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Knockdown of CDC2 expression inhibits proliferation, enhances apoptosis, and increases chemosensitivity to temozolomide in glioblastoma cells

Baosheng Zhou · Guoyun Bu · Yipin Zhou · Yue Zhao · Wei Li · Mu Li

Abstract Cell division cycle 2 (CDC2) is always overexpressed in malignant tumor cells and is correlated with chemosensitivity, but it is unclear whether CDC2 overexpression contributes to the chemoresistance potential of glioma cells. The aim of study was to determine the relationship of CDC2 expression with the prognosis and chemoresistance of glioblastoma. In this study, the glioblastoma U87 and U251 cell lines were steadily transfected with a lentivirus vector expressing a short hairpin RNA-targeting CDC2. Expression of CDC2 was evaluated in glioblastoma and cell lines by immunohistochemistry and Western blot analysis. The relationship between CDC2 expression and clinicopathological characteristics was analyzed. Using RNA interference, the effects of CDC2 on chemosensitivity to temozolomide (TMZ) were investigated in U87 and U251 cell lines in vitro. Combined CDC2 knockdown and TMZ treatment inhibited cell proliferation and induced apoptosis in vitro more effectively than either treatment alone. qRT-PCR and Western blot analysis showed that cells underexpressing CDC2 revealed lower expression of the anti-apoptotic protein B cell lymphoma-2 and increased expression of the apoptosis effector caspase-3 compared to U87 and U251 cells transfected with a control vector. Furthermore, expression levels of CDC2 in U87 and U251 cells were related to the IC50 of the antitumor drug TMZ. Knockdown of CDC2 expression was associated with decreased expression of Ral-binding protein 1, a classical chemotherapy drugs transporter. These results indicate that the ability to suppress the malignant phenotype by down-regulating CDC2 expression may provide a new gene therapy approach for overcoming CDC2-associated chemoresistance in patients with malignant glioma.

Keywords Glioma · CDC2 · Chemoresistance · Apoptosis · Temozolomide · Proliferation · Prognosis

Introduction

Malignant astrocytomas (glioblastoma multiforme and anaplastic astrocytoma) are the most frequent primary neoplasms in adults [1]. With aggressive interventions, including surgery, chemotherapy, and radiotherapy, malignant astrocytomas are still associated with significant morbidity and mortality [2]. Currently, improved chemotherapeutics play a critical role in improving prognosis for patients with malignant glioma, but chemoresistance is a fundamental hallmark of malignant glioma [3].

Cell division cycle 2 (CDC2) is an active sub-unit of the M-phase promoting factor (MPF) [4]. Recent studies have shown that CDC2 could promote cellular transformation, apoptosis, and tumor growth [5, 6]. It has been linked to aggression in a variety of human cancers, including pancreatic, esophageal, breast, and lung cancers [7–10]. Moreover, diffuse large B cell lymphoma cells

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overexpressing CDC2 were more resistant to chemotherapy-induced apoptosis [11]. Previous study found that CDC2 expression was positively correlated with the pathologic grades of human gliomas [12]. Furthermore, CDC2 enhances Ral-binding protein 1 (RLIP76) transporter activity in H358 cells and modulates cellular response to chemotherapy [13]. RLIP76 acts as a primary regulator of protein synthesis and cell growth and functions as a multifunctional transporter that transports various chemotherapy drugs across the plasmalemma, which in turn is the most frequently hyperactivated pathway in glioblastoma (GBM) [14, 15]. In light of these findings, we studied the effect of CDC2 expression levels on the progression of human malignant glioma cells and the effect of CDC2 knockdown on the sensitivity of glioma cells to the chemotherapy drug temozolomide (TMZ).

Here, we define a novel molecular mechanism for TMZ resistance in human glioblastoma that depends on overexpression of the RLIP76 protein secondary to over-activation of the CDC2 signaling pathway. Furthermore, our findings highlight CDC2 as a novel molecular target for glioma therapy that can be specifically inhibited to sensitize glioma cells to TMZ, thereby facilitating antitumor therapy.

Materials and methods

Tissue samples

Tissue samples were obtained from patients with gliomas who underwent surgical treatment at the first and the third central hospital of Tianjin, respectively, from January 1999 to December 2011. All samples were histologically verified by two experienced pathologists. Tumor samples were immediately frozen to −70 °C until they were used. The follow-up was carried out in all patients, with survival time being completed in July 2012. The follow-up period was conducted every 6 months by telephone with the last follow-up being January 2013. The tumor size, location, extent of surgical resection, and time free from recurrence were recorded. The clinicopathological characteristics of the patients are summarized in Table 1. The selection criteria were described previously [14]. Patients receiving radiotherapy prior to the surgery were excluded. According to the selection criteria, 96 patients were enrolled in this study.

Cell culture

The malignant glioma cell lines U87 and U251 were obtained from the Chinese Academy of Sciences (Shanghai, China). All cell lines were grown in DMEM supplemented

Table 1 Demographic and clinicopathological characteristics of glioblastoma multiforme patients

Characteristics	Value	CDC2 expression Low	CDC2 expression High
No. patients	96	57	39
Age (years)			
<60	68 (70.8 %)	41	27
≥60	28 (29.2 %)	16	12
Gender			
Male	60 (62.5 %)	24	26
Female	36 (37.5 %)	23	13
IICP			
Present	44 (45.8 %)	25	19
Absent	52 (54.2 %)	32	20
Cystic degeneration			
Present	26 (27.1 %)	17	9
Absent	70 (72.9 %)	40	30
Necrosis on MRI			
Present	20 (20.8 %)	12	8
Absent	76 (79.2 %)	45	31
MTD (cm)			
<5	64 (66.7 %)	36	28
≥5	32 (33.3 %)	11	21
Chemotherapy			
Present	68 (70.8 %)	39	29
Absent	28 (29.2 %)	18	10
Radiotherapy			
Present	58 (60.4 %)	34	24
Absent	38 (39.6 %)	23	15
Survival status			
Live	11 (11.5 %)	11	0
Dead	85 (88.5 %)	46	39

IICP increased intracranial pressure, *MTD* mean tumor diameter

with 8 % fetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 µg/ml) and maintained in monolayer culture at 37 °C in humidified air with 5 % CO_2. Viability of the cells was monitored with trypan blue staining.

Constructs and transfection

CDC2 constructs and transfection followed a previously described protocol [12]. The U87 and U251 cells were stably transfected with GFP-siRNA, or CDC2-siRNA according to the manufacturer's protocol (Invitrogen). After 2–3 weeks, single independent clones were randomly isolated, and each individual clone was plated separately. After clonal expansion, cells from each independent clone were tested for CDC2 expression by immunoblotting. Four

U87 and U251 lines were isolated, one expressing GFP, and another expressing the CDC2-specific shRNA that led to stable CDC2 underexpression.

Immunohistochemistry and expression analysis

The immunohistochemistry was performed as previously described [16]. Tissue samples were provided by the Department of Neurosurgery,the first and the third central hospital of Tianjin, respectively. CDC2 primary monoclonal antibody (1:500; Abcam, ab56815) or Ki-67 primary antibody (1:75; Dako, Glostrup, Denmark) was used in this procedure. Staining intensity in the cytoplasma was graded, and the percentage of positively stained cells was scored as previously described [14]. The final composite score was the product of intensity and percentage scores and was classified as strong (+++, final score >6), moderate (++, final score = 4–6), or weak (+, final score = 1–3), or null (−, final score = 0). For statistical analysis, CDC2 expression was divided into "high" (++ and +++) versus "low" (+ and −).

Quantitative RT-PCR analysis

Quantitative RT-PCR analysis was measured as described previously [12]. The primer sequences used were CDC2 5′-CAGTCAGACCAAAATACCTACTGGGT-3′ (F) and 5′-ACACCAACCAGCTGCAGCATCTTCTT-3′ (R). The PCR conditions were 5 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

Viability and apoptosis detection

The cell proliferation rate was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric cell viability assay. Briefly, cells were incubated with 0.2 mg/ml MTT for 4 h in the dark at 37 °C. After removal of the medium, the formazan crystals produced from MTT by live cells were dissolved in 150 μl dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm. Apoptosis was measured using an Annexin V/fluorescein isothiocyanate (FITC) apoptosis detection kit (Bender Med System, CA) according to the manufacturer's instructions.

Colony formation assay

The single cells were seeded in DMEM with 10 % FBS at a density of 2,000 cells/well on 6-well plates that were pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA). After removing the media and washing the cells with cold PBS, colonies were fixed with glutaraldehyde (6.0 % v/v), stained with crystal violet (0.5 % w/v), and counted using a stereomicroscope. Only colonies containing >50 cells were photographed. The parental cells were plated at the same density as the control.

Statistics

Kaplan–Meier survival analysis was used to compare overall survival times in glioma patients. The Kruskal–Wallis test was used to analyze the CDC2 expression and clinicopathological characteristics. Univariate survival analysis was performed using the Kaplan–Meier method and analyzed by the log-rank test to assess survival differences between groups. The Cox proportional hazards model for multivariate survival analysis was used to assess predictors of survival. A two-tailed P value of less than 0.05 was considered statistically significant. Analyses were performed using the SPSS 10.0 statistical software for Windows (SPSS, USA).

Results

CDC2 expression level was higher in poor prognosis GBM

Two groups of glioblastoma tissue samples were collected, one group from patients with good prognosis, as defined by 12-month survival or longer, and the other from patients with poor prognosis (<12 months). We first estimated the expression level of CDC2 by immunohistochemical staining. Glioma with poor prognosis exhibited greater immunoreactivity for CDC2 proteins compared to glioma with good prognosis (Fig. 1a). Statistical analysis showed increased CDC2 immunoactivity in poor prognosis gliomas versus good prognosis gliomas (45.23 ± 3.02 vs. 4.87 ± 1.77 %, $P < 0.001$) (Fig. 1b). Consistent with these immunohistochemical results, poor prognosis gliomas exhibited dramatically higher CDC2 mRNA expression than good prognosis gliomas (Fig. 1c). Western blotting confirmed that CDC2 protein levels were higher in poor prognosis gliomas than good prognosis gliomas (Fig. 1d).

In light of these findings, we used univariate and multivariate Cox regression analysis to further explore the correlation between prognosis and the CDC2 expressing of the tumors. Univariate survival analysis revealed significant relationships between expression of CDC2 ($P = 0.007$, Table 2), age ($P = 0.021$, Table 2) and survival rate, while no significant associations were found between survival rate and other clinical features (age, sex, and tumor size). Multivariate analysis using the Cox proportional hazards model for all variables included in univariate analysis revealed that high CDC2 expression at
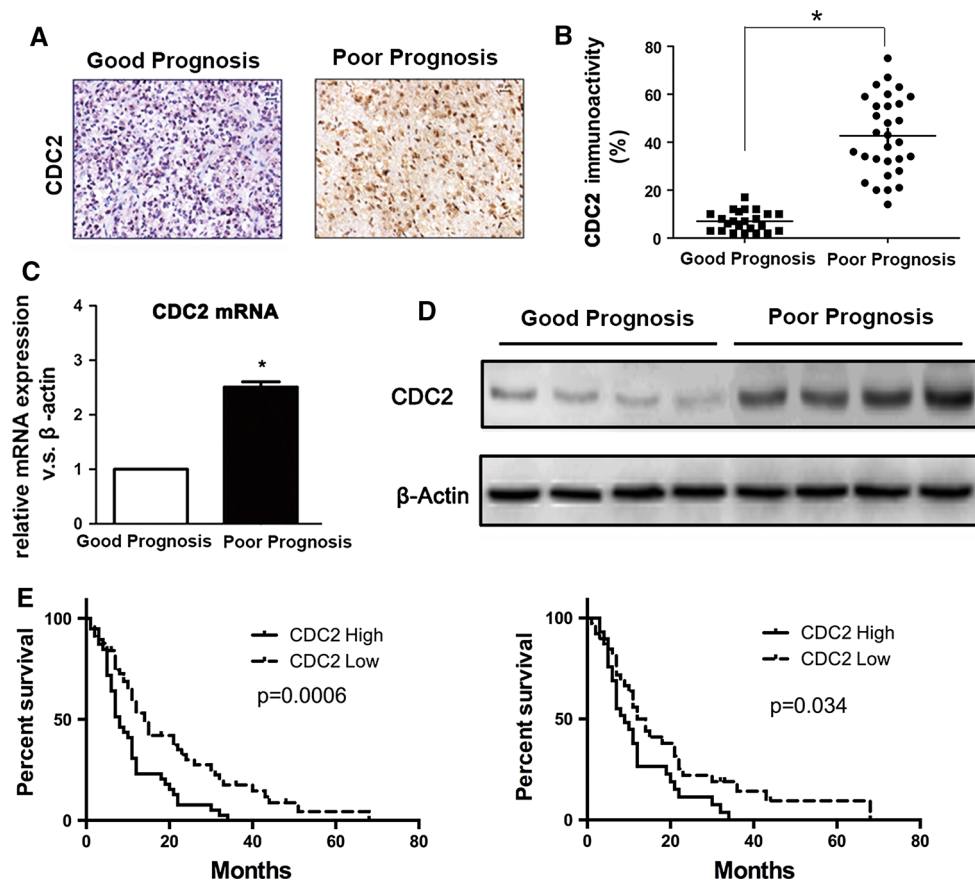
Fig. 1 a Immunohistochemical expression of CDC2 in patients with poor prognosis and good prognosis. Few CDC2-positive tumor cells are found in patients with good prognosis, while strong staining intensity is detected in patients with poor prognosis. b Statistical analysis revealed a significant increase in CDC2 immunoreactivity in poor prognosis versus good prognosis. c mRNA levels of CDC2 measured by quantitative real-time PCR. *$P < 0.05$. d Expression of CDC2 proteins in good prognosis (*lanes* 1–4) and poor prognosis (*lanes* 5–8) measured by Western blot. e *Left* Kaplan–Meier survival curves according to CDC2 expression. Patient tumors were analyzed by immunohistochemistry for RLIP76 expression and divided into high and low expression groups as described in "Materials and methods" section. ($P = 0.001$). *Right* the Kaplan–Meier survival curve shows that patients with high CDC2 expression receiving chemotherapy after surgery had shorter overall survival than these with low CDC2 expression receiving chemotherapy

diagnosis was independent prognostic factors for patients with glioma (Fig. 1e left, $P = 0.006$). These results are consistent with previous data (Fig. 1a–d) that good prognosis patients with GBM have low CDC2 expression.

Moreover, the Kaplan–Meier survival curve shows that patients with high CDC2 expression receiving chemotherapy after surgery had shorter overall survival than these with low CDC2 expression receiving chemotherapy (Fig. 1e right, $P = 0.034$). These results suggest that CDC2 expression may be a key prognostic index for glioma patient survival and a possible indicator in predicting the effects of the chemotherapy.

CDC2 shRNA decreased the expression level of CDC2 in the human glioma cell lines

Transfection of U251 and U87 glioblastoma cell lines with a CDC2-targeted shRNA resulted in an approximately 54.8 % decrease in RLIP76 mRNA expression in U87 cells and an 47.8 % decrease in U251 cells compared to untransfected wild type parental cells and cells transfected with GFP-shRNA (Fig. 2a, b). We also confirmed the decrease in CDC2 protein expression in U87 and U251 shRNA transfectants by Western blotting (Fig. 2a, b).

Knockdown of CDC2 increased the chemosensitivity to TMZ

The fact that CDC2 overexpression conferred a poor prognosis, especially for advanced glioma patients who are more likely to receive chemotherapy or chemoradiotherapy, suggested it might contribute to chemotherapeutic resistance. To test this hypothesis, U87 and U251 cells transfected with CDC2-shRNA were used. We found that U87 cells treated with combined TMZ and the CDC2-targeted shRNA showed a significantly greater growth

Table 2 Univariate and multivariate analysis of different prognostic parameters in patients with glioblastomas by Cox regression analysis

Variable	No. patient	Univariate log-rank test (P)	Cox multivariable analysis (P)	Relative risk
Age (years)				
<60	68	0.021	0.043	5.278
≥60	28			
Gender				
Male	60	0.951	0.774	NS
Female	36			
MTD (cm)				
<5	64	0.659	0.803	NS
≥5	32			
Chemotherapy	68	0.38	0.214	NS
Radiotherapy	58	0.486	0.398	NS
CDC2				
Low	57	0.007	0.021	5.471
High	39			
IICP	44	0.975	0.768	NS
Cystic degeneration	26	0.438	0.698	NS
Necrosis on MRI	20	0.582	0.389	NS

IICP increased intracranial pressure, *MTD* mean tumor diameter, *NS* no significant

inhibition than cells treated with TMZ alone by daily MTT cell viability assays (Fig. 3a). The IC50 value for shRNA-transfected U87 cells was 4.45 ± 0.28 μg/ml, whereas IC50 values for GFP-transfected and control U87 cells were 7.33 ± 1.45 and 7.56 ± 1.23 μg/ml, respectively. Similar results were found in U251 glioma cells. The IC50 value for shRNA-transfected U251 cells was 7.26 ± 0.69 μg/ml, and IC50 values for GFP-transfected and control U251 cells were 9.84 ± 0.97 and 10.18 ± 0.78 μg/ml, respectively (Fig. 3b). Therefore, down-regulation of CDC2 increased the sensitivity of U87 and U251 glioma cells to TMZ.

Previous study has shown that knockdown of CDC2 expression reduced the rate of glioma cell proliferation and triggered apoptosis [12]. These results prompted us to investigate whether U87 and U251 cells underexpressing CDC2 were more sensitive to the antitumor drug TMZ by enhancing apoptosis. Incubation with TMZ resulted in an increase in apoptosis rate, as indicated by flow cytometric analysis, and shRNA-transfected cells exhibited enhanced TMZ sensitivity compared to control and GFP-transfected U87 cells (Fig. 3c). In light of the enhanced rate of apoptosis in cells underexpressing CDC2, we measured the expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic effector caspase-3. We found that the combination of shCDC2 and TMZ decreased Bcl-2 expression and increased caspase-3 expression when compared with control and GFP cells treated with TMZ alone by Western blot analysis. RT-PCR revealed the similar results at mRNA level (Fig. 3d). These data might explain that the knockdown of CDC2 contributes to the improvement of chemoresistance, at least partially by enhancing apoptosis.

CDC2 knockdown decreased RLIP76 expression

The multifunctional drug transporter RLIP76 confers chemoresistance to GBM, suggesting that CDC2 may alter TMZ sensitivity by regulating RLIP76 expression. We
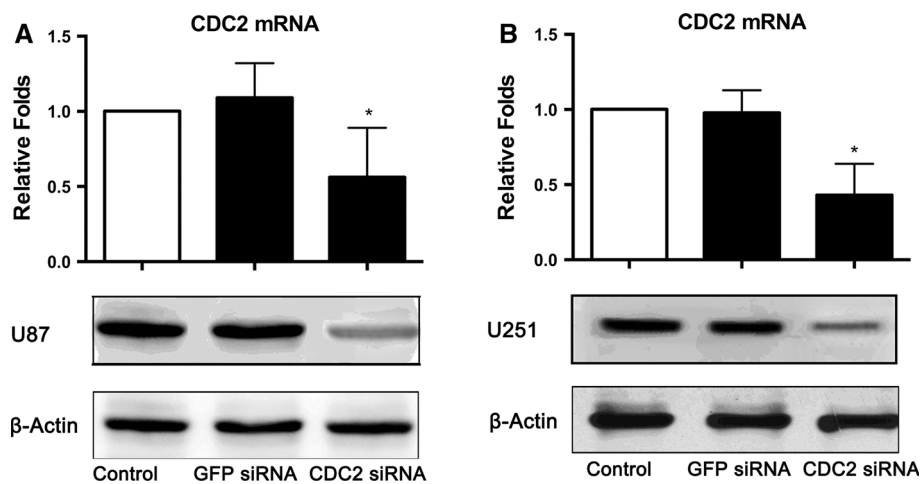

Fig. 2 a *UP* the mRNA level of CDC2 is measured in U87 by real-time PCR. *Bottom* Western blotting analysis demonstrates that CDC2 protein was significantly lower suppressed in U87 cells stably transfected with CDC2-targeted siRNA compared to untransfected glioblastoma cells and cells stably transfected with GFP-siRNA. **b** *UP* real-time PCR reveals that CDC2 mRNA was significantly decreased in U251 cells transfected with CDC2-targeted siRNA. *Bottom* Western blotting analysis measures the protein level of CDC2 in U251
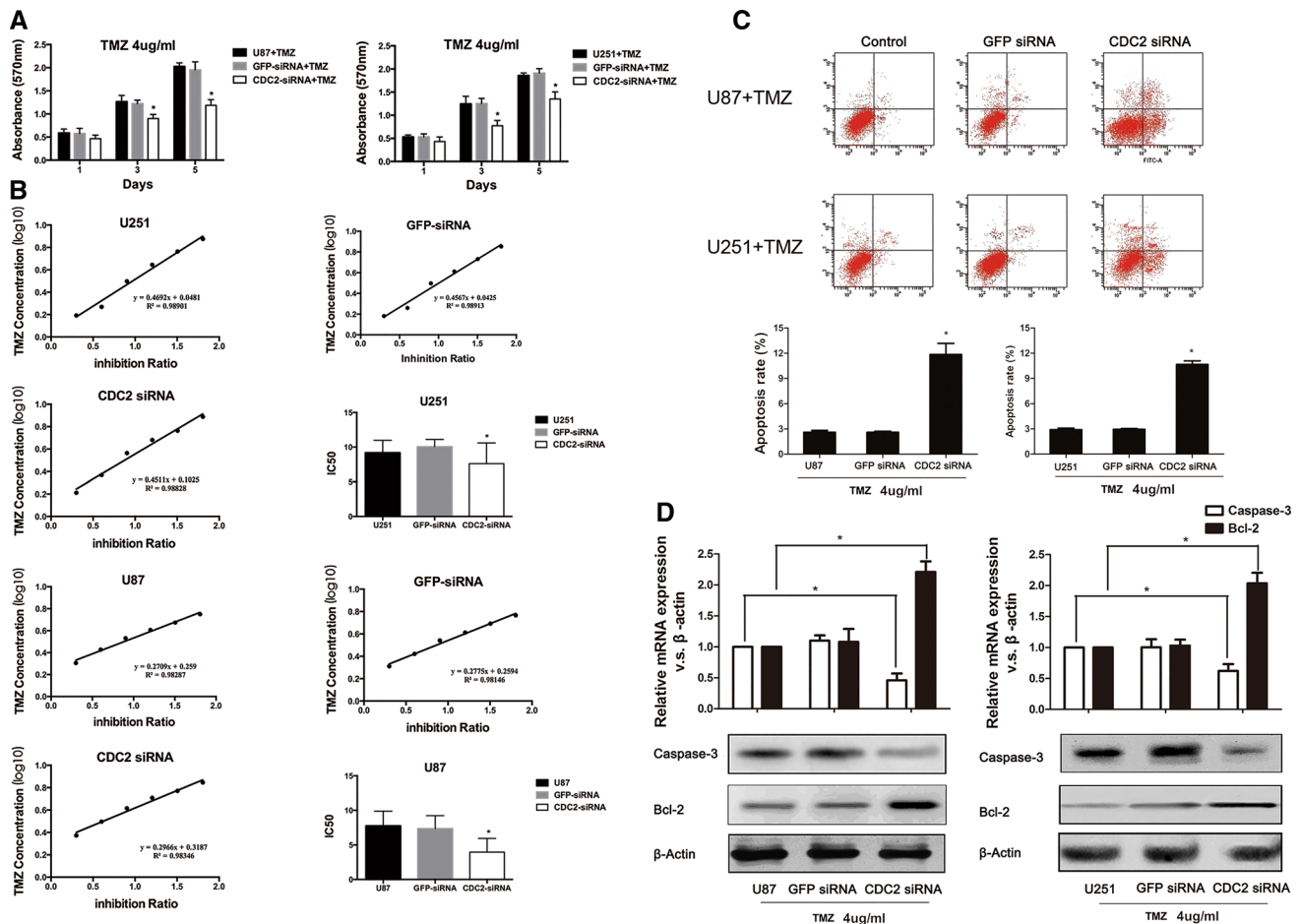
Fig. 3 a Knockdown of CDC2 resulted in increased growth inhibition by TMZ (4 ug/ml). Control and GFP-transfected U87 and U251 cells were significantly more resistant to TMZ than shRNA-transfected cells as revealed by MTT cell viability assays. Combining TMZ with CDC2 shRNA induced significantly greater inhibition of proliferation than TMZ alone (*$P < 0.05$ compared to control and lenti-GFP cells treated with TMZ alone). b CDC2 siRNA- and GFP-transfected cells were treated with varying concentrations of TMZ. Incubation with TMZ resulted in concentration-dependent growth inhibitory effects in all cells with increased TMZ sensitivity in the CDC2 siRNA-transfected cells. The IC50 value for TMZ in shRNA-transfected U87 cells was significantly lower than that in control and

GFP-transfected cultures, indicative of higher TMZ sensitivity in U87 and U251 cells underexpressing CDC2 (*$P < 0.05$ compared to control and GFP-transfected cultures). c Combining TMZ with CDC2 shRNA induced significantly greater apoptosis than TMZ alone in U87 and U251 cells as measured by PI staining and flow cytometry (*$P < 0.05$ compared to control and lenti-GFP cells treated with TMZ alone). d Western blots revealed that caspase-3 protein levels were significantly higher, while Bcl-2 protein expression was markedly lower in the combination of shRNA-transfected U87 cells and TMZ compared to control and lenti-GFP cells treated with TMZ alone (*$P < 0.05$ vs. control + TMZ or lenti-GFP group + TMZ). Quantitative RT-PCR demonstrated the similar results

found that reduced expression of CDC2 in shRNA-transfected U87 and U251 cells decreased RLIP76 mRNA and protein expression. Furthermore, the combination of shRNA and TMZ decreased RLIP76 expression on mRNA and protein level, while the combination of GFP and TMZ and TMZ alone did not influence RLIP76 mRNA and protein expression (Fig. 4a, b). These results strongly suggest that the enhanced susceptibility to TMZ in U87 and U251 cells underexpressing CDC2 was due to reduced RLIP76 protein expression and a concomitant decrease in RLIP76-mediated TMZ efflux.

Discussion

Affymetrix microarray analysis revealed many differentially expressed genes that affected the chemical sensitivity of patients with glioblastoma, including CDC2 and other kinases [17]. Furthermore, Chen et al. [12] revealed that CDC2 was overexpressed in glioma and was associated with the pathological grade. In this study, we confirmed that expression of CDC2 is positively correlated with poor prognosis in patients with GBM. Cox regression analysis further revealed that CDC2 was an

Fig. 4 a Quantitative RT-PCR analysis showed that CDC2 knockdown decreased the mRNA levels of RLIP76 and the combination of CDC2 knockdown and TMZ (4 ug/ml) also down-regulated the mRNA levels of RLIP76 in U87 cells (*$P < 0.05$ compared to control and lenti-GFP cells). Western blot analysis revealed that CDC2 knockdown decreased RLIP76 protein expression and the combination of CDC2 knockdown and TMZ (4 ug/ml) decreased RLIP76 protein expression in U87 cells as well. b Similar results were found in U251 cells (*$P < 0.05$ compared to control and lenti-GFP cells)
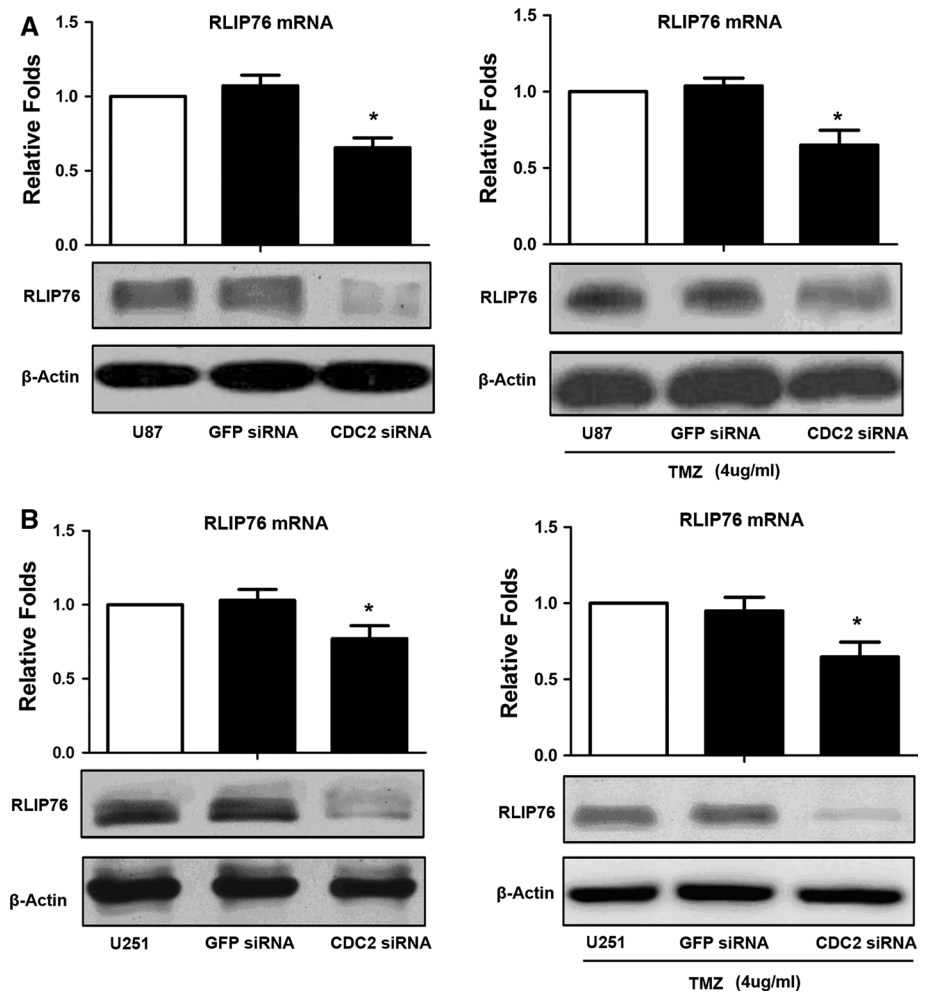
independent factor for survival. Our results indicated that high CDC2 expression was a prognostic factor for poor outcome. Moreover, we found that low expression of CDC2 in patients who received chemotherapy after surgery was correlated with better prognosis. It indicated that CDC2 expression level could be used to predict the effect of the chemotherapy.

In the present study, we have demonstrated that down-regulation of CDC2 expression decreased the IC50 of TMZ in malignant gliomas. We found that combined TMZ and CDC2 inhibition exerted greater inhibition on proliferation than TMZ treatment alone, suggesting that CDC2 is an attractive target for enhancing the antitumor efficacy of TMZ. Moreover, we found that the combination of shCDC2 and TMZ resulted in an increase in apoptosis than TMZ treatment alone. To investigate the mechanisms of apoptosis induced by CDC2 inhibition, we used qRT-PCR and Western blot to measure Bcl-2 and caspase-3 mRNAs and proteins expression. CDC2 inhibition decreased Bcl-2 expression and increased caspase-3 expression at both the

mRNA and protein levels, implying a functional interaction between CDC2 and the Bcl-2 and caspase-3 pathways.

Resistance to chemotherapy depends on enhanced expression of RLIP76 that transports many classical chemotherapy drugs across the plasmalemma in several cancer types [18]. Moreover, RLIP76 is highly expressed in malignant glioma cell lines and in clinical specimens and associated with chemoresistance both in vitro and in vivo [14]. We then further explore the mechanism underlying these changes in proliferation and TMZ sensitivity. Specifically, we investigated whether CDC2 regulated the expression of RLIP76 as there is compelling evidence that binding of CDC2 to RLIP76 is essential to shut off endocytosis during mitosis and CDC2 inhibits RLIP76 transport activity in concentration-dependent manner [13, 19]. In this study, we showed that the expression of RLIP76 protein was significantly decreased in malignant glioma cells following CDC2 knockdown. These results are consistent with previous study that the Cdk inhibitor flavopiridol could enhance TMZ-induced cytotoxicity in human glioma

cells [20]. Thus, we speculated that CDC2-dependent overexpression of RLIP76 may further decrease chemosensitivity, while reduced CDC2 signaling could lower RLIP76 expression and ensuing drug efflux.

In summary, our results indicate that decreased CDC2 expression in malignant glioma cells inhibits proliferation, induces apoptosis by increased caspase-3 and decreased Bcl-2 expression, and enhances the chemosensitivity to TMZ by decreasing the expression of the RLIP76 protein. However, the mechanisms through which CDC2 regulates proliferation, apoptosis, and drug resistance require further investigation. These data indicate that knockdown of CDC2 protein expression may represent a potential gene-targeting approach to treat glioma and may increase the chemosensitivity of gliomas to TMZ and similar alkylating agents.

Conflict of interest   The authors declare no conflict of interest.

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