lower than those in the control group. However, due to budget constraints and limited laboratory equipment, the number of mice included in the present experiments was relatively small, and glioma cells were not directly inoculated into the mouse brains, which should be conducted in future research.

Finally, to investigate the specific mechanism by which CNN3 affects glioma growth, the online tool STRING was used to analyze other genes that may interact with CNN3. The results revealed that CNN3 had a strong potential to interact with ERK. Previous studies showed that ERK could affect the migration and invasion of cells by activating the MAPK/ERK signaling pathway (23, 24). Both ERK1 and ERK2 can be phosphorylated simultaneously in glioma cells(25, 26), and phosphorylated ERK1/2 can further activate glioma growth-related pathways. Therefore, the present study calculated the correlation between CNN3 and the expression of related genes (MEK1/2 and ERK1/2) in the MAPK signaling pathway using Pearson's correlation analysis and the mRNA expression data of GBM and LGG in TCGA. The results showed that CNN3 was significantly correlated with the mRNA transcription levels of MEK1/2 and ERK1/2 ($P < 0.05$). Therefore, it was concluded that CNN3 may affect the occurrence and development of glioma through the MEK/ERK signaling pathway, but this hypothesis needs further experimental verification.

In conclusion, the present study demonstrated that CNN3 functioned as an oncogene in glioma *in vitro* and *in vivo*. Bioinformatics analysis suggested that CNN3 had a negative correlation with the prognosis of patients with glioma and had a significant correlation with the MEK/ERK signaling pathway in glioma. Overall, the present results suggest that CNN3 may potentially be a novel target and biomarker for the treatment of glioma.

Declarations

Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Harbin Medical University Cancer Hospital (April 29, 2021/No KY2021-22). Informed consent was obtained from all individual participants included in the study.

All animal procedures performed in the present study met the requirements of the National Institutes of Health (CAB International, 2011) and were approved by the Ethics Committee of Harbin Medical University Cancer Hospital (Ethical code KY2021-22). The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org).

Consent for publication

Not applicable

Availability of data and materials

The datasets generated during and/or analysed during the current study are available in GEO GSE16011 dataset, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16011; GEO GSE111260 dataset, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111260; GEPIA database, http://gepia.cancer-pku.cn/detail.php?gene=ERBB2; TCGA database, https://portal.gdc.cancer.gov/; CGGA database, http://www.cgga.org.cn/.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Xu Xiao, Yu Wang, Chenlong Li and Han Xiao. The study was supervised by Peng Liang. The first draft of the manuscript was written by Xu Xiao and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

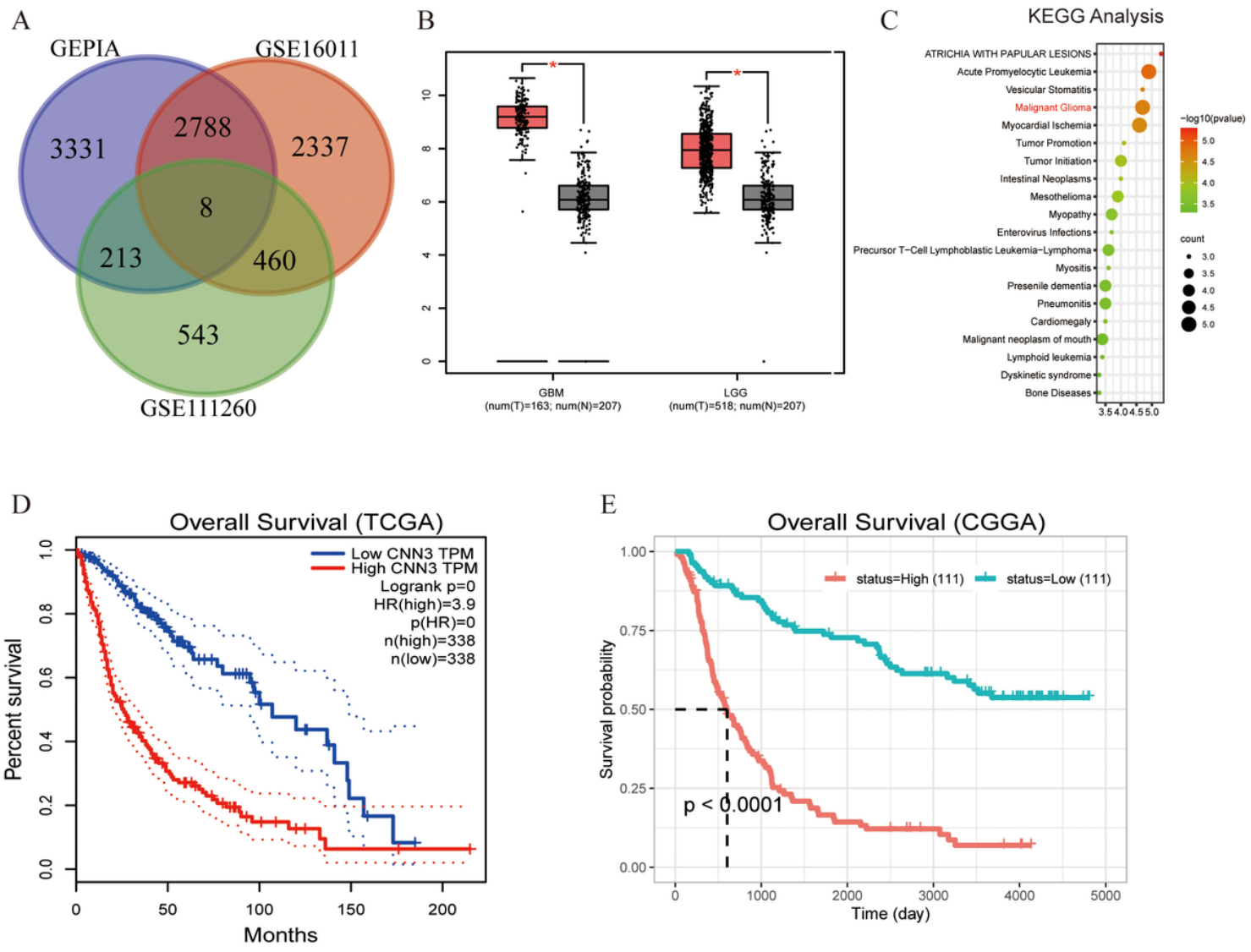

Figure 1

Bioinformatics indicates that CNN3 expression is elevated in glioma and predicts poor prognosis. (A) A Venn diagram was constructed according to the differentially expressed genes between glioma and normal tissues using the data from GEO database (GSE16011 and GSE111260) and GEPIA database. (B) The mRNA levels of CNN3 in GBM and LGG tumor and normal brain tissues were analyzed with the online tool Gene Expression Profiling Interactive Analysis. (C) Kyoto Encyclopedia of Genes and Genomes enrichment analysis was performed to predict the function of CNN3 in bioprocesses. (D and E) The prognostic potential of CNN3 expression in GBM was detected by Kaplan-Meier curves using clinical data from The Cancer Genome Atlas and Chinese Glioma Genome Atlas, respectively (P<0.001). CNN3, calponin 3; GBM, glioblastoma multiforme; LGG, low grade glioma..

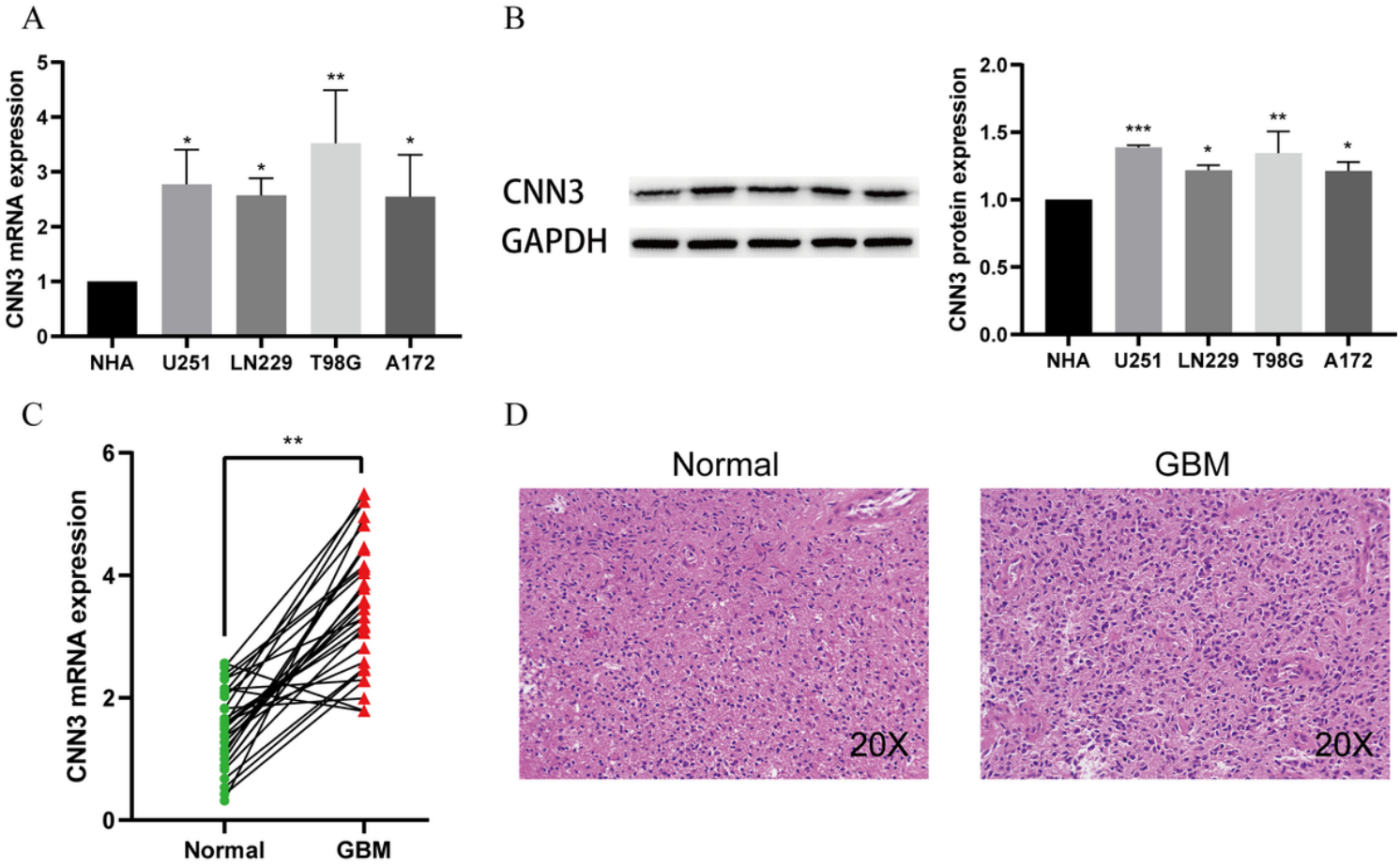

Figure 2

CNN3 is overexpressed in glioma cell lines and tissues. (A and B) RT-qPCR and western blotting showed that CNN3 was overexpressed at the mRNA and protein level in glioma cell lines (U251, LN229, T98G and A172). (C and D) RT-qPCR and immunohistochemistry showed that the expression levels of CNN3 were elevated in glioblastoma multiforme tissues. CNN3, calponin 3; RT-qPCR, reverse transcription-quantitative PCR.

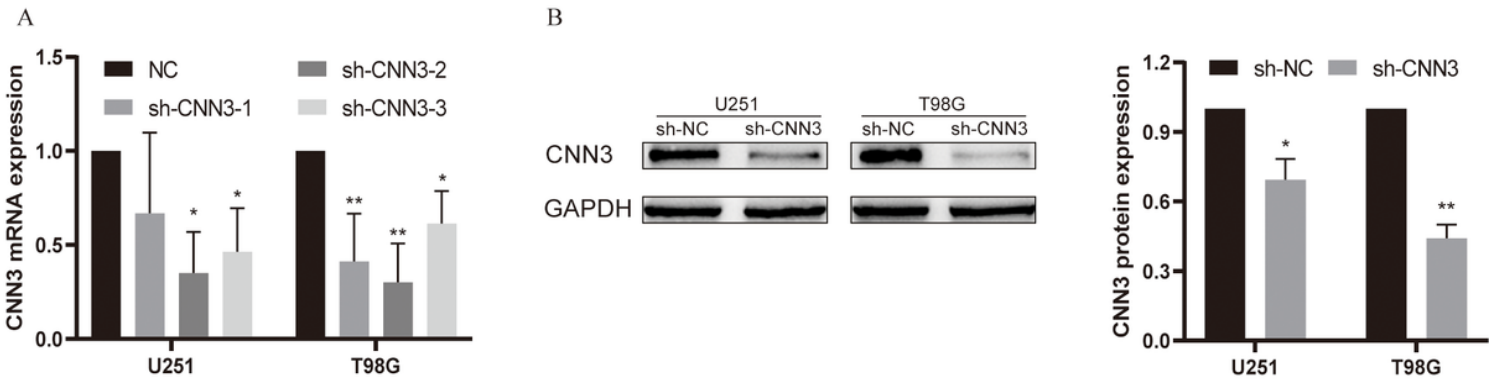

Figure 3

Construction of glioma cell lines with low expression of CNN3. (A) Reverse transcription-quantitative PCR showing the CNN3 mRNA level in U251 and T98G cell lines treated with sh-NC, sh-CNN3-1, sh-CNN3-2 or sh-CNN3-3. (B) Western blotting showed that CNN3 at the protein level was detected in U251 and T98G cells treated with sh-NC or sh-CNN3-2. CNN3, calponin 3; sh, short hairpin; NC, negative control.

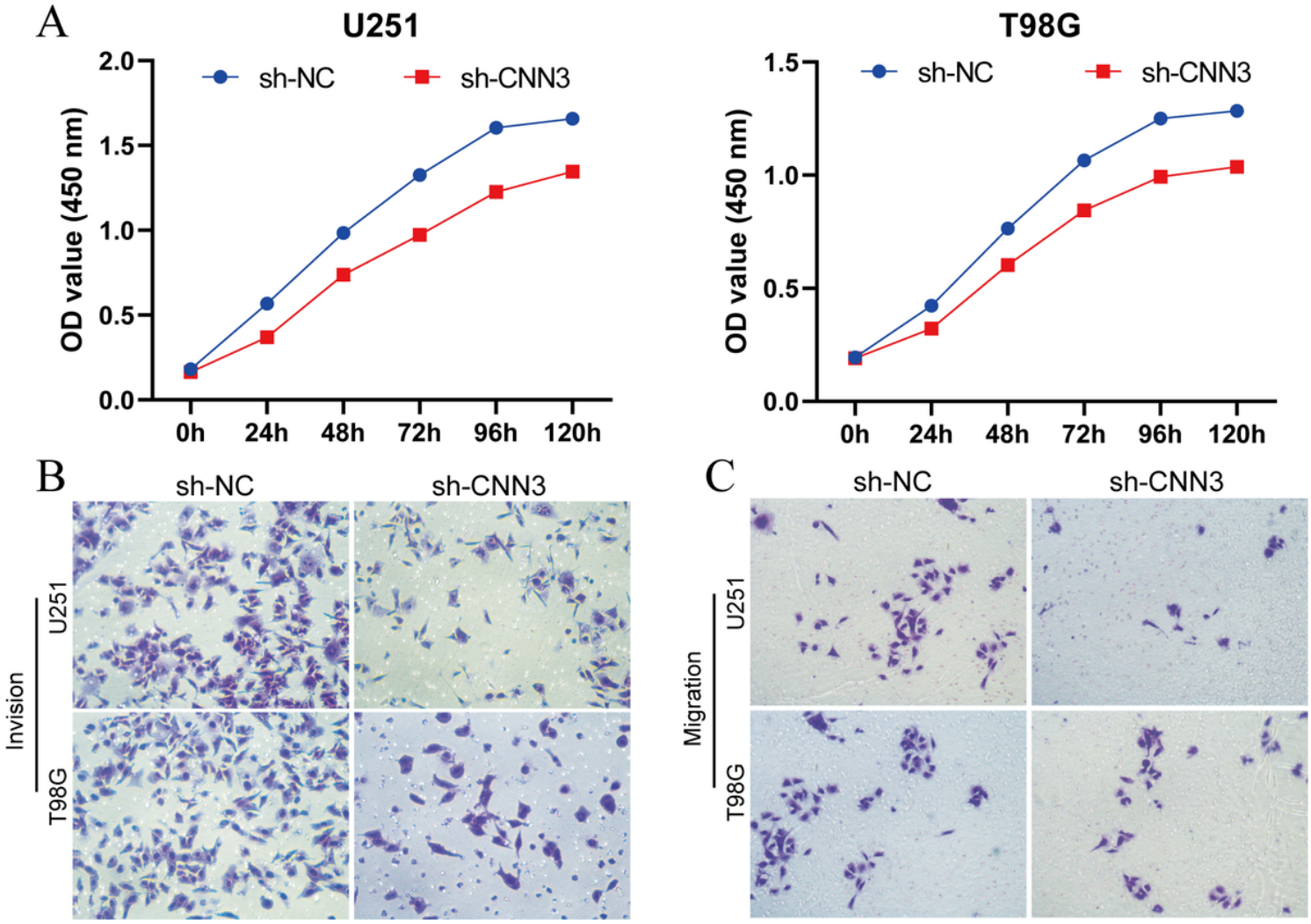

Figure 4

Knocking down the expression of calponin 3 inhibits the proliferation, invasion and migration abilities of glioma cells. (A) Cell Counting Kit-8 assay was used to examine the proliferation ability of glioma cells. (B and C) Transwell assays were employed to examine the invasion and migration abilities of glioma cells.

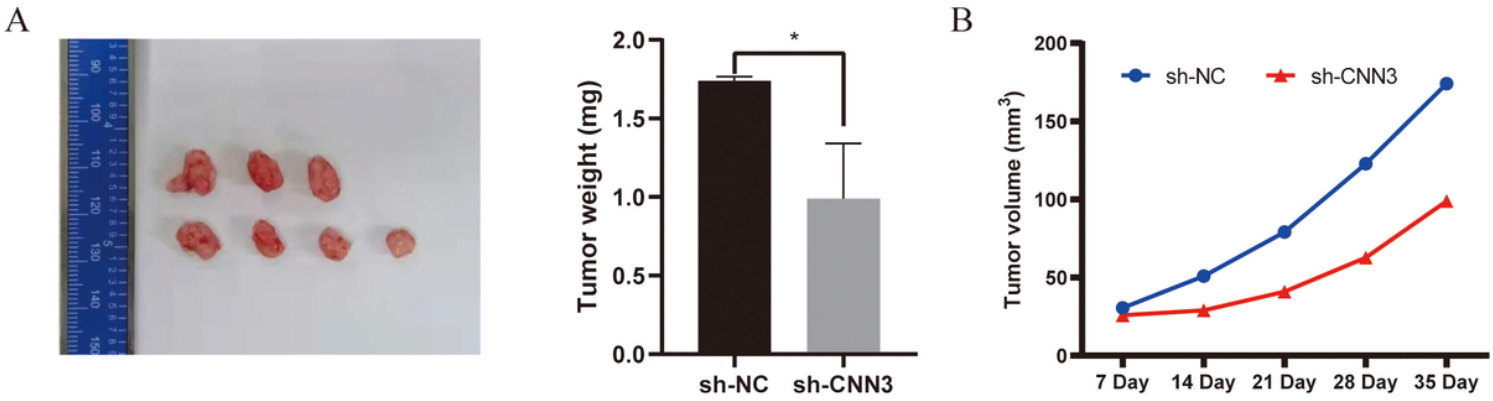

Figure 5

Knockdown of CNN3 inhibits glioma growth *in vivo*. (A) Images and weight of glioma xenografts derived from sh-CNN3 U251 cells and sh-NC U251 cells after 35 days. (B) Tumor growth curves of the sh-NC and sh-CNN3 groups. CNN3, calponin 3; sh, short hairpin; NC, negative control.

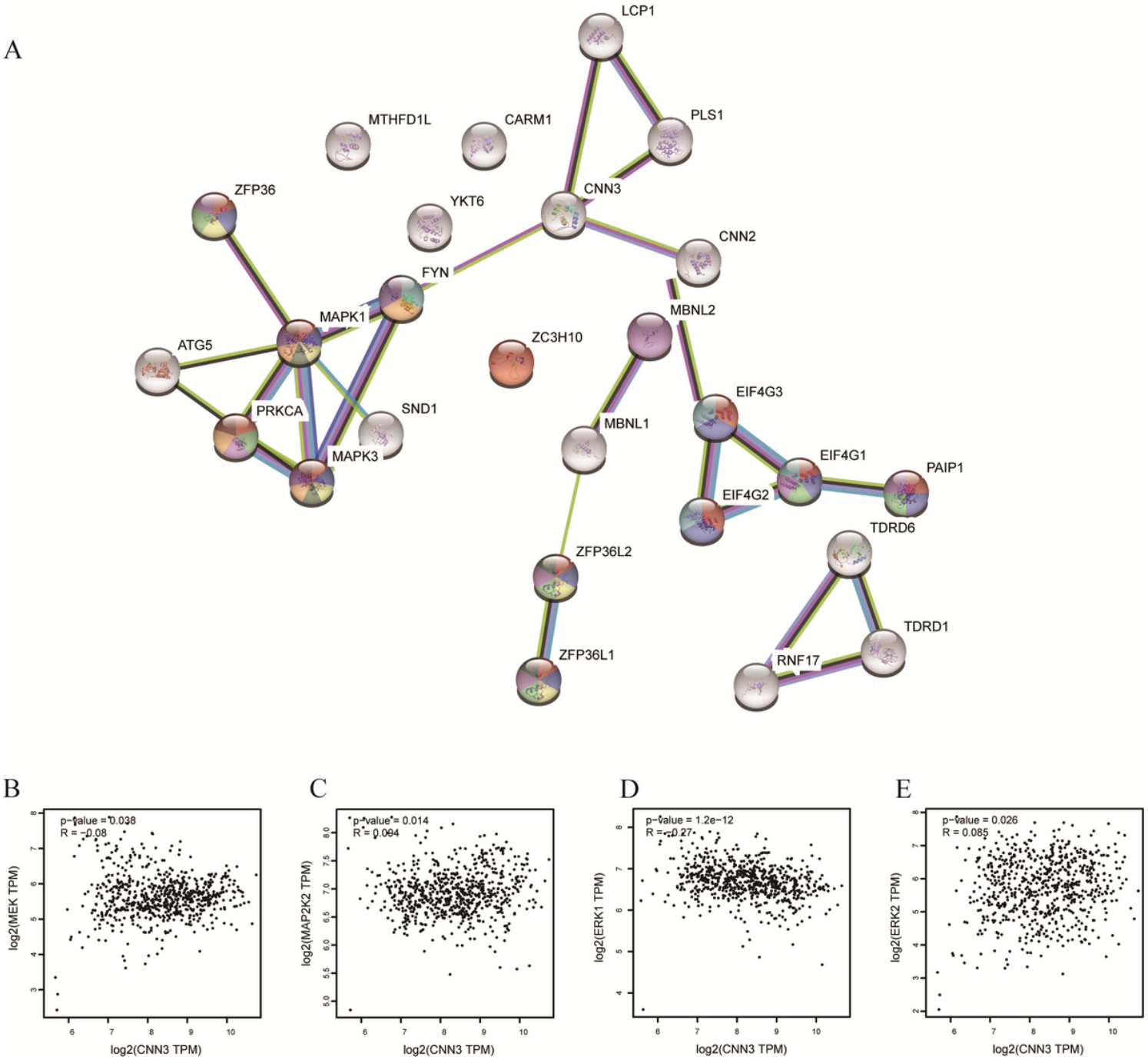

Figure 6

Bioinformatics suggests that CNN3 affects the MEK/ERK signaling pathway.

(A) The online tool Search Tool for the Retrieval of Interacting Genes/Proteins suggested that CNN3 had a correlation with the MAPK signaling pathways. (B-E) Pearson's correlation analysis of the mRNA expression levels in GBM tissues by GEPIA between CNN3 and MEK1, MEK2, ERK1 and ERK2. CNN3, calponin 3; GEPIA, Gene Expression Profiling Interactive Analysis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile.pdf
- Supplementaryfiles.docx