

## Original Article

# Overexpression of CAMK2N1 indicates good prognosis for glioma and regulates androgen receptor-associated cell proliferation and apoptosis

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**Abstract:** Glioma is the most common form of primary central nervous system tumors. The present study aimed to identify the prognostic roles of Calcium/calmodulin-dependent protein kinase II inhibitor 1 (CAMK2N1) and its regulation of glioma cell proliferation and apoptosis. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) were used to detect expression of CAMK2N1 in glioma. Kaplan-Meier survival analysis was used to analyze the association of CAMK2N1 with overall survival (OS) of glioma patients. Cell growth was detected by MTT assay and cell apoptosis was tested by flow cytometry and Western blot analysis. Results revealed that expression of CAMK2N1 was significantly lower in high-grade glioma tissue compared with low-grade glioma. High expression of CAMK2N1 indicates better outcomes for glioma patients. Univariate and multivariate analysis suggested that CAMK2N1 expression was an independent risk factor for OS. Overexpression of CAMK2N1 may inhibit proliferation and promote apoptosis in glioma cell U87 by activating apoptosis regulatory kinases BAX, Bcl-2, Cleaved-caspase3, and Cleaved-PARP. This could be reversed by synthetic androgen (R1881), a standard androgen receptor (AR) signaling agonist. These findings characterize CAMK2N1 as a tumor suppressor gene that regulates glioma cell proliferation and apoptosis, most likely through AR signaling pathways.

**Keywords:** CAMK2N1, glioma, androgen receptor, proliferation, apoptosis

## Introduction

Glioma accounts for almost half of primary central nervous system tumors and is the most prevalent malignancy of brain tumors [1]. The World Health Organization (WHO) has classified glioma into grades I-IV, according to their cellular origin and degree of malignancy [2]. Current therapeutic approaches include surgery, chemotherapy, radiotherapy, and molecular targeted therapy. However, the most malignant gliomas, grade IV gliomas, also called glioblastomas (GBMs), only have a 5-year survival rate of 9.8% at best [3]. Genetic and epigenetic alterations are major culprits of cellular transformation and therapy resistance [4]. Recent genomic studies have unveiled the complexity of tumor heterogeneity in glioblastomas, providing new insight into the genomic landscape of tumor cells that survive and initiate tumor recurrence

[5]. Therefore, it is critical to develop novel and effective molecular makers to assist early diagnosis and accurate prediction of prognosis in patients suffering from glioma.

Ca<sup>2+</sup>-calmodulin stimulated protein kinase II (CaMKII) is a multifunctional Ser/Thr protein kinase which is enriched in the brain [6]. Its biological function is cell type and cell context dependent [7]. It plays a central role in synapse formation, neurotransmitter synthesis and secretion, receptor and ion channel function, synaptic plasticity, and memory [8]. However, in cancer cells, it participates in the activation of ERK pathways by oncogenic Ras and RET rearrangements (RET/PTC), thus modulating tumor cell proliferation [9].

The CAMK2N1 gene, characterized as an inhibitor of CaMKII (calcium/calmodulin-dependent

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protein kinase II), has recently been shown to affect tumorigenesis and tumor growth [10-12]. A previous study suggested that it inhibited human colon adenocarcinoma cell growth and was negatively correlated with severity of human colon adenocarcinoma, indicating a pivotal role of CAMK2N1 in the development and progression of carcinomas [12]. An integrated analysis of genome-wide DNA methylation and RNA expression profiles using cervical cancer tissues identified 19 novel cervical cancer-related genes, including CAMK2N1, which suppressed mRNA expression regulated by DNA methylation [13]. Genome-wide miRNA expression profiling also revealed that CAMK2N1 may regulate cell apoptosis and the cell-cycle process in osteosarcoma [14]. Recently, CAMK2N1 was found to inhibit prostate cancer progression through androgen receptor (AR)-dependent signaling in vitro and in vivo, indicating cross-regulation of AR and CAMK2N1 in cancers [11]. Data suggests that CAMK2N1 may play an important role in cancer progression. However, the molecular mechanisms and functional link between CAMK2N1 and glioma remain unknown.

The present study aimed to detect expression levels of CAMK2N1 in glioma and to identify its correlation with clinicopathologic features in glioma patients. Moreover, this study investigated its regulatory role in proliferation and apoptosis of glioma cells and explored its relationship with AR signaling.

### Methods

#### *Patients and samples*

For reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay, eight fresh glioma tissues with low grade, eight fresh glioma tissues with high grade, and eight normal non-cancerous tissue samples were obtained from patients with glioma, undergoing surgical resection at Anhui Provincial Hospital Affiliated to Anhui Medical University, between January 2016 to January 2017. Human glioma tissue microarray (n=180) was purchased from Outdo Biotech (Shanghai, China) for Kaplan-Meier survival analysis. The operation time of patients was from February 2008 to October 2011, with follow-ups ending in July 2017. No patients received chemotherapy or radiothera-

py prior to surgery. Patient clinical features were obtained from medical records. The median age of this cohort was 23 years (range, 3-80 years). Written informed consent was obtained from each patient for the use of tissue samples for research purposes. This study was carried out with the approval of the Ethics Committee of Anhui Provincial Hospital Affiliated to Anhui Medical University.

#### *Reagents*

Human anti-CAMK2N1 antibody and anti-GAPDH antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human anti-BAX, anti-Bcl-2, anti-caspase3, and anti-PARP antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). R1881, also known as methyltrienolone, was purchased from SIGMA.

#### *Cell culture*

Human glioma U87 cells were obtained from ATCC and were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>.

#### *Lentiviral construction and transfections*

Human CAMK2N1 genes were polymerase chain reaction (PCR) amplified from normal genomic DNA and cloned into lentiviral vector GV166 (Ubi-MCS-3FLAG-IRES-puromycin; Shanghai Genechem, Shanghai, China) for ectopic expression of CAMK2N1. Expression of CAMK2N1 was confirmed by Western blot analysis.

#### *Quantitative real-time PCR (qRT-PCR)*

Total RNA was isolated and reversely transcribed to cDNA using TRIzol Reagent (Invitrogen) and the PrimeScript RT Reagent kit (RR037A; Takara), respectively, according to manufacturer instructions. qRT-PCR was carried out via Bio-Rad CFX96 Real-Time PCR Detection System with SYBR Green Supermix (Bio-Rad). Relative gene expression was normalized and calculated by the 2<sup>-ΔΔCt</sup> method. Primers for PCR were designed by Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA), as shown in **Table 1**.

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**Table 1.** Primer sequences used for quantitative reverse transcription-polymerase chain reaction

Gene	Forward or reverse	Sequences
GAPDH	Forward	GGTCGGAGTCAACGGGTGAG
	Reverse	GGAAGATGGTGTGGGATTTTC
CAMK2N1	Forward	GACACCAACAACCTTCTTCGGC
	Reverse	CCCTCCATCCCTGCGTCTCAG
Androgen Receptor	Forward	GACGACCAGATGGCTGTCATT
	Reverse	GGGCGAAGTAGAGCATCCT

### *Immunohistochemistry (IHC) staining and Western blot analysis*

Immunohistochemical analysis of human glioma tissues was conducted using human primary antibody anti-CAMK2N1. Human glioma tissue microarray was purchased from Outdo Biotech (Shanghai, China). Western blot was performed on U87 cell lines, as previously indicated. Cells were pelleted and lysed in buffer supplemented with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Immunoblots were performed by incubating polyvinylidene fluoride membranes with 5% non-fat milk in TRIS-buffered saline and 2.5% Tween-20 for 1 hour at room temperature. Each membrane was then incubated with primary antibodies at 4°C overnight and incubated with secondary antibodies at room temperature for 1.5 hours. Each blot was stripped and re-probed with a GAPDH antibody as an internal control. Images were captured with SynGene G: Box Chemi XRQ (Alpha Metrix Biotech, Germany). Experiments were performed in triplicate and repeated at least 3 times.

### *Cell proliferation and apoptosis analysis*

First,  $2 \times 10^3$  stable cells were seeded in 96-well plates in normal growth medium. Cell growth was measured daily by MTT assays using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide. The experiment was performed at least 3 times. PE-Annexin-V Apoptosis Detection Kit (BD Biosciences) was used to detect apoptosis by flow cytometry, according to manufacturer instructions. Each sample was analyzed by flow cytometry with a FACScan Flow Cytometer (Becton-Dickinson Biosciences,

Mansfield, MA) using a 488 nm laser. A minimum of 10,000 events were collected to maximize the statistical validity of compartmental analysis. The experiment was performed at least 3 times.

### *Statistical analysis*

SPSS software (version 19.0; SPSS, Chicago, IL) was used to carry out statistical analyses. Results

are presented as means of at least 3 independent experiments. Statistical comparisons were performed using Student's t-test, with  $P$  values  $<0.05$  considered statistically significant. Kaplan-Meier survival analysis was used to illustrate the prognostic relevance of CAMK2N1 in glioma patients.  $P$  values  $<0.05$  are considered statistically significant.

## Results

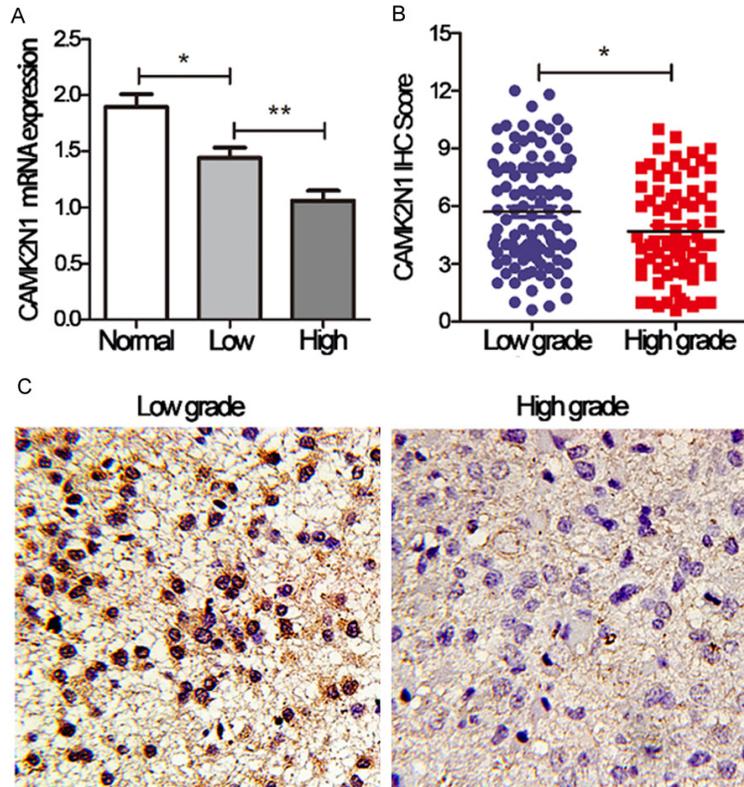
### *Expression of CAMK2N1 in glioma of different grades*

CAMK2N1, characterized as an inhibitor of CaMKII, plays a critical role in tumorigenesis in different cancers. CAMK2N1 mRNA expression of para-cancerous normal tissue and glioma specimens was measured by performing quantitative RT-PCR (qRT-PCR). As shown in **Figure 1A**, mRNA levels of CAMK2N1 were significantly reduced as tumor malignancy degree increased. Moreover, protein expression of CAMK2N1 was significantly decreased in tumor tissues with high grade compared to those with low grade. Next, CAMK2N1 protein expression in human glioma specimens was analyzed by performing immunohistochemical (IHC) staining using tissue microarray. CAMK2N1 showed higher IHC scores in low grade (**Figure 1B**) than in high grade, consistent with mRNA expression in gliomas. **Figure 1C** shows representative IHC pictures from the tissue microarray. CAMK2N1 showed whole cell distribution, with stronger staining in the nucleus, in low grade when compared with high grade.

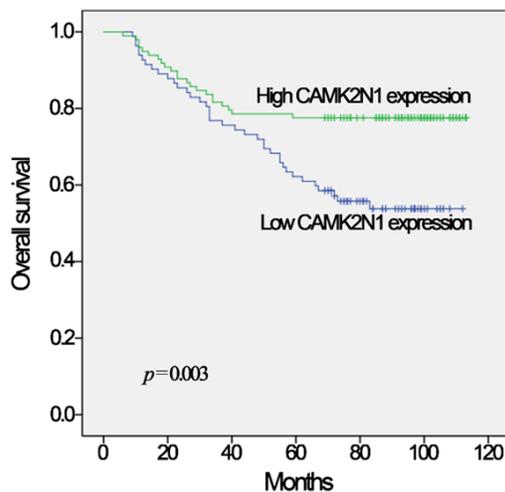
### *Relationship between CAMK2N1 expression and overall survival in glioma*

Kaplan-Meier survival analysis showed that patients with glioma expressing high expression of CAMK2N1 had good overall survival ( $P=0.003$ , **Figure 2**). Univariate analyses were

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**Figure 1.** Expression of CAMK2N1 in glioma of different grades. A. Reverse transcription-polymerase chain reaction detection of CAMK2N1 mRNA in normal tissues (n=8), low grade glioma tissues (n=8), and high-grade glioma tissues (n=8). B. CAMK2N1 IHC score in the glioma tissue microarray (N=180). C. Representative examples of immunohistochemical staining for CAMK2N1 in low and high grade of glioma tissue as indicated. Values are expressed as mean  $\pm$  SEM (\* $P$ <0.05, \*\* $P$ <0.01\*\*\* $P$ <0.001).



**Figure 2.** Significant positive correlation between CAMK2N1 expression and overall survival in glioma. In Kaplan-Meier survival analysis, patients with glioma expressing high expression of CAMK2N1 had good overall survival ( $P=0.003$ ).

performed using age, gender, WHO grade, and CAMK2N1 expression as possible variables. Results identified age ( $P$ <0.01), WHO grade ( $P$ <0.01), and CAMK2N1 expression ( $P$ <0.01) as significant predictors of overall survival (OS). Multivariate analysis confirmed that WHO grade (HR=3.3-64; 95% CI=2.1464.844;  $P$ <0.01) and CAMK2N1 expression (HR=1.175; 95% CI=0.3-71-2.597;  $P$ <0.01) were independent risk factors for OS (Table 2). Results revealed that high expression of CAMK2N1 is associated with good prognosis for glioma patients.

### *CAMK2N1 inhibits cell proliferation and promotes apoptosis*

To explore the biological roles of CAMK2N1 in glioma, U87 was chosen for the construction of CAMK2N1-overexpressed glioma cell lines. CAMK2N1 stably overexpressing U87 (U87-CAMK2N1) and U87 expressing the empty vector (U87-vector) by lentiviral transfection was established. Western blot analysis of CAMK2N1 expression demonstrated the upregulation of CAMK2N1 in U87-CAMK2N1 (Figure 3A). As shown in Figure 3B, MTT assay showed that CAMK2N1 inhibited proliferation in U87-CAMK2N1, compared to U87-vector. Moreover, it was found that CAMK2N1 could upregulate the proportion of apoptosis of glioma cells, as shown in Figure 3C. After upregulating the expression of CAMK2N1, the proportion of apoptotic cells was significantly increased, compared with Mock cells. Differences were statistically significant ( $P$ <0.05). As shown in Figure 3D, 3E, lentiviral-mediated transfection of CAMK2N1 overexpression induced visual increases of protein expression of BAX ( $P$ <0.01), Bcl-2 ( $P$ <0.001), Cleaved-Caspase3 ( $P$ <0.01), and Cleaved-PARP ( $P$ <0.05), representing cell apoptosis in the U87 cell line. Results suggest that upregulating CAMK2N1 could inhibit the proliferation of glioma through promoting apoptosis in glioma cells.

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**Table 2.** Univariate and multivariate analyses were performed to analyze clinical characteristics and overall survival in glioma

Variable	Univariate Cox		Multivariate Cox	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
Age				
Increasing years	1.146 (1.078-1.253)	<0.01	1.023 (0.964-1.137)	0.221
Gender				
Female vs Male	0.945 (0.809-1.146)	0.355		
WHO Grade				
High vs Low	4.681 (3.021-6.181)	<0.01	3.364 (2.146-4.844)	<0.01
CAMK2N1				
Low vs High	2.446 (1.268-4.353)	<0.01	1.175 (0.371-2.597)	<0.01

*Effects of CAMK2N1 on cell proliferation and apoptosis could be reversed by androgen receptor-associated signaling (AR) signaling activation*

To explore the association of CAMK2N1 with AR signaling in glioma, U87 cells were treated with 10 nm R1881 (AR signaling activator) for 0-48 hours. Expression of CAMK2N1 decreased with time after R1881 was treated (**Figure 4A**). Next, expression of AR in U87-CAMK2N1 and U87-vector was detected. It was found that AR was down-expressed in U87-CAMK2N1, compared with U87-vector (**Figure 4B**,  $P < 0.01$ ). As shown in **Figure 4C**, proliferation of U87-CAMK2N1 was rescued when treated with R1881. Flow cytometry demonstrated that R1881 could inhibit apoptosis of CAMK2N1 over-expressed glioma U87 cells (**Figure 4D and 4E**,  $P < 0.01$ ). As shown in **Figure 4F, 4G**, R1881 induced a visual decrease on protein expression of BAX, Cleaved-Caspase3 ( $P < 0.05$ , respectively), Bcl-2, and Cleaved-PARP ( $P < 0.01$ , respectively). Results suggest that proliferation inhibition and apoptosis promotion effects of CAMK2N1 could be reversed by activation of androgen receptor-associated signaling in glioma.

### Discussion

CAMK2N1, an inhibitor of CaMKII (calcium/calmodulin-dependent protein kinase II), plays a critical role in tumorigenesis in different cancers [10-13, 15, 16]. For instance, prostate cancer patients loss of CAMK2N1 is always accompanied with poor outcomes. Loss of CAMK2N1 in advanced prostate cancer could be the primary cause of enhanced kinase signaling. Moreover, overexpression of CAMK2N1

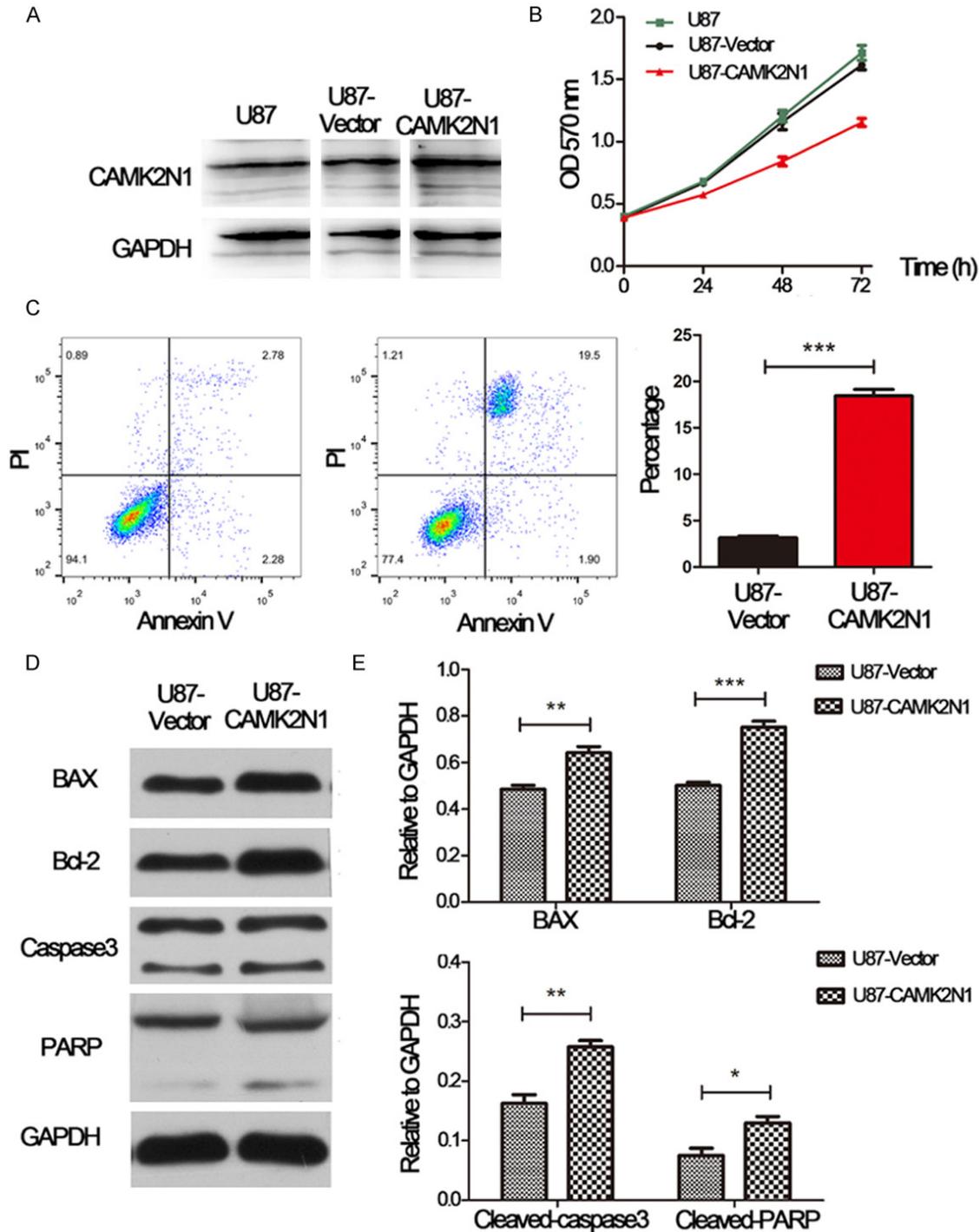
has been reported to suppress prostate cancer progression depending on androgen receptor (AR)-associated signaling [11]. In ovarian cancer, hypermethylation of CAMK2N1 resulting in loss of CAMK2N1 is associated with poor clinical outcomes in Type II EOC, also after macroscopic complete resection [15]. Previous research has revealed that

inhibition of CaMKII in medullary thyroid carcinoma (MTC) cells induced a reduction of Raf-1, MEK, and ERK phosphorylation, cyclin D expression, and cell proliferation. Moreover, mRNA expression of CAMK2N1 inversely correlates with severity of MTC [16].

The present study provides evidence that, in human glioma cancer tissue, endogenous CAMK2N1 expression was positively correlated with prognosis of glioma. It was found that CAMK2N1 showed higher IHC scores in low grade than in high grade, consistent with mRNA expression in glioma. Patients with high CAMK2N1 levels had longer overall survival. Univariate analyses and multivariate analysis were conducted, finding that CAMK2N1 expression was an independent risk factor for OS. Results were consistent with those in other cancers. At the cellular level, it was confirmed that CAMK2N1 inhibited cell proliferation and promoted apoptosis through activating BAX, Bcl-2, caspase3, and PARP proteins.

Recent studies have shown that CAMK2N1 and AR signaling formed an auto-regulatory negative feedback loop, where CAMK2N1 was down regulated by AR activation in response to androgen, while CAMK2N1 inhibited AR expression and activity through CAMKII pathways [11]. Researchers have also found significantly upregulated AR expression in GBM tissue, compared to the periphery normal brain tissue, in patients. Further in vitro experiments have suggested that AR signaling might promote tumorigenesis of GBM in adult men by inhibiting TGF-beta receptor signaling [17]. To study the relationship between CAMK2N1 and AR in glioma, the present study detected the expression

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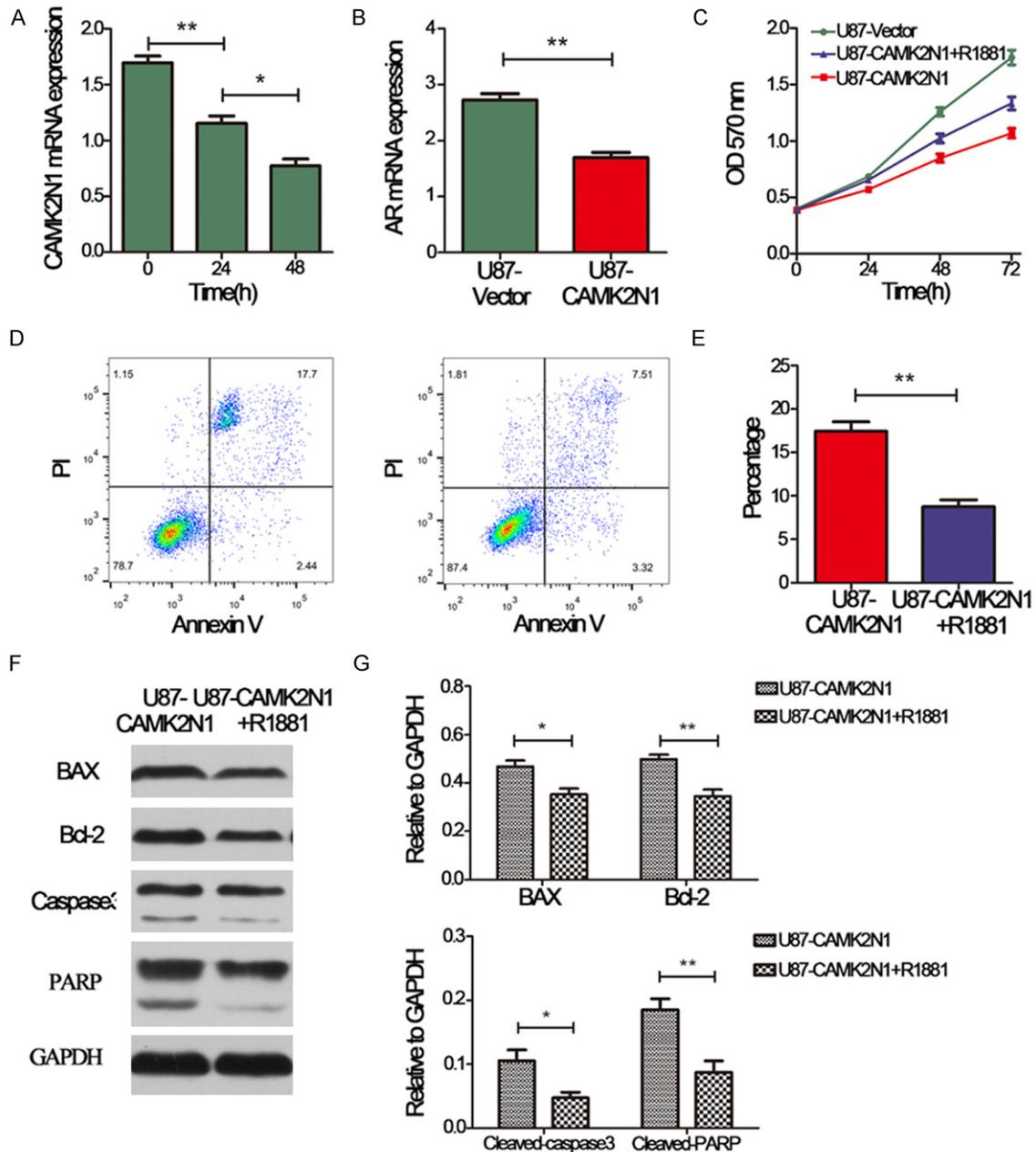


**Figure 3.** CAMK2N1 inhibits cell proliferation and promotes apoptosis. A. CAMK2N1 expression was detected in U87, U87-vector, and U87-CAMK2N1 cells by Western blots. B. Cell proliferation activity of U87, U87-vector, and U87-CAMK2N1 were measured daily by MTT assays for three days. C. U87-vector and U87-CAMK2N1 were then analyzed for cell apoptosis by flow cytometry. D, E. Western blot analysis of apoptosis related protein expression demonstrated upregulation of BAX, Bcl-2, Cleaved-Caspase3, and Cleaved-PARP in U87-CAMK2N1 cells compared with U87-vector cells. Values are expressed as mean  $\pm$  SEM (\* $P$ <0.05, \*\* $P$ <0.01\*\*\* $P$ <0.001).

of AR in U87-CAMK2N1 and U87-vector, finding that AR was down regulated in CAMK2N1-

overexpressed glioma cells. Next, this study used R1881, known as a synthetic androgen,

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**Figure 4.** Effects of CAMK2N1 on cell proliferation and apoptosis could be reversed by androgen receptor-associated signaling (AR) signaling activation. A. CAMK2N1 gene expression was detected by quantitative PCR in U87 treated with 10 nm R1881 for 0-48 hours. B. Androgen receptor expression was measured by quantitative PCR in U87-vector and U87-CAMK2N1 cell line. C. Cell proliferation activity of U87-vector and U87-CAMK2N1 treated with or without R1881 was measured daily by MTT assays for three days. Experiments were performed at least 3 times. D, E. U87-CAMK2N1 treated with or without R1881 for 48 hours were then analyzed for cell apoptosis by flow cytometry. F, G. Western blot analysis of apoptosis related protein expression indicated downregulation of BAX, Bcl-2, Cleaved-Caspase3, and Cleaved-PARP in U87-CAMK2N1 cells with R1881 treated for 48 hours when compared with U87-CAMK2N1 cells without R1881 treated. Values are expressed as mean  $\pm$  SEM (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

to activate AR, finding that activation of AR rescued CAMK2N1 related cell proliferation inhibition and apoptosis increased as expected. This could prove that CAMK2N1 interacts with AR in glioma cells.

AR plays a critical role in the development of prostate cancer [18, 19]. In prostate cancer cells, AR promotes proliferation and invasion to promote progression of prostate cancer through inhibiting apoptosis associated regulato-

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ry kinases [20], including BAX/Bcl-2, Bad, caspase4, caspase7, and activating PI3K/AKT and MEK/ERK signaling pathways [14]. In this study, it was observed that CAMK2N1 activated apoptosis regulatory kinases BAX, Bcl-2, caspase3, and PARP protein expression through blocking AR signaling pathways in glioma. Collectively, results revealed the tumor suppressive role of CAMK2N1 in glioma.

In conclusion, the presents study identified a possible functional link between CAMK2N1 and androgen receptor signaling in glioma. Overexpression of CAMK2N1 countered glioma growth, indicating good prognostic outcomes in glioma patients. Present results may provide a novel and convincing method of treating glioma by targeting the functional interaction between CAMK2N1 and AR associated signaling.

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### Disclosure of conflict of interest

None.

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