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Knockdown of ADAMDEC1 inhibits the progression of glioma *in vitro*

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Summary. Background. Glioma is one of the most lethal malignant tumors all over the world. The prognosis of patients with high-grade glioma remains very poor. Therefore, it is urgent to find a novel strategy for the treatment of glioma. It has been reported that ADAMDEC1 could regulate the progression of multiple diseases, including cancers. However, the role of ADAMDEC1 during the tumorigenesis of glioma remains largely unknown. Methods, Gene expression of ADAMDEC1 in glioma tissues or in cells was detected by qRT-PCR. Western blot was performed to measure the protein expressions of p53, active caspase3, active caspase9, CDK2 and Cyclin A in glioma cells. Cell proliferation was detected by CCK-8 assay. Cell apoptosis or cycle was tested by flow cytometry. Transwell was used to test the invasion of glioma cells. Results. The expression of ADAMDEC1 in glioma tissues or cells was significantly upregulated. In addition, downregulation of ADAMDEC1 notably inhibited the proliferation and induced apoptosis of glioma cells through upregulation of active caspase 3 and active caspase 9. Besides, silencing of ADAMDEC1 obviously induced G1 arrest in glioma cells via modulation of cell cycle-related proteins. Finally, knockdown of ADAMDEC1 significantly inhibited the migration and invasion of glioma cells. In contrast, overexpression of ADAMDEC1 promoted cell proliferation, migration and invasion of glioma cells. Conclusion. Downregulation of ADAMDEC1 could significantly inhibit the tumorigenesis of glioma *in vitro*, which may serve as a novel target for the treatment of glioma.

Key words: Glioma, ADAMDEC1, Apoptosis, Invasion

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Introduction

Glioma is one of the most common and aggressive malignant brain tumors all over the world (Chen et al., 2017; Malta et al., 2018). According to a previous report, about 296,851 new tumor cases occurred and 241,037 died from this disease in 2018 (Bray et al., 2018). It has been regarded that glioma is divided into grade I-IV according to the World Health Organization (WHO) (Rees, 2011; Lima-Oliveira et al., 2019). The clinical outcome of glioma patients remains limited despite the great advances in diagnosis and treatment over the past decades (Ho et al., 2014). At present, surgery and chemotherapy are major treatments for glioma. However, there is still a lack of effective treatment for patients with glioma. Therefore, it is necessary to find a novel therapeutic strategy for the cure of glioma.

A disintegrin and metalloproteinase domain-like protein decysin 1 (ADAMDEC1) is a member of a disintegrin and metallo-proteinase (ADAM) family. It has been previously reported that ADAMDEC1 could be

involved in the tumorigenesis and metastasis of malignant tumors (Lund et al., 2013). Crouser et al also found that ADAMDEC1 could act as an important marker for the diagnosis of lung diseases, including pulmonary sarcoid (Crouser et al., 2009). In addition, Hwang et al indicated that ADAMDEC1 played a key role in the metastasis of hepatoma (Hwang and Kim, 2009). These data suggested that ADAMDEC1 might act as a promoter during the progression of cancers. However, the biological function of ADAMDEC1 in the tumorigenesis of glioma remains unclear.

In this study, we aimed to investigate the effect of ADAMDEC1 on the progression of glioma. As expected, our finding showed that silencing of ADAMDEC1 could notably inhibit the tumorigenesis of glioma *in vitro*, which might provide us a new strategy for treatment of glioma.

Material and methods

Tissue samples

A total of 81 tissue samples (5 Grade I glioma tissues, 23 Grade II glioma tissues, 23 Grade III glioma tissues, 25 Grade IV glioma tissues and 5 paired normal brain tissues) were collected from patients with glioma who underwent surgery in Affiliated Hospital of Southwest Medical University. Each tissue sample was stored at -80°C until RNA extraction. The pathologic diagnosis and classification of each tissue sample was reviewed and confirmed by at least two pathologists according to World Health Organization Glioma Classification (Louis et al., 2016). Association between ADAMDEC1 expression and WHO grading of gliomas is recorded in Table 1. This study was reviewed and approved by the Ethics Committee of Affiliated Hospital of Southwest Medical University. All patients in this study signed and provided written informed consent.

Cell culture

U87MG and U251 cell lines were obtained from Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium (Thermo Fischer Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS, Thermo Fischer Scientific), 1% penicillin (Thermo Fischer Scientific) and streptomycin (Thermo Fischer Scientific) at 37°C, 5% CO_2.

Cell transfection

U87MG or U251 cells were seeded at 3×10^5 cells/well in a 6-well plate and cultured until 70% confluence. Then, cells were transfected with siADAMDEC1, si ADAMDEC1-2 or negative control using Lipofectamine 2000 reagent (Thermo Fischer Scientific). For siRNA knockdown, the sequence of siRNA targeting ADAMDEC1 (siADAMDEC1 or siADAMDEC1-2) was designed and synthesized from Genepharma (Shanghai, China) with negative control (siControl). The efficiency of transfection was detected by fluorescence immunoassay, qRT-PCR and western blot, respectively.

For ADAMDEC1 overexpression, glioma cells were transfected with the pcDNA vector (NC) or pcDNA-ADAMDEC1 (ADAMDEC1-OE) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The pcDNA vector and pcDNA-ADAMDEC1 were purchased from GenePharma (Shanghai, China). After transfection, the cells were incubated at 32°C for 48 h. Finally, q-PCR was performed to detect the efficiency of cell transfection.

Quantitative real-time PCR (qRT-PCR)

Trizol reagent (Takara, Tokyo, Japan) was performed to extract total RNA from the tissues or from cells. Tue quantities of RNA were evaluated with the Nanodrop technology (Thermo Fisher Scientific). Then, cDNA was synthesized using the reverse transcription kit (TaKaRa, Ver.3.0) according to the manufacturer's protocol. Real-Time qPCRs were performed in triplicate under the following protocol: 2 minutes at 94°C, followed by 35 cycles (30 s at 94°C and 45 s at 55°C) (EnTurbo™ SYBR Green PCR SuperMix, Wuhan China). All the primers used were designed and synthesized by Genepharma (Shanghai, China). ADAMDEC1: forward, 5'-TGGAGAAGAAATCATTCTCTCCC-3' and reverse 5'-GAAGTATCCTCTCAACCCGTCAC-3'. GAPDH: forward, 5'-CATCATCCCTGCCTCTACTGG-3' and reverse 5'-GTGGGTGTCGCTGTTGAAGTC-3'. The relative expression of ADAMDEC1 was calculated with the $2^{-\Delta\Delta Ct}$ method, and GAPDH was used as internal control.

Western blot

Total protein was isolated from tissue or from cell lysates by using RIPA buffer, and quantified by BCA protein assay kit (Beyotime, Shanghai, China). Proteins were isolated with 10% SDS-PAGE, which were then transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% skim milk for 1 h, the membranes were incubated with primary antibodies

Table 1. Association between ADAMDEC1 expression and WHO grading of gliomas.

Non-cancerous brain and WHO grading	N	ADAMDEC1		Positive expression rate(±s) %	P
		-	+		
Non-cancerous brain	5	5	0	0	<0.001
Grade I gliomas	5	5	0	0	
Grade II gliomas	5	5	0	0	
Grade III gliomas	23	6	17	0.683±0.037	
Grade IV gliomas	25	8	17	0.680±0.040	

at 4°C overnight. Then, the membranes were incubated with secondary anti-rabbit antibody (Abcam, Cambridge, MA, USA; 1:5000) at room temperature for 1 h. After that, membranes were scanned using an Odyssey Imaging System and analyzed with Odyssey v2.0 software (LICOR Biosciences, Lincoln, NE, USA). The primary antibodies used in this study: anti-ADAMDEC1 (Abcam, Cambridge, MA, USA; 1:1000), anti-p53 (Abcam; 1:1000), anti-cleaved caspase3 (Abcam; 1:1000), anti-CDK2 (Abcam; 1:1000), anti-Cyclin A (Abcam; 1:1000), anti-active caspase9 (Abcam; 1:1000) and anti-GAPDH (Abcam; 1:1000). GAPDH was used as an internal control.

Immunohistochemical staining

Glioma or normal brain tissues from patients were collected and the expression of ADAMDEC1 in glioma or in normal brain tissues was detected by immunohistochemistry. The tissue sections were deparaffinized and then blocked. Then, the samples were incubated with the primary antibody ADAMDEC1 (1:50; Abcam) at 4°C overnight, and incubated with secondary anti-rabbit antibody (1:100; Abcam) at room temperature for 30 min. After that, tissue sections were stained with DAB and the images were captured using a photomicroscope.

Cell transfection and fluorescence immunoassay

ADAMDEC1 siRNA1 (siADAMDEC1) or ADAMDEC1 siRNA2 (siADAMDEC1-2) was transfected into U87MG or U251 cells, respectively. Then, cells were cultured for 72 h. Fluorescent microscope (Millipore, Darmstadt, Germany) was used to observe the expression of green fluorescent protein to assess the infection efficiency.

CCK-8 assay

U87MG or U251 cells were seeded in 96-well plates (5×10^3 per well) overnight. Then, U87MG cells were transfected with lenti-vector (NC) or ADAMDEC1 siRNA (siADAMDEC1) for 16, 24, 36, 48, 60 and 72 h, respectively. On the other hand, U251 cells were transfected with negative control (NC), siADAMDEC1-2 or pcDNA-ADAMDEC1 (ADAMDEC1-OE) for 72 h. Then, 10 μl CCK-8 reagents were added to each well and further incubated for 2 h at 37°C. Finally, the absorbance of glioma cells was measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

Cell apoptosis analysis

Glioma cells were trypsinized, washed with phosphate buffered saline and resuspended in Annexin V Binding Buffer (Thermo Fisher Scientific), followed by staining with 5 μl FITC and 5 μl propidium (PI) in the system for 15 min. Cells were analyzed using flow cytometer (BD, Franklin Lake, NJ, USA) to test the cell apoptosis rate.

Cell cycle detection

U87MG cells were transfected with NC or siADAMDEC1 for 72 h, respectively. Then, the cell cycle distribution was detected by flow cytometry as previously described (Menbari et al., 2020). The cell cycle distribution key was used (YEASEN, Shanghai, China).

Wound healing assay

Glioma cells were plated into a 24-well Cell Culture Cluster, and were allowed to grow to 80-90% confluence. Then, cells were underlined perpendicular to the cell culture plate with a small pipette head. After washing with PBS 3 times, serum-free medium was used for further culture, and the scratch widths at 0 and 48 h were recorded under an optical microscope.

Transwell assay

For cell invasion analysis, transwell assay (BD, Franklin Lake, NJ, USA) was performed in this study. The upper chamber is pre-treated with 100 μl of Matrigel (BD, Franklin Lake, NJ, USA). U87MG cells were seeded into the upper chamber in media with 1% FBS, and the cell density was adjusted to 1.0×10^6 cells per chamber. Then, RPMI1640 medium with 10% FBS was added in the lower chamber. After 48 h of incubation at 37°C, the non-invading cells in the upper chamber and on the Matrigel were removed with a cotton swab. Then, cells in the lower chamber were stained with 0.1% crystal violet and counted at 3 different fields under a microscope (LEICADMLB2, Frankfurt, Germany).

Statistical analysis

Three independent experiments were performed and all data were expressed as mean ± standard deviation (SD). Graphpad Prism7 (La Jolla, CA, USA) was used for all statistical analyses. One-way analysis of variance (ANOVA) followed by Tukey's test was used for comparisons between multiple groups. A value of $p<0.05$ was considered a significant difference.

Results

ADAMDEC1 was upregulated in tumor tissues of patients with advanced glioma

First, to investigate the role of ADAMDEC1 during the progression of glioma, qRT-PCR and western blot were performed. As shown in Fig. 1A, the gene expression of ADAMDEC1 in grade III/IV glioma tissues of patients was significantly higher than that in grade I/II glioma or in normal tissues. Consistently, the

protein expression of ADAMDEC1 in grade I/II glioma or normal tissues was notably downregulated compared with grade III/IV glioma tissues (Fig. 1B). Next, immunohistochemical staining was used to verify these results. As we expected, the protein expression of ADAMDEC1 in grade III/IV glioma tissues was significantly up-regulated compared with that in grade I/II glioma or in para-carcinoma tissues (Fig. 1C). Finally, the level of ADAMDEC1 in tumor tissues was positively corrected with the grade of glioma (Table 1). All these data indicated that ADAMDEC1 was upregulated in tumor tissues of patients with grade III/IV glioma.

ADAMDEC1 siRNAs and ADAMDEC1 pcDNA3.1 were successfully transfected into glioma cells

To further investigate the effect of ADAMDEC1 on the tumorigenesis of glioma, ADAMDEC siRNAs or siNC were transfected into glioma cells. As indicated in Fig. 2A, more than 90% efficiency of transfection for glioma cells was observed after fluorescence staining. To verify this result, qRT-PCR and western blot were performed. As shown in Fig. 2B-E, the expression of ADAMDEC1 in glioma cells was obviously downregulated by ADAMDEC1 siRNAs. These data demonstrate that ADAMDEC1 siRNAs were stably transfected into glioma cells. To confirm the function of ADAMDEC1 during the tumorgenesis of glioma, U251 cells were transfected with ADAMDEC1 pcDNA3.1 (ADAMDEC1-OE). As expected, ADAMDEC1 was notably upregulated in glioma cells in the presence of ADAMDEC1-OE (Fig. 2F,G). The result suggests that pcDNA3.1- ADAMDEC1 was stably transfected into glioma cells.

Knockdown of ADAMDEC1 significantly inhibited the growth of glioma cells

For the purpose of exploring the effect of

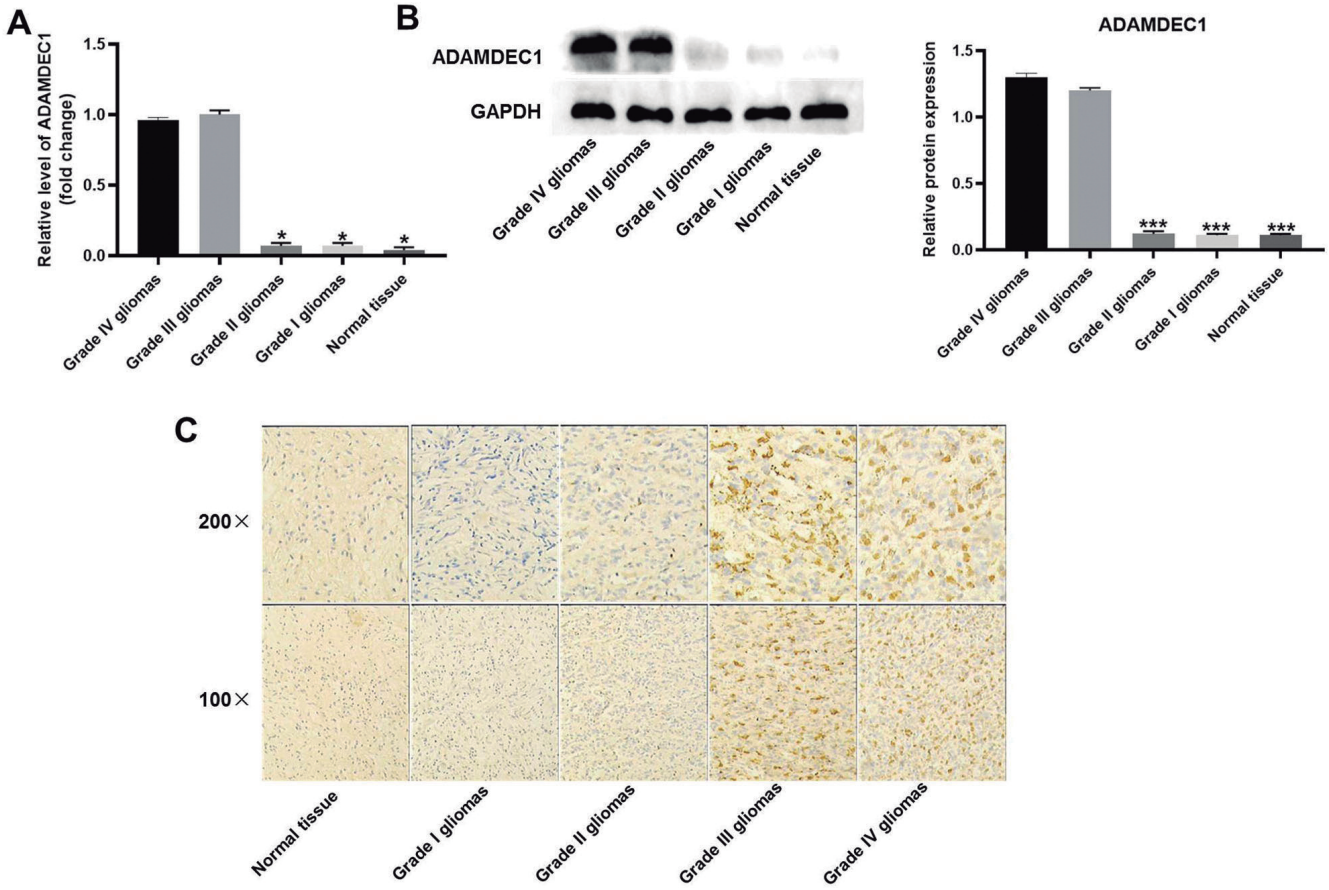

Fig. 1. ADAMDEC1 was upregulated in grade III/IV glioma tissues of patients. **A.** The gene expression of ADAMDEC1 in grade I/II/III/IV glioma tissues or in normal tissues of patients was detected by qRT-PCR. **B.** The protein expression of ADAMDEC1 in grade I/II/III/IV glioma tissues or in normal tissues of patients was detected by western blot. The relative expression of ADAMDEC1 was quantified normalizing to GAPDH. **C.** Glioma or normal brain tissues from patients were collected and the expression of ADAMDEC1 in glioma or in normal brain tissues was detected by immunohistochemistry. *P<0.05, ***P<0.01 compared to grade III/IV glioma.

ADAMDEC1 on proliferation of glioma cells, CCK-8 assay was used. As illustrated in Fig. 3A,B, the OD value of glioma cells was notably decreased in the presence of ADAMDEC1 siRNAs. In contrast, overexpression of ADAMDEC1 significantly increased the OD value of U251 cells (Fig. 3C). Next, to investigate the apoptosis and cycle distribution of glioma cells, flow cytometry was performed. The results revealed that silencing of ADAMDEC1 notably induced the apoptosis of glioma cells (Fig. 3D-G). Besides, downregulation of ADAMDEC1 also induced G1 arrest in U87MG cells (Fig. 3H,I). Taken together, downregulation of ADAMDEC1 could significantly inhibit the growth of glioma cells.

Downregulation of ADAMDEC1 notably inhibited the invasion of glioma cells

To confirm the effect of ADAMDEC1 on the progression of glioma, cell invasion was tested by transwell assay. As revealed in Fig. 4A,B, the invasion of glioma cells was significantly decreased by downregulation of ADAMDEC1. Similarly, silencing of ADAMDEC1 notably suppressed the migration of glioma cells (Fig. 4C-F). In contrast, overexpression of ADAMDEC1 greatly promoted the migration of U251 cells (Fig. 4G,H). These results confirmed that knockdown of ADAMDEC1 could notably inhibit the migration and invasion of glioma cells, while overexpression of ADAMDEC1 exerts the opposite effect.

Knockdown of ADAMDEC1 inhibited the growth of glioma cells via mediation of apoptotic and cell cycle-related proteins

In order to explore the mechanism by which si-ADAMDEC1 downregulated the growth of glioma *in vitro*, western blot was performed. As demonstrated in Fig. 5A-F, the expression of p53, active caspase 3 and active caspase 9 in glioma cells were significantly upregulated by knockdown of ADAMDEC1. In contrast, silencing of ADAMDEC1 notably decreased the protein expression of CDK2 and Cyclin A in U87MG cells. Taken together, knockdown of ADAMDEC1 inhibited the growth of glioma cells via mediation of apoptotic and cell cycle-related proteins.

Discussion

Previous studies have indicated that ADAMDEC1 is involved in multiple diseases (Lund et al., 2018; Sugimoto et al., 2018; Ueno et al., 2018). In our study, knockdown of ADAMDEC1 suppressed the growth of glioma cells. Our findings supplemented the biological function of ADAMDEC1, suggesting that ADAMDEC1 could be an oncogene during the tumorigenesis of glioma. However, Sugimoto et al. found that ADAMDEC1 was downregulated in non-eosinophilic chronic rhinosinusitis (Sugimoto et al., 2018). This discrepancy may due to different types of diseases.

Our findings showed that ADAMDEC1 could induce the apoptosis of glioma cells. In addition, we found that

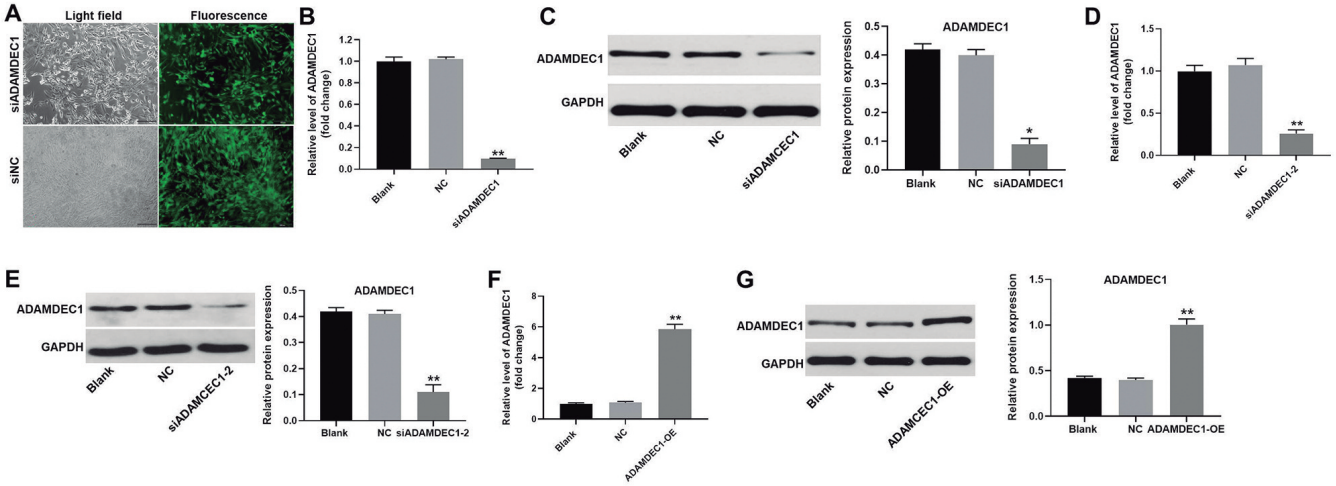

Fig. 2. ADAMDEC1 siRNA and pcDNA3.1 ADAMDEC1 were successfully transfected into glioma cells. siADAMDEC1 or siNC was transfected into U87MG cells. Then, transfection efficiency was examined by fluorescence staining **(A)**. **B.** The expression of ADAMDEC1 in glioma cells was detected by qRT-PCR. GAPDH was used as an internal control. **C.** The protein expression of ADAMDEC1 in glioma cells was detected by western blot. The relative expression of ADAMDEC1 was quantified normalizing to GAPDH. siADAMDEC1-2 or siNC was transfected into U87MG cells. Then, the expression of ADAMDEC1 in glioma cells was detected by qRT-PCR. GAPDH was used as an internal control **(D)**. **E.** The protein expression of ADAMDEC1 in glioma cells was detected by western blot. The relative expression of ADAMDEC1 was quantified via normalizing to GAPDH. pcDNA3.1 ADAMDEC1 or pcDNA3.1 vector (NC) was transfected into U251 cells. **F.** The gene expression of ADAMDEC1 in glioma cells was detected by qRT-PCR. GAPDH was used as an internal control. **G.** The protein expression of ADAMDEC1 in glioma cells was detected by western blot. The relative expression of ADAMDEC1 was quantified via normalizing to GAPDH. *P<0.05, **P<0.01 compared to Blank.

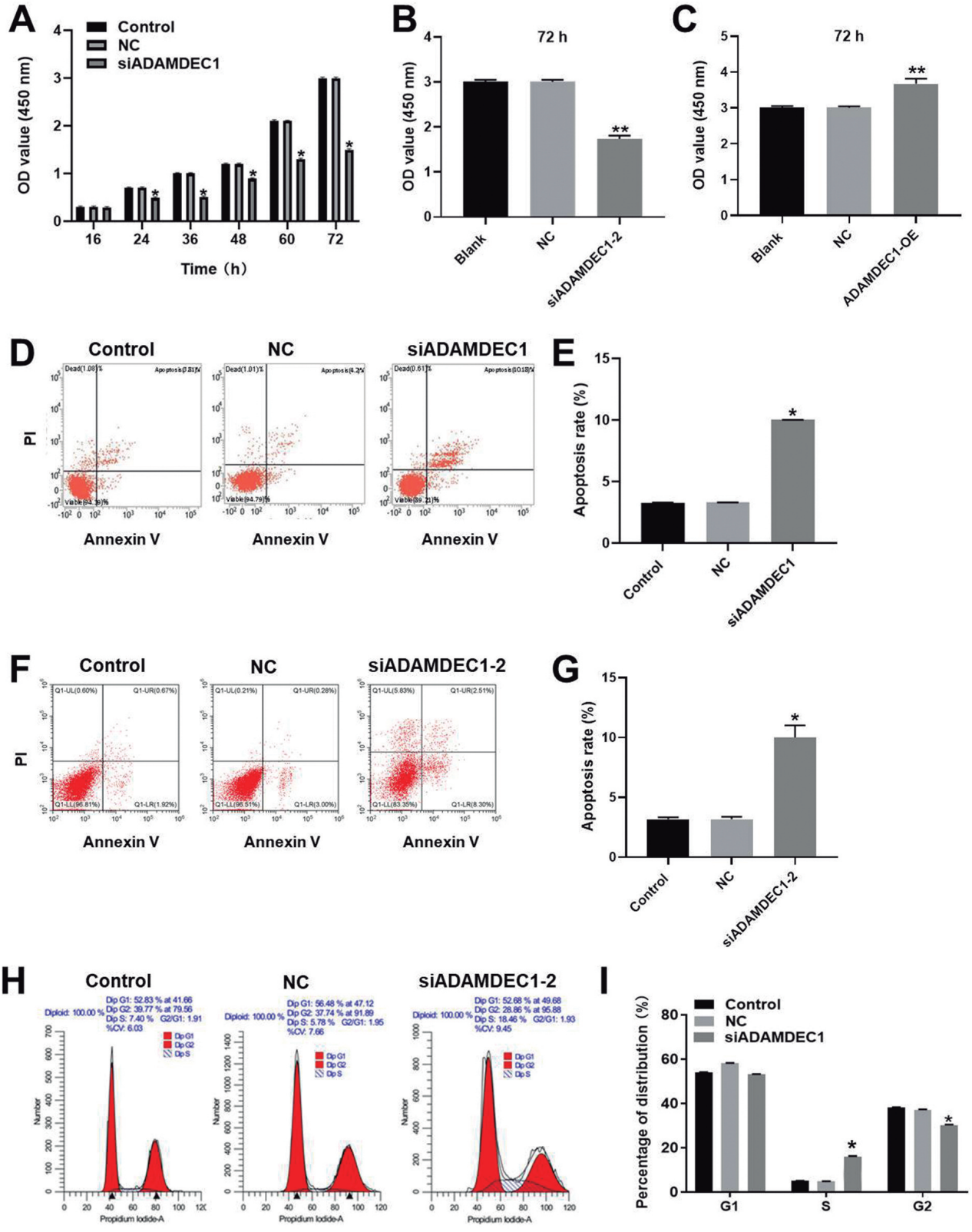

Fig. 3. Knockdown of ADAMDEC1 significantly induced glioma cell growth inhibition. **A.** The proliferation of U87MG cells at 16, 24, 36, 48, 60 and 72 h time point was tested by CCK-8 assay. **B, C.** U251 cells were transfected with siADAMDEC1-2 or pcDNA3.1 ADAMDEC1. Then, OD value of U251 cells was tested by CCK-8 assay. The apoptotic rate of U87MG **(D, E)** or HU251 **(F, G)** cells was detected by FACS after double staining with Annexin V and propidium iodide (PI). X axis: the level of Annexin-V FITC fluorescence; Y axis: the PI fluorescence. **H, I.** The cell cycle distribution in G0/G1, S, and G2 phase of U87MG cells transfected with siADAMDEC1 were determined by FACS. *P<0.05, **P<0.01 compared to control.

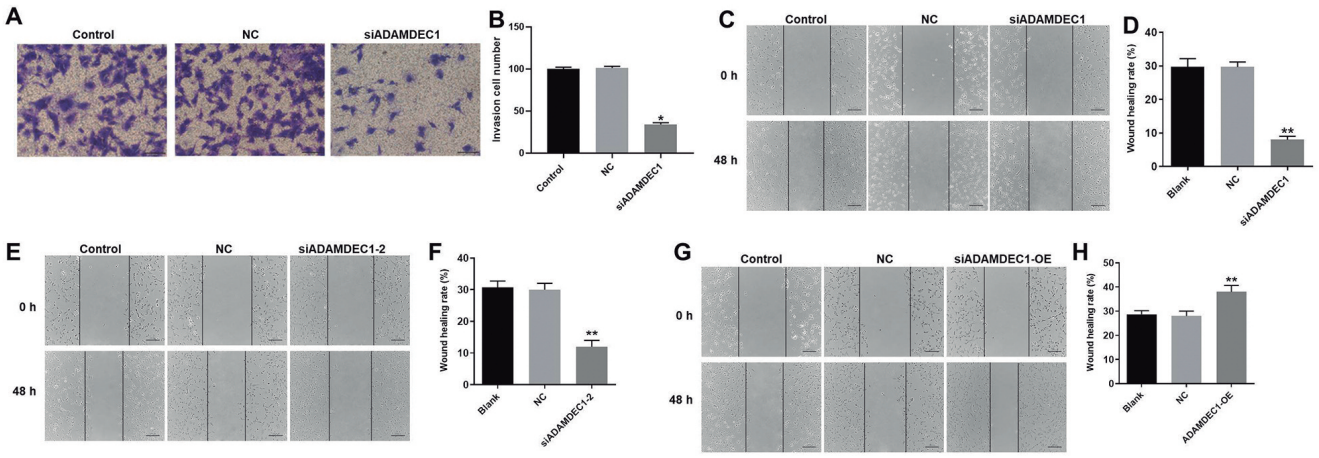

Fig. 4. Downregulation of ADAMDEC1 notably inhibited the migration and invasion of glioma cells. **A.** U87MG cells were transfected with siNC or siADAMDEC1 for 24 h. Then, cell invasion of glioma was tested using transwell migration assay; ×200 magnification. **B.** The invasion number of glioma cells was calculated. **C.** The migration of glioma cells was detected by wound healing. **D.** The wound healing rate was calculated. siADAMDEC1-2 or pcDNA3.1 ADAMDEC1 was transfected into U251 cells. Then, the migration of U251 cells was detected with wound healing assay **(E-H)**. Wound healing rate was calculated. *P<0.05, **P<0.01 compared to Blank.

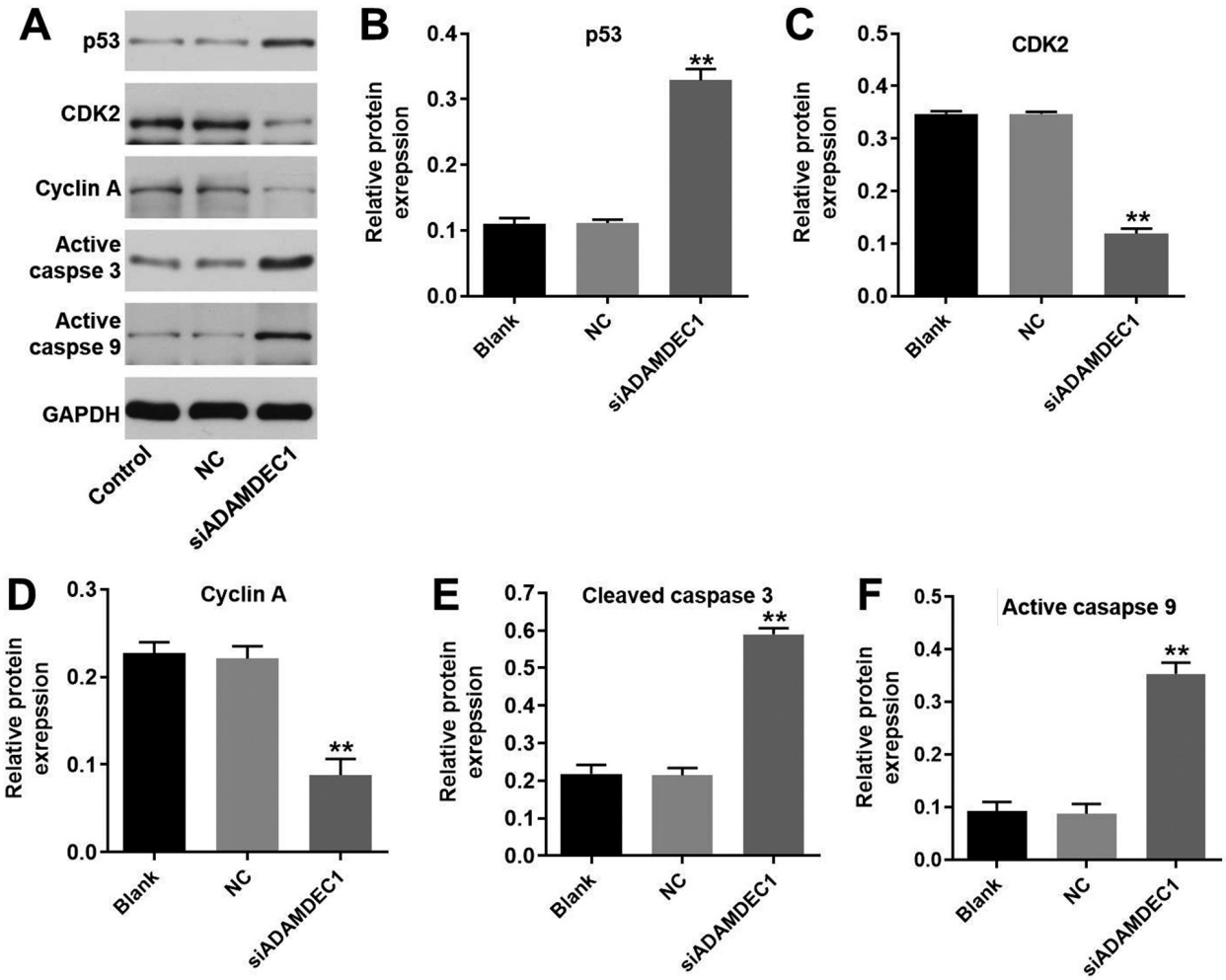

Fig. 5. Knockdown of ADAMDEC1 inhibited the growth of glioma cells via mediation of apoptotic and cell cycle-related proteins. **A.** The protein expressions of p53, CDK2, Cyclin A, active caspase 3 and active caspase 9 in glioma cells were detected by western blot. **B.** The relative protein expression of p53 was quantified via normalizing to GAPDH. **C.** The relative protein expression of CDK2 was quantified via normalizing to GAPDH. **D.** The relative protein expression of Cyclin A was quantified via normalizing to GAPDH. **E.** The relative protein expression of active caspase 3 was quantified via normalizing to GAPDH. **F.** The relative protein expression of active caspase 9 was quantified via normalizing to GAPDH. **P<0.01 compared to Blank.

active caspase 3 and active caspase 9 were upregulated by knockdown of ADAMDEC1. Active caspase3 and active caspase 9 have been regarded as two apoptotic proteins during the cellular process (Ge et al., 2015). Zhou L et al reported that active caspase 3 and active caspase 9 are key mediators in cell apoptosis (Zhou et al., 2020). Our data were consistent with these findings, suggesting that knockdown of ADAMDEC1 induced the apoptosis of glioma cells via upregulation of active caspase 3 and active caspase 9. Additionally, we investigated the effect of ADAMDEC1 on glioma cell proliferation by CCK-8 assay, the result of which suggested that knockdown of ADAMDEC1 could inhibit the proliferation of glioma cells. Xu et al. found thatTamoxifen (TMX) inhibited the growth of craniopharyngioma cells by downregulation of ADAMDEC1 (Xu et al., 2012). Our present study was consistent with this result, further confirming that ADAMDEC1 could act as a promoter during the progression of glioma. Besides, we also found that downregulation of ADAMDEC1 could inactivate CDK2 and Cyclin A and increase the expression of p53 in glioma cells. P53, CDK2 and Cyclin A participate in cell growth (You et al., 2019). P53 has been confirmed to be upregulated during cell cycle arrest, while Cyclin A and CDK2 have been regarded to be suppressed with the induction of cell cycle arrest (Liu et al., 2019; Narahara et al., 2020; Tao et al., 2020). Our data further confirmed these results, indicating that inhibition of ADAMDEC1 induced cell cycle arrest in glioma via mediation of p53, CDK2 and Cyclin A. Frankly speaking, this research only focused on the effect of ADAMDEC1 on apoptotic and cell cycle-related proteins so far. Since inactivation of PI3K/Akt signaling could cause the apoptosis of cancer cells (Fresno Vara et al., 2004), we will further investigate the effect of ADAMDEC1 on this signaling pathway. On the other hand, we will explore the role of ADAMDEC1 in the pathogenesis of other malignant tumors in future.

In conclusion, downregulation of ADAMDEC1 could inhibit the progression of glioma *in vitro*, which may serve as a potential novel target for the treatment of glioma.

Conflict of interest. These authors declared no competing interests in this research.

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