Pathology - Research and Practice 215 (2019) 564–570

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

LncRNA CASC7 inhibits the progression of glioma via regulating Wnt/β-catenin signaling pathway

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ARTICLE INFO

Keywords:
CASC7
Glioma
Wnt/β-catenin signaling pathway
Proliferation

ABSTRACT

Increasing evidence reveal the important role of long non-coding RNAs (lncRNAs) in the initiation and progression of glioma. However, the role of lncRNA cancer susceptibility candidate 7 (CASC7) in glioma is largely unknown. At first, the expression level of CASC7 was tested in glioma tissues and cell lines by using qRT-PCR. We applied Kaplan-Meier method to analyze the correlation between the expression level between CASC7 expression and the overall survival rate of glioma patients. We found that CASC7 was downregulated in glioma tissues and cell liens and predicted poor prognosis for patients with glioma. To determine the involvement of CASC7 in the biological processes of glioma, we conducted gain or loss-of function assays in two glioma patients. We found that CASC7 suppressed glioma cell proliferation and induced glioma cell apoptosis. Mechanistically, the expression level of CASC7 was negatively correlated with the expression levels of core factors of Wnt/β-catenin signaling pathway in glioma cells. Moreover, TOP flash luciferase activity further revealed the negative effect of CASC7 on the activity of Wnt/β-catenin signaling pathway. Finally, rescue assays were carried out to determine that Wnt/β-catenin signaling pathway involved in CASC7-mediated glioma progression. Taken together, all research findings suggested that CASC7 inhibited the progression of glioma via regulating Wnt/β-catenin signaling pathway.

1. Introduction

Glioma has been acknowledged as one of the most common and aggressive malignant tumors in the central nervous system with high recurrence and mortality [17,27]. Despite considerable advances in the diagnosis and treatment in recent years, the overall survival of glioma patients remains unfavorable [6,7,36,45]. Therefore, there is an urgent requirement to find the novel molecular diagnostic or therapeutic targets for glioma [29]. In this regard, we investigated the molecular mechanism underlying the tumorigenesis of glioma.

Long non-coding RNAs (lncRNAs), a subtype of non-coding RNAs (ncRNAs) with more than 200 nucleotides in length, have been reported as essential diagnostic or prognostic factors and biological regulators in malignant tumors, including glioma [9,10,18,21–23,30,33,40]. Increasing evidence indicate the involvement of lncRNAs in diverse biological processes such as cell proliferation, apoptosis, and differentiation [25,34]. LncRNA cancer susceptibility candidate 7 (CASC7) has been reported in colon cancer [43] due to its tumor-suppressive role. To our knowledge, the function and mechanism of CASC7 in glioma remains unclear. In this study, we examined the expression level of CASC7 in glioma tissues and cell lines. The prognostic effect of CASC7 on the overall survival rate of glioma patient was analyzed by Kaplan-Meier survival analysis. In addition, gain or loss-of-function assays were performed to investigate the biological role of CASC7 in glioma cells. Accordingly, we identified the tumor-suppressive role of CASC7 in glioma.

Wnt/β-catenin signaling pathway is regarded to be of great importance in regulating cell growth, apoptosis and differentiation [8,26]. Previous studies have demonstrated that Wnt/β-catenin signaling pathway played an essential role in the biological process of various cancers, including glioma [15,32,35,37]. In the present study, we analyzed the correlation between lncRNA CASC7 and Wnt/β-catenin signaling pathway. Rescue assays were performed to validate the involvement of Wnt/β-catenin signaling pathway in CASC7-mediated glioma progression. In summary, this study revealed that CASC7 suppressed glioma progression via inactivation of Wnt/β-catenin signaling pathway.

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https://doi.org/10.1016/j.prp.2019.01.018
Received 13 August 2018; Received in revised form 26 December 2018; Accepted 12 January 2019
0344-0338/

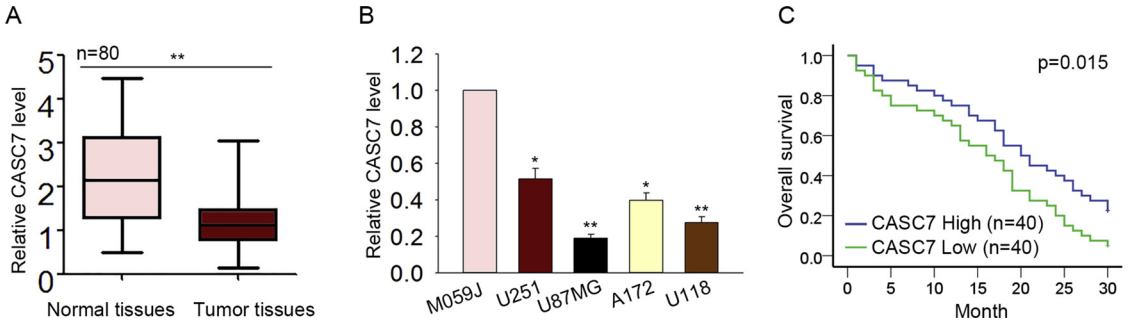

Fig. 1. Downregulation of CASC7 was associated with the poor prognosis of patients with glioma (A–B) The expression level of CASC7 in glioma tissues and cell lines were detected by qRT-PCR. (C) Kaplan-Meier analysis of the overall survival of glioma patients with high or low CASC7 expression. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group.

Table 1
Correlation between the expression of CASC7 and clinicopathological features of glioma patients. (n = 80).

Variable	CASC7 expression		P-value
	Low	High	
Gender			
Male	16	18	0.821
Female	24	22	
Age			
≤40	22	19	0.655
> 50	18	21	
Tumor Size (cm)	22	8	0.002**
< 3	18	32	
≥3			
KPS			
Present	26	13	0.007**
Absent	14	27	< 0.001***
WHO Stage	25	9	
I-II	15	31	
III-IV			

Low/high by the sample mean. Pearson χ^2 test. $^{**}P < 0.01$, $^{***}P < 0.001$ was considered statistically significant.

Table 2
Multivariate analysis of prognostic parameters in patients with glioma by Cox regression analysis.

Variable	Category HR	CI (95%)	P-value
Age	≤40 0.862	0.524-1.418	0.558
	> 40		
Gender	Male 0.811	0.48-1.368	0.432
	Female		
KPS	Present 0.642	0.379-1.089	0.1
	Absent		
WHO stage	WHO I-II 1.902	1.068-3.386	0.029*
	WHO III-IV		
Tumor Size	< 3 0.689	0.395-1.203	0.19
	≥3		
CASC7 expression	Low 1.958	1.061-3.615	0.032*
	High		

Proportional hazards method analysis showed a positive, independent prognostic importance of CASC7 expression (P = 0.032*), in addition to the independent prognostic impact of WHO stage (P = 0.029*). $^{*}P < 0.05$ was considered statistically significant.

2. Materials and methods

2.1. Tissue samples and cell culture

80 human glioma samples were obtained from patients who were diagnosed with glioma at Hunan Provincial People's Hospital. None of the patients received chemotherapy or radiotherapy before the surgery. Patients enrolled in this study signed the informed consent. This study was approved by the Ethics Committee of Hunan Provincial People's Hospital.

Primary normal human astrocytes (M059J) were purchased from the Sciencell Research Laboratories (Carlsbad, CA) and cultured under the conditions as instructed by the manufacturer. Four glioma cell lines (A172, U251, U87MG and U118) were obtained from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL Co. Ltd., USA) which was supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C with 5% CO_2.

2.2. Cell transfection

The constructed pcDNA3.1-CASC7 plasmid (pcDNA3.1 vector containing the whole sequence of CASC7) or the empty vector (Addgene, Inc., Cambridge, MA, USA) was transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Moreover, short hairpin RNAs targeted CASC7 (sh-CASC7#1, sh-CASC7#2, sh-CASC7#3) and negative control shRNA (sh-NC) were purchased from RiboBio (Guangzhou, China). The cultured cells (5×10^5 cells/well) were seeded in 6-well culture plates and were maintained in RPMI 1640 medium with 10% FBS for 24 h before transfection. qRT-PCR was applied to determine the transfection efficiency.

2.3. qRT-PCR analysis

Total RNA was extracted from glioma tissues or cells with TRIzol reagents (Invitrogen, Carlsbad, CA). RNA was reversely transcribed into cDNAs using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Indianapolis, IN, USA) in line with the manufacturer's instructions. qRT-PCR reactions were performed using a qPCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and SYBR-Green PCR Master Mix (Roche Diagnostics). The relative expression level was calculated by the relative quantification ($2^{-\Delta\Delta Ct}$) method. GAPDH was used as an endogenous control.

2.4. CCK-8 assay

Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assay was used to evaluate cell viability. Glioma cell lines were seeded in DMEM with 10% FBS at a density of 5×10^3 cells per well and then cultivated in 96-well plates. At 24, 48, 72 and 96 h following transfection, 10 µl CCK-8 solution was added into each well. The cells were then cultured at 37 °C for 2 h. The absorbance was measured at 450 nm using a microplate reader.

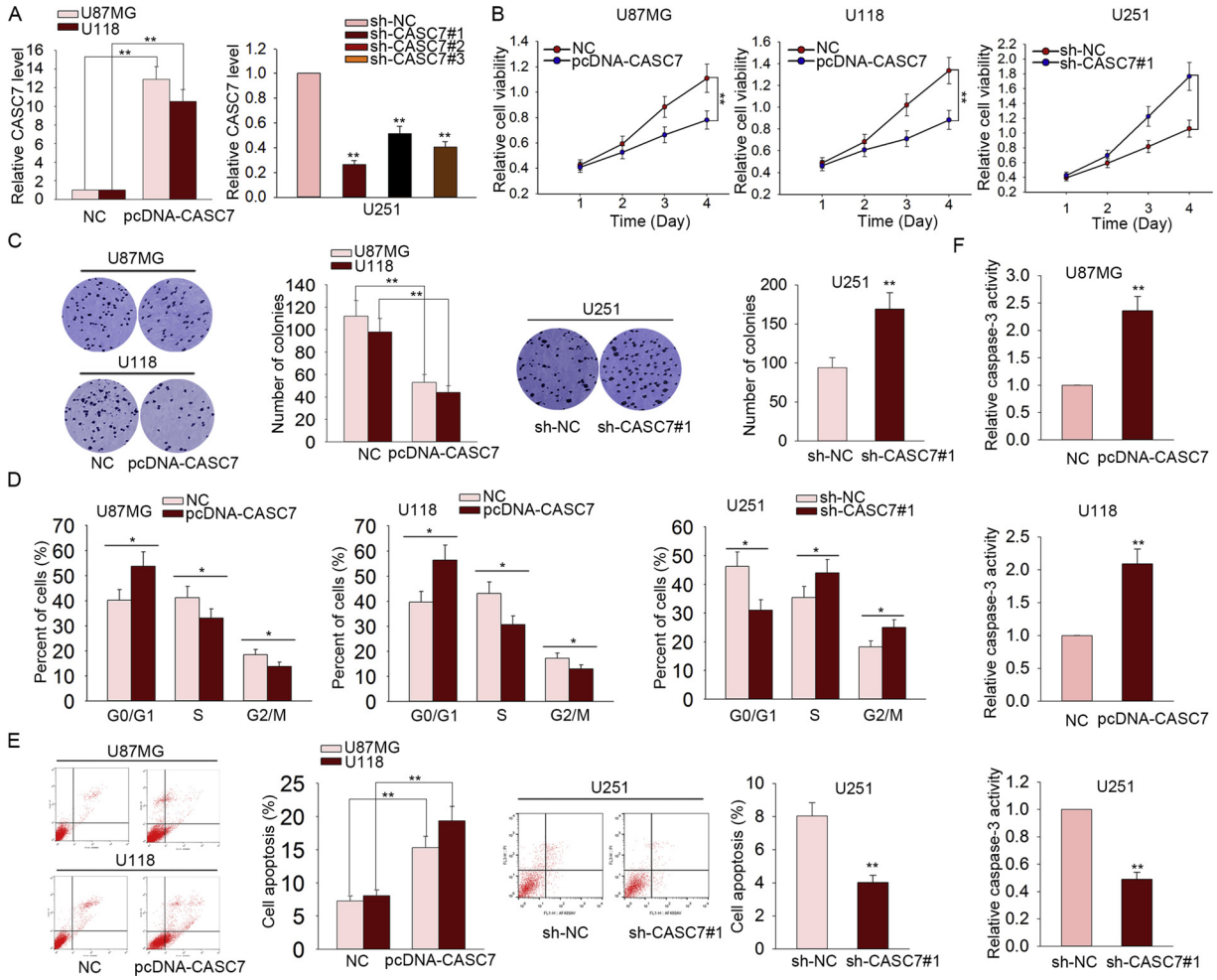

Fig. 2. CASC7 inhibited cell proliferation and induced apoptosis in glioma (A) qRT-PCR was performed to detect the expression level of CASC7 in U87MG and U118 cells transfected with pcDNA-CASC7 or U251 cell transfected with CASC7-specific shRNAs (sh-CASC7#1, sh-CASC7#2, sh-CASC7#3). (B–C) CCK-8 assay and colony formation assay were conducted to determine the cell proliferation in U87MG and U118 cells transfected with pcDNA-CASC7 or U251 cell transfected with sh-CASC7#1. (D–E) The cell cycle and cell apoptosis in glioma cells were detected by flow cytometry assay. (F) The of caspase-3 activity detection in indicated glioma cells. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group.

2.5. Colony formation assay

Glioma cell lines were plated in 6-well plates at 500 cells per well and maintained in DMEM containing 10% FBS for 2 weeks. The medium was replaced for every four days. 2 weeks later, the colonies were washed twice with PBS, fixed with methanol and stained with crystal violet (Sinopharm Chemical Reagent, China). The number of colonies was counted under a microscope.

2.6. Flow cytometry analysis

For cell cycle analyses, glioma cells were harvested after transfection with pcRNA plasmids, washed once with PBS, and fastened in 70% cold ethanol for 2 h. The cells were added with 100 µl of RNase and incubated at 37 °C for 30 min and added with 400 µl of PI and incubated at 4 °C for 30 min away from light. The samples were detected by flow cytometer (FACScan, BD Biosciences, USA). The early and late apoptotic cells were measured using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmigen, USA) by Accuri C6 flow cytometer analysis 72 h after transfection.

2.7. Caspase-3 activity assay

The caspase 3 activity was determined by using a colorimetric caspase 3 assay kit (Abcam, USA) in light of the manufacturer's advice. The absorbance at 405 nm was measured by an absorbance reader (Biotek, USA).

2.8. Luciferase reporter assay

For the luciferase reporter assay, glioma cells were seeded in 96-well plates and then transfected with TCF/β-catenin reporter plasmid (Promega, USA) as well as 10 ng Renilla using Lipofectamine 2000 transfection system. After incubation for 48 h, the luciferase activities of firefly and Renilla were analyzed using the dual-luciferase reporter assay system (Promega, Madison, WI) from the cell lysates.

2.9. Western blot analysis

Proteins were isolated from tissues by lysing frozen tissues in radio immunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was measured by using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 µg) were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Thermo Fisher Scientific, Inc.). The membrane was blocked in 5% non-fat milk in PBS overnight and incubated with primary antibodies. Primary antibodies included rabbit anti-β-catenin (1:5000), rabbit anti-c-Myc (1:10,000), mouse anti-β-actin (1:5000; Abcam). β-

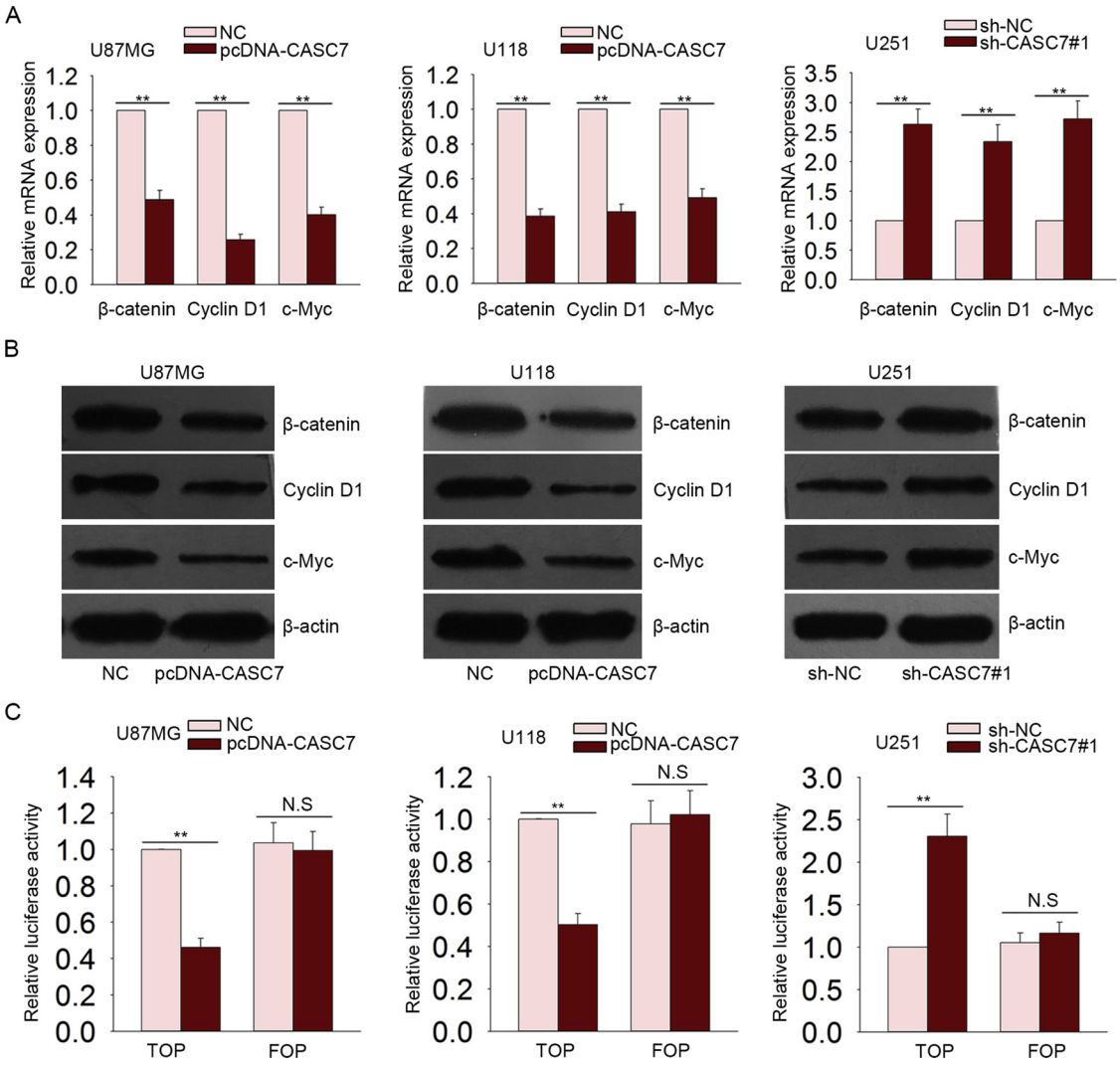

Fig. 3. CASC7 inactivated the Wnt/β-catenin signaling pathway in glioma cells (A–B) qRT-PCR and western blot assay were used to determine the relative mRNA levels or protein levels of β-catenin, cyclin D1 and c-Myc in U87MG and U118 cells transfected with pcDNA-CASC7 and U251 cell transfected with sh-CASC7#1. (C) The luciferase activities in TOP flash or FOP flash was measured in indicated glioma cells. $^{**}P < 0.01$ vs control group. N.S: no significance.

actin was utilized as internal reference. Then, the blots were incubated with an appropriate second antibody that conjugated with the horseradish peroxidase for 2 h. Finally, the complexes were detected using chemiluminescence.

2.10. Statistical analysis

Statistical analyses were conducted using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean ± SD. The significance of differences between groups was assessed by either Student's *t*-test or one-way analysis of variance (ANOVA). The overall survival was analyzed using the Kaplan-Meier method and the differences were analyzed by the log-rank test. The correlation between CASC7 expression and clinic-pathological features was analyzed using Chi-squared test. $P < 0.05$ was considered to have a statistically significant difference. Each experiment was performed at least three times.

3. Results

3.1. Downregulation of CASC7 was associated with the poor prognosis of patients with glioma

To investigate the role of CASC7 in glioma, we firstly tested the expression level of CASC7 in glioma tissues and cell lines using qRT-PCR. Compared with the normal controls, CASC7 was significantly expressed lower in glioma tissues and cell lines (Fig. 1A–B). Based on the median value of CASC7 expression levels in 80 glioma tissues, all glioma samples were classified into CASC7 high or low expression group. Then, we analyzed the clinical potential in glioma patients. As listed in Table 1, the expression of CASC7 was associated with the KPS, WHO stage and tumor size. Moreover, multivariate analysis demonstrated that CASC7 and WHO stage might be two independent prognostic factors for glioma patients (Table 2). Kaplan-Meier survival analysis revealed the positive relevance between CASC7 expression and the overall survival of glioma patients (Fig. 1C).

3.2. CASC7 inhibited cell proliferation and induced apoptosis in glioma

To investigate whether CASC7 can regulate the biological processes in glioma, we designed and performed gain or loss-of function assays in glioma cells. According to the result of qRT-PCR analysis, CASC7 was expressed highest in U251 cell but was expressed lowest in U87MG and U118 cells. Thus, we overexpressed CASC7 in U87MG and U118 cells by transfecting with pcDNA-CASC7, and we silenced CASC7 in U251 cells with CASC7-specific shRNAs (sh-CASC7#1, sh-CASC7#2, sh-CASC7#3). The transfection efficiency was determined by qRT-PCR

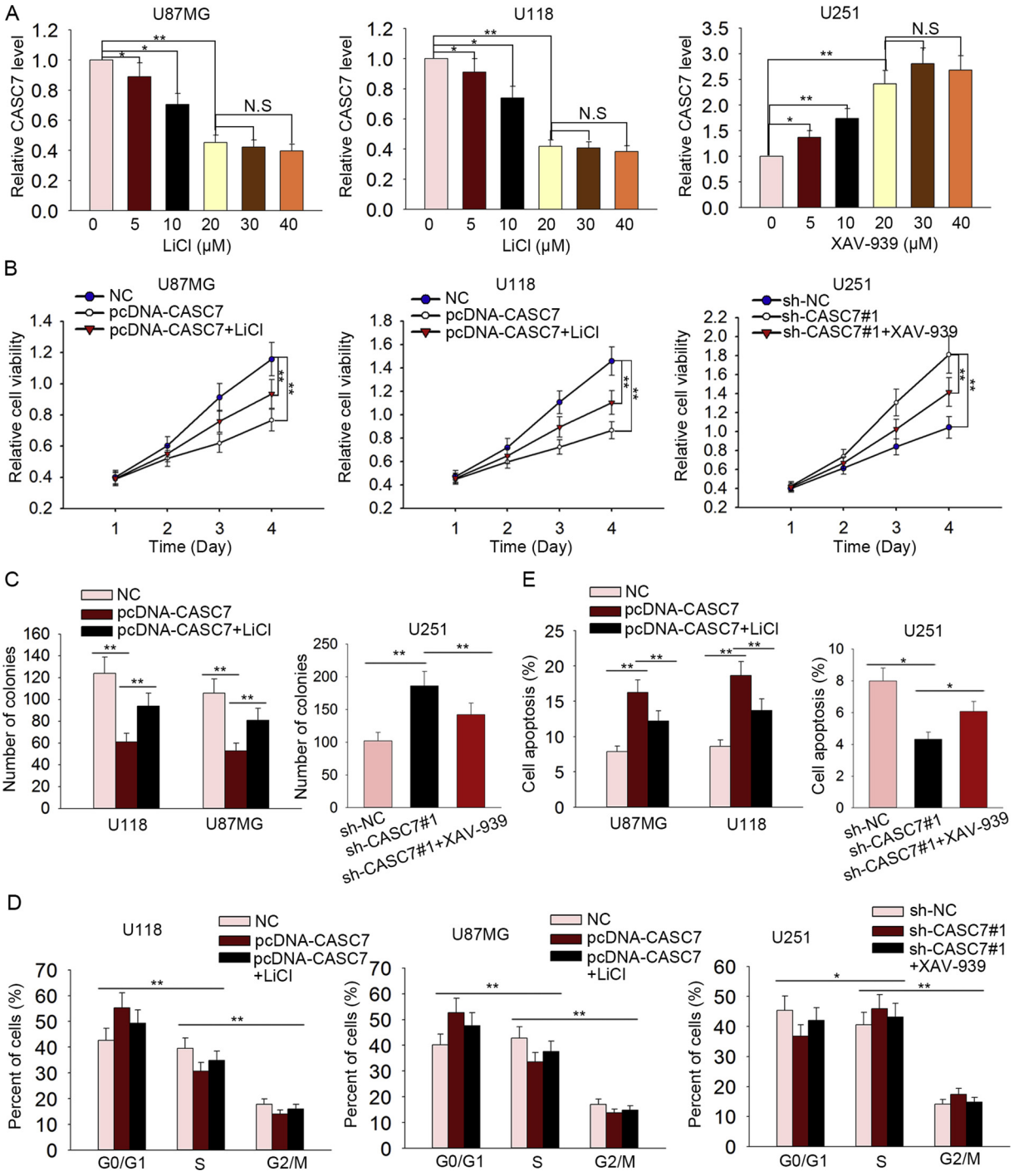

Fig. 4. CASC7 functioned as a tumor suppressor in glioma via inactivating Wnt/β-catenin signaling pathway (A) Relative CASC7 expression was tested in glioma cells treated with different doses of LiCl or XAV-939. (B–C) CCK-8 assay and colony formation assay were conducted to detect the cell proliferation in CASC7-overexpressed or CASC7-downregulated glioma cells by adding 20 μM LiCl or XAV-939. (D–E) Cell cycle and apoptosis were determined by flow cytometry in CASC7-overexpressed or CASC7-downregulated glioma cells after the treatment of LiCl or XAV-939. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group. N.S: no significance.

(Fig. 2A). Since sh-CASC7#1 most efficiently silenced the expression of CASC7, we chose it for subsequent experiments. Then, CCK-8 and colony formation assays were performed to detect the effect of CASC7 overexpression or knockdown on cell proliferation. Results showed that CASC7 overexpression significantly suppressed cell proliferation in U87MG and U118 cells, while CASC7 knockdown promoted cell proliferation in U251 cell (Fig. 2B–C). Furthermore, flow cytometry analyses showed that overexpression of CASC7 induced G0/G1 cell cycle arrest and accelerated apoptosis in U87MG and U118 cells. Whereas, downregulation of CASC7 promoted cell cycle progress and inhibited apoptosis in U251 cell (Fig. 2D–E). In addition, caspase-3 activity assay showed that the activity of caspase 3 was increased in U87MG and U118 cells transfected with pcDNA-CASC7, while decreased in U251 cell transfected with sh-CASC7 (Fig. 2F).

3.3. CASC7 inactivated the Wnt/β-catenin signaling pathway in glioma cells

Wnt/β-catenin signaling participated in the progression of various cancers, including glioma [2,13,38]. Previous studies revealed that lncRNAs can regulate tumor progression by activating or inactivating

Wnt/β-catenin signaling pathway [3,11,31]. Therefore, we investigated whether lncRNA CASC7 exerted its function in glioma by regulating the activity of Wnt/β-catenin signaling pathway. We measured the mRNA and protein levels of β-catenin, Cyclin-D1 and c-Myc (core factors of Wnt/β-catenin signaling pathway) in cells transfected with pcDNA-CASC7 or sh-CASC7. The levels of these factors were found to be negatively regulated by CASC7 (Fig. 3A–B). Similarly, TOP flash luciferase activity was negatively modulated by CASC7 (Fig. 3C). All these findings indicated that CASC7 inactivated Wnt/β-catenin signaling pathway in glioma.

4. CASC7 functioned as a tumor suppressor in glioma via inactivating Wnt/β-catenin signaling pathway

To determine whether CASC7 exerted functions in glioma by regulating the activity of Wnt/β-catenin signaling pathway, we conducted rescue assays in two glioma cells. LiCl and XAV-939 which can activate or inhibit the Wnt/β-catenin pathway [20,28]. In this study, we examined the expression level of CASC7 in cells treated with different concentration of LiCl and XAV-939. It was found that the expression level of CASC7 was significantly decreased in U87MG and U118 cells treated with 20 μM LiCl and was increased in U251 cell treated with 20 μM XAV-939 (Fig. 4A). Subsequently, CCK-8 assay and colony formation assay indicated that the effect of CASC7 overexpression or knockdown on cell proliferation was rescued by the addition of 20 μM LiCl or XAV-939 (Fig. 4B–C). Moreover, flow cytometry analyses demonstrated that cell cycle and apoptosis induced by CASC7 overexpression or knockdown were reversed with the addition of LiCl or XAV-939 (Fig. 4D–E). These findings indicated that CASC7 suppressed glioma progression through inactivating the Wnt/β-catenin pathway.

5. Discussion

As a common intracranial tumor, glioma accounts for more than 50% of all primary brain tumors [14,19]. Despite advances in surgical and medical therapy for glioma in recent years, the prognosis of patients with malignant glioma remains extremely poor [16,44]. Therefore, exploring the mechanisms underlying glioma progression and development is essential for developing more novel effective therapeutic targets. Recent studies have indicated that upregulation and downregulation of lncRNAs might be closely associated with multiple types of tumors [1,12,39]. LncRNA CASC7 has been reported to function as tumor suppressor in colon cancer. However, it is unclear whether CASC7 can regulate glioma progression. In current study, CASC7 was discovered to be downregulated in glioma tissues and cell lines. Moreover, low CASC7 expression was associated with the poor prognosis of patients with glioma, indicating the potential involvement of CASC7 in glioma progression. Gain-of-function and loss-of-function assays were separately conducted in glioma cells. Overexpression of CASC7 had negative effect on cell proliferation and cell cycle progress. Whereas, cell apoptosis was increased by knockdown of CASC7. Thus, we confirmed the tumor-suppressive role of CASC7 in glioma.

Wnt/β-catenin pathway can involve in the progression of human cancers [42]. β-catenin, c-Myc and Cyclin D1 are three core factors of Wnt/β-catenin signaling pathway [41]. Moreover, previous studies have shown that Wnt/β-catenin signaling pathway can involve in tumor progression induced by lncRNAs [4,5,24]. In our present study, we investigated the potential regulation of CASC7 on the activity of Wnt/β-catenin signaling pathway. Both mRNA and protein levels of β-catenin, c-Myc and Cyclin D1 were negatively regulated by CASC7, indicating the inactivation of Wnt/β-catenin signaling pathway in CASC7-expressed glioma cells. Luciferase activity analysis further demonstrated the negative effect of CASC7 on Wnt/β-catenin signaling. Thus, we hypothesized that CASC7 might exert its functions by activating Wnt/β-catenin signaling pathway. At last, rescue assays were carried out to make further confirmation. It was found that the inhibitory effect of CASC7 overexpression on glioma cell growth was partially attenuated by the treatment of LiCl (Wnt/β-catenin signaling pathway activator). Whereas, glioma cell growth promoted by the knockdown of CASC7 was partly recovered by the introduction of XAV-939 (Wnt/β-catenin signaling pathway inhibitor). Accordingly, we confirmed that Wnt/β-catenin signaling pathway involved in CASC7-mediated glioma progression. In conclusion, our current study revealed the tumor-suppressive role of CASC7 in glioma progression and demonstrated the potential correlation between CASC7 and Wnt/β-catenin signaling pathway. CASC7 exerted as a tumor suppressor in glioma via inactivation of Wnt/β-catenin signaling pathway. Although the mechanism between CASC7 and Wnt/β-catenin signaling was not fully investigated. We will make a deeper investigation on the specific mechanism in future. This study may extend our knowledge on the mechanism underlying glioma progression and provide a potential therapeutic perspective for the treatment of glioma.

Conflicts of interests

The authors declare that there are no competing interests associated with this study.

Acknowledgements

The authors appreciate all laboratory members. This study was supported by a grant from Hunan Provincial Health and Wellness Committee Research Project (NO.C2019054 and NO.C2019053).

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