Original Paper

Inhibition of Cyclin D1 Expression in Human Glioblastoma Cells is Associated with Increased Temozolomide Chemosensitivity

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Key Words

Glioblastoma • Chemoresistance • Temozolomide • CCND1 • MDR1

Abstract

Background/Aims: Cyclin D1 (CCND1) is frequently overexpressed in malignant gliomas. We have previously shown ectopic overexpression of CCND1 in human malignant gliomas cell lines. **Methods:** Quantitative reverse transcriptase PCR (qRT-PCR) and Western Blot (WB) was performed to investigate the expression of CCND1 in glioma tissues and cell lines. The biological function of CCND1 was also investigated through knockdown and overexpression of CCND1 *in vitro*. **Results:** Here we reported that CCND1 expression was positively associated with the pathological grade and proliferative activity of astrocytomas, as the lowest expression was found in normal brain tissue (N = 3) whereas the highest expression was in high-grade glioma tissue (N = 25). Additionally, we found that the expression level of CCND1 was associated with IC₅₀ values in malignant glioma cell lines. Forced inhibition of CCND1 increased temozolomide efficacy in U251 and SHG-44 cells. After CCND1 overexpression, the temozolomide efficacy decreased in U251 and SHG-44 cells. Colony survival assay and apoptosis analysis confirmed that CCND1 inhibition renders cells more sensitive to temozolomide treatment and temozolomide-induced apoptosis in U251 and SHG-44 cells. Inhibition of P-gp (MDR1) by Tariquidar overcomes the effects of CCND1 overexpression on inhibiting temozolomide-induced apoptosis. Inhibition of CCND1 inhibited cell growth *in vitro* and *in vivo* significantly more effectively after temozolomide treatments than single temozolomide treatments. Finally, inhibition of CCND1 in glioma cells reduced tumor volume in a murine model. **Conclusion:** Taken together, these data indicate that CCND1 overexpression upregulate P-gp and induces chemoresistance in human malignant gliomas cells and that inhibition of CCND1 may be

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an effective means of overcoming CCND1 associated chemoresistance in human malignant glioma cells.

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Introduction

Malignant gliomas (anaplastic astrocytoma and glioblastoma multiforme) occur more frequently than other types of primary central nervous system (CNS) tumors. The life expectancy of GBM patients is on average of only 14 months after diagnosis even with the aggressive surgery, radiation, and chemotherapies [1, 2].

Temozolomide is the second generation imidazotetrazine prodrug that doesn't need hepatic metabolism for activation and is able to penetrate the blood-brain barrier [3, 4]. Temozolomide can clearly improve overall survival and has the advantage of wide applicability with its relatively simple administration and favorable toxicity profile compared with older agents such as carmustine [5]. However, the emergence of temozolomide resistance hampers its use in GBM patients. Temozolomide chemoresistance might due to the variations in multidrug resistance genes (MDR1) and DNA repair proteins such as O6-methylguanine-DNA methyltransferase, and apoptosis-regulating genes [6, 7].

As a key cell cycle regulator of the G1 to S phase progression [8], the binding of CCND1 to cyclindependent kinase (cdk4 or cdk6) would lead to the phosphorylation of retinoblastoma protein (pRb), which subsequently triggers the release of E2F transcription factors to allow transcription of genes required for the G1 to S phase progression of the cell cycle [9, 10]. Moreover, overexpression of CCND1 has been found in a variety of cancers such as breast cancer, head and neck cancer, hepatocellular carcinoma [11, 12].

CCND1 overexpression contributes to the resistance of several tumor cells to chemotherapeutic agents [13], including fibrosarcoma cell line [14], pancreatic cancer cell lines [15-17], squamous cell carcinoma (OSCC) cell lines [18], and ovarian and prostate cancer cell lines [19]. Combined CCND1 knockdown using small-interfering RNA and cisplatin treatment is more effective in inhibiting cell growth than any of these single treatments [19]. Altered expressions of chemoresistance genes such as MDR-1 and MRP may attribute to the effect that CCND1 inhibition enhances their responsiveness to multiple chemotherapeutic agents [15-17].

Using Affymetrix microarray, we previously revealed that CCND1 up-regulation was related to the survival duration of human glioblastoma and might influence chemosensitivity of GBM [20]. Then we demonstrated CCND1 overexpression could upregulate p-gp expression and contribute to temozolomide chemoresistance [21]. Herein, we studied the dysregulation of CCND1 in human astrocytoma tissues by immunohistochemical and Western blot analysis. Furthermore, we investigated the functional role of CCND1 in glioblastoma cell lines examined *in vitro*. These results presented the first evidence that alteration of CCND1 expression could be functionally associated with chemosensitivity, and CCND1 might serve as a molecular target for therapeutic applications.

Materials and Methods

Tumor samples

The current study included 40 patients who attended our institute from 2014 to 2015. Overall, 25 high-grade astrocytomas (WHO grade III-IV), 12 low-grade astrocytomas (WHO grade I-II) and 3 normal brain samples were collected via surgical resection. The gliomas were graded in accordance with the WHO pathological diagnostic standard [22]. None of the patients had received radiotherapy or chemotherapy before tumor resection. Normal brain tissues were taken from peripheral nontumor glial brain tissue from patients. The clinicopathological features of patients included are detailed in Table 1. Samples were divided and either frozen in liquid nitrogen and stored at -80°C or kept in RNAlater (Ambion, Austin, TX, USA) at -20°C. The study was conducted in accordance with the Declaration of Helsinki and approved by the

Institutional Review Board of Shanghai Second Military Medical University. Informed consent was returned from all patients included in the current study or their direct relatives.

Cell culture

The human malignant glioma cell lines U87, U138, U251, LN-18, T98G, A-172 and SHG-44 purchased from the Cell Bank at Chinese Academy of Sciences (Shanghai, China), were grown in a complete medium composed of DMEM supplemented with 8% FBS, penicillin G (100 U/mL), and streptomycin (100 mg/mL), and maintained in monolayer culture at 37°C in humidified air with 5% CO₂. Viability of the cells was determined by trypan blue staining.

Immunohistochemistry

Immunohistochemical staining was performed using a method described previously. Briefly, thawed samples were fixed in 4% formalin and embedded in paraffin for histopathological analysis. Samples were deparaffinized with xylol and then sliced into 4-μm sections. Sections were rehydrated using a graded ethanol series. A heat-induced epitope protocol was used for antigen-retrieval (95°C for 40 min). Samples were incubated in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase. Samples were blocked with protein serum (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA) and then incubated (overnight at 4°C) with polyclonal rabbit anti-human CCND1 or Ki67 antibody at 1:1000 (MBL International Corporation, Nagoya, Japan). After washing three times in TBST (150 mM NaCl, 10 mM Tris-HCl, pH 7.6), sections were incubated with secondary antibody for 20 min at room temperature. Peroxidase-conjugated biotin-streptavidin complex (Dako, Glostrup, Denmark) was then applied to the sections for 20 min. Sections were visualized with 3, 3'-diaminobenzidine and counterstained with hematoxylin. The negative control used nonimmune serum instead of primary antibody.

Constructs and transfection

CCND1 inhibition and overexpression transfectants were generated by co-transfection of U251 or SHG-44 cells with pLVTHM lentivirus vector (Invitrogen) harbouring shRNA targeting CCND1 or BISVECTOR harbouring the CCND1 gene described previously [23].

Protein extraction and Western blotting.

Proteins were extracted from a subconfluent culture of cells and then subjected to Western blot analysis. After blocking with 5% nonfat milk in PBS-T for 1 h at room temperature, the membranes were blotted with primary antibody, followed by incubation with a peroxidase-conjugated secondary antibody. Bound antibodies were visualized using enhanced chemiluminescence (Bio-Rad). The primary antibodies used were rabbit polyclonal antibody to CCND1 (Santa Cruz, sc-717, 1:1000 dilution), mouse monoclonal antibody to MDR-1 (Santa Cruz, sc-59593, 1:1000), goat polyclonal antibody to Caspase 3 (Santa Cruz, sc-1225, 1:1000), mouse monoclonal antibody to Bcl-2 (Santa Cruz, sc-130307, 1:1000), and rabbit polyclonal antibody to β-actin (Santa Cruz, sc-130656, 1:1000).

RNA extraction and real-time PCR.

Total RNA was extracted using the TRIzol reagent (Life Technologies) following the manufacturer's protocol. RNA was reverse-transcribed to cDNA with the Super-Script First-Strand cDNA System (Invitrogen, Carlsbad, CA, USA), and amplified by Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). We prepared a master mix including Platinum SYBR Green qPCR SuperMix-UDG, forward and reverse primer, template cDNA (10 ng) for qPCR test. The reaction conditions were 95°C for 5 min, and then 32 cycles at 95°C for 15 secs and 1 min at 60°C and 30 secs at 72°C. Primers are listed in Table 2. The β-actin was used as an internal control. The relative expression levels of mRNA were quantified using the ΔΔCt method.

Table 1. Relationships between CCND1 expression in human glioma tissues and clinicopathological features. WHO = World Health Organization

Clinicopathological features	No. of cases	CCND1 (n, %)		p Value
		High	Low	
Gender				
Male	18	7 (39)	11 (61)	>0.05
Female	19	9 (47)	10 (53)	
Age				
<45	21	11 (52)	10 (48)	>0.05
≥45	16	7 (44)	9 (56)	
WHO grade				
I	6	1 (17)	5 (83)	<0.05
II	6	2 (33)	4 (67)	
III	11	7 (64)	4 (36)	
IV	14	12 (86)	2 (14)	

Growth inhibition

Growth inhibition was determined by an MTT assay. In brief, cells were seeded in 96-well plates at a density of 5×10^3 cells/well (200 μ L/well), 24 hr before temozolomide treatment. The culture medium was then replaced with fresh medium containing different concentrations of temozolomide ranging from 0 to 80 μ g/ml and the cells were cultured in this medium for 72 hr followed by MTT assay. The IC_{50} values (concentration of drug that results in 50% reduction in absorbance compared with control) were calculated as previously described [24].

Clonogenic survival assays

Cell proliferation was assessed by a soft agar colony formation assay. A 6-well plate containing a 1.5 ml bottom layer and 0.5 ml top layer of agar was used (5.1 mg/mL, Difco Laboratories, Detroit, MI, USA). Cells (10^4 /well) were transferred onto the bottom layer and then overlaid with a top layer and cultured at 37°C in 5% CO₂. Giemsa staining was used to quantify the formation of colonies on the 7th day.

Drugs and drug treatment

TMZ (Schering-Plough, Kenilworth, NJ, USA) stocks were prepared by dissolving the drug in ethanol and diluting it with sterile dH₂O. Stock solutions were filtered, aliquoted and stored at -80°C. U251 or SHG-44 were treated with increasing concentrations of Temozolomide and then harvested after 144 h to determine apoptosis. For the time responses, the glioma cells were treated with temozolomide and then harvested at the appropriated times. Tariquidar was provided by Merck and Co., Inc. (Kenilworth, NJ, USA). Tariquidar was dissolved in DMSO for *in vitro* experiments.

Apoptosis assay

Apoptosis was measured using an Annexin V/FITC apoptosis detection kit (Bender Med System, CA). Briefly, cells cultured in 6-cm dishes were trypsinized, washed and stained with FITC-conjugated anti-Annexin V antibody in darkness for 15 min at room temperature, and then analyzed with a flow cytometer (FACSCalibur; Becton Dickinson).

Tumor xenograft model and tumorigenicity assay

A xenograft tumor model was generated using 6-week-old male BALB/c nude mice (n = 20) obtained from Shanghai Laboratory Animal Company (SLAC, Shanghai, China). U251 cells were transfected with an empty vector or shCCND1 and then resuspended in PBS. Mice were randomly divided into four groups of five mice. Each mouse was inoculated with 6×10^6 cells in 100 μ L of PBS subcutaneously into the left armpit. Tumor diameters were measured with a vernier caliper every 7 days and tumor volumes were calculated using the following equation: tumor volume = $1/2$ (length $\times$ width²). Mice were given TMZ by oral gavage (60 mg/kg daily for 5 days/week). After 5 weeks, mice were sacrificed to retrieve tumors. All experiments were approved and performed according to the guidelines of the Ethics Committee of the Institutional Review Board of Shanghai Second Military Medical University (Shanghai, China), conformed to the Principles of Laboratory Animal Care (National Society for Medical Research), and were conducted according to the National Institutes of Health guidelines.

Statistical Analysis

Results are expressed as mean value SD. Statistical significance was taken as $P < 0.05$ using a one-tailed Student's *t* test. Analysis of variance (ANOVA) was also used. Kaplan-Meier survival curves were plotted for the intracranial experiment and analyzed by the log-rank method.

Table 2. Sequences of primers for quantitative real-time PCR

Gene name	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
Cyclin D1	GGGAGGAACAGAAAGTGC	GAGTTGTGCGGTAGATGC
MDR1	CCCATCATTGCAATAGCAGG	TGTTCAAACCTTCTGCTCCTGA
MRP1	ATGTCACGTGGAATACCAGC	GAAGACTGAACCTCCTTCTCT
MMP2	CTGACCCCAAGTCTATCTGCC	TGTTGGGAACGCTGACTTCAG3
MMP9	GCCTGGCACATAGTAGGCC	TTCTAGCCAGCCGGCATC
Caspase-3	TGTCATCTCGCTCTGGTACG	AAATGACCCCTTCATCACCA
Bcl-2	GGTGGAGGAGCTCTTCAGG	ATAGTTCCACAAAGGCATCC
p53	AATCCCGTGGGATTTCTCT	AGGCTGCCACTTCTTGAT
FASL	GCACACAGCATCATCTTTGG	GGACCTTGAGTTGGACTTGC
β -actin	CTCCATCTGGCCTCGCTGT	GCTGTCACCTTCACCGTTCC

Results

CCND1 is highly expressed in human glioma and associated with decreased patient survival

Using astrocytoma tissues, quantitative real time PCR analysis demonstrated high expression levels of CCND1 in high-grade astrocytoma tissue compared with low-grade or normal brain tissues (Fig. 1A). Expression of CCND1 was also determined by immunohistochemistry in all tissues. Normal tissue sections were stained fewer indicating a lower presence of CCND1 than in low and high grades of astrocytoma (Fig. 1B). Further western blotting assay confirmed the CCND1 expression was significantly increased as astrocytoma progressed (Fig. 1C). Kaplan–Meier survival curve suggested that the expression of CCND1 was reflected in patient survival rates with longer survival associated with a lower expression of CCND1 (Fig. 1D).

CCND1 expression was associated with the temozolomide IC50 in GBM cell lines

The mRNA expression and protein level of CCND1 were assessed in glioblastoma cell lines U87, U138, U251, LN-18, T98G, A-172 and SHG-44 (Fig. 2A, B). IC50 values are shown in Fig. 1C. Significantly higher levels of CCND1 expression and IC50 were found in U251 and SHG-44 compared to the other cell lines. The U251 and SHG-44 cell lines were subjected to further analysis. Meanwhile, the correlation between the expression level of CCND1 and temozolomide IC50 in these 7 GBM cell lines were analyzed. Spearman Rank Correlation suggested a positive correlation between the CCND1 expression and temozolomide IC50 ($r^2=0.859$, $p=0.036$).

Combined CCND1 knockdown and temozolomide treatment inhibited cell growth significantly more effectively

We further investigate the effect of CCND1 on the cell viability of U251 and SHG-44 cell lines in response to temozolomide treatment using shRNA or overexpression mediated transient transfection. We determined the effect of altering CCND1 expression first. We knocked down or overexpression CCND1 both at the mRNA and protein level in U251 and

Fig. 1. CCND1 expression of patients with astrocytoma. (A) CCND1 mRNA and protein levels were detected by RT-PCR in low- (n = 12) and high- (n = 25) grade astrocytoma surgical samples compared with normal brain tissues (n = 3). (B) Expression of CCND1 was determined by

immunohistochemistry staining in all tissues. (C) CCND1 protein levels were detected by western blotting in low- and high-grade astrocytoma surgical samples compared with normal brain tissues. (D) Kaplan–Meier survival curve of patients with low- and high-expression of CCND1. * $P < 0.05$, ### $P < 0.001$ by unpaired two-tailed Student's t-test.

Fig. 2. The correlation between CCND1 expression in glioblastoma cell lines and TMZ IC₅₀ values. (A and B) CCND1 expression levels in seven glioblastoma cell lines were analyzed by RT-PCR and western blotting. (C) TMZ IC₅₀ values in seven glioblastoma cell lines. (D) Spearman Rank Correlation suggested a positive correlation between the CCND1 expression and TMZ IC₅₀ values.

Fig. 3. Overexpression or knockdown of CCND1 promotes or inhibits cell proliferation in U251 and SHG-44 cells. (A and B) RT-PCR and western blot detection of CCND1 expression levels in U251 and SHG-44 control cells or transfected with shCCND1 or CCND1 or shNC. Cell proliferation was detected by MTT assay in (C) U251 and (D) SHG-44 after different TMZ concentration treatment. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ by unpaired two-tailed Student's t-test.

SHG-44 cell lines (Fig. 3A and B). Furthermore, cell proliferation detected by an MTT assay was found to be reduced in U251 and SHG-44 cells transfected with CCND1 or shCCND1 in different TMZ concentration (Fig. 3C and D).

The combined treatment with temozolomide inhibited cell growth and killed more efficiently than temozolomide (together with shNC or control) alone dose-dependent manner in U251 and SHG-44 cell lines which were analyzed by MTT assay (Fig. 3C and D). Colony formation assays in U251 and SHG-44 cells showed that across all concentrations of Temozolomide treatment, cells transfected with shRNA targeting CCND1 are clearly more sensitive to the killing effect of temozolomide than negative control cells transfected with shNC or control. On the other hand, we found that glioblastoma cells with high CCND1 levels exhibited greater Clonogenic survival across all concentrations of temozolomide treatment (Fig. 4).

Fig. 4. Effects of CCND1 on cell proliferation in U251 and SHG-44 cells detected by colony formation. Cell proliferation was detected by colony formation assay in (A) U251 and (B) SHG-44 after different TMZ concentration treatment. (C) The typical figures were select in 0 and 80 $\mu\text{g/ml}$ TMZ. ** $P < 0.01$, *** $P < 0.001$ by unpaired two-tailed Student's t-test.

Fig. 5. Effects of CCND1 on cell apoptosis in U251 and SHG-44 cells. (A and B) Analysis of apoptosis rates by flow cytometry in U251 and SHG-44 after different TMZ concentration treatment. (C) The typical figures were select in 80 $\mu\text{g/ml}$ TMZ. * $P < 0.05$ and *** $P < 0.001$ by unpaired two-tailed Student's t-test.

Influence of CCND1 inhibition on Temozolomide-triggered apoptosis

To show that the inhibiting effect observed after temozolomide treatment in U251 glioblastoma cells is related to the induction of apoptosis, the apoptotic response was measured as a function of drug concentration. U251 and SHG-44 cells were transfected with shCCND1, CCND1, shNC or control with temozolomide (0, 20, 40, 60 and 80 µg/ml). Flow cytometry analysis of apoptosis rates were measured at after temozolomide treatment. As shown in Fig. 5, our results demonstrated that both shRNA and temozolomide can induce apoptosis, and combined CCND1 inhibition and temozolomide treatment triggered apoptosis significantly more effectively than control.

Effects of CCND1 knockdown or overexpression on cell proliferation in vitro

The effect of knockdown or overexpressed CCND1 on protein level and mRNA expression of the drug-resistant proteins MDR1 (P-gp) and MRP1, the apoptosis-related proteins Bcl-2, Caspase-3, MMP-2, MMP-9, p53 and FasL were determined in U251 and SHG-44 cells (Fig. 6). Western blot analysis indicated that MDR1 (P-gp), MRP1 and Bcl-2 protein levels were reduced in both U251 and SHG-44 cells transfected with shCCND1 whereas levels of Caspase-3 were increased while after CCND1 overexpression had the opposite results. Caspase-3 is required for cell apoptosis and normal brain development. An increase in Caspase-3 activity indicates increased apoptosis when CCND1 is knockdown in the glioma cell lines and Bcl-2 is down-regulated. The number of apoptotic cells in the CCND1 knockdown group was greater than that of the blank and negative control group. These findings revealed that CCND1 knockdown

Fig. 6. Effect of CCND1 on protein and mRNA expression of MDR1 (p-gp), MRP1, MMP-2, MMP-9, Caspase-3, Bcl-2, p53 and FasL in U251 and SHG-44 cells. (A and B) RT-PCR analysis the mRNA levels of MDR1 (p-gp), MRP1, MMP-2, MMP-9, Caspase-3, Bcl-2, p53 and FasL in U251 and SHG-44 cells. (C) Western blot analysis the protein levels of MDR1 (P-gp), MRP1, Bcl-2, CASPASE-3 and CLEAVED-CASPASE-3 in U251 and SHG-44 cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ by unpaired two-tailed Student's t-test.

Fig. 7. Inhibition of p-gp inhibits cell proliferation in U251 and SHG-44 cells. (A and B) RT-PCR and western blot detection of MDR1 expression levels in U251 and SHG-44 cells after Tariquidar treatment. Cell proliferation was detected by MTT assay in (C) U251 and (D) SHG-44 after different MDR1 inhibitor Tariquidar treatment.

may induce apoptosis by activating Caspase-3 and downregulating Bcl-2, MDR1, and MRP1 in U251 and SHG-44 cells. The effect of overexpressing or knockdown CCND1 was confirmed by mRNA analysis. RT-PCR confirmed that mRNA levels of MDR1 (P-gp), MMP-2, MMP-9, p53 and Bcl-2 were significantly increased or reduced in U251 and SHG-44 cells whereas MRP1, Caspase-3 and FasL had the opposite results.

Inhibition of MDR1 (p-gp) prevents cell proliferation in U251 and SHG-44 GBM cells

To substantiate the role of p-gp in CCND1 overexpression or knockdown cells, MDR1 was inhibited in both cell lines using the specific inhibitor Tariquidar. We detected mRNA and protein levels of MDR1 in CCND1 overexpression or knockdown U251 and SHG-44 cells treated with the Tariquidar (Fig. 7A and B). In U251 and SHG-44 CCND1 overexpression cells, inhibition of p-gp caused a significant decrease in the apoptotic response following TMZ treatment whereas Tariquidar had no significant effect in CCND1 knockdown glioblastoma cells (Fig. 7C and D).

CCND1 in vivo tumor formation from U251 cells in nude mice after temozolomide treatment

U251 cells transfected with shCCND1 or empty vector were implanted subcutaneously in the left flank of nude mice. Fig. 8A shows the difference in the volume of tumors after 42 days. shCCND1 significantly reduces the proliferation of cells in tumors (Fig. 8B). Furthermore, the number of apoptotic cells, as determined by a TUNEL assay, was increased in tumor cells overexpressing CCND1 (Fig. 8C). Ki67 is used as a marker of cell proliferation as it is absent in resting cells. The number of proliferating cells detected by a Ki67 immunohistochemistry assay was lower in tissue overexpressing CCND1 (Fig. 8D). The mRNA levels of CCND1, MDR1, MRP1, MMP-2, MMP-9, Caspase-3, BCL-2, p53 and FasL and protein levels of CCND1, MDR1, Caspase-3 and Bcl-2 in tumor tissues from shCCND1 or shNC mice were assessed and were found to be similar to *in vitro* results (Fig. 8E and F). These results indicate that glioma cells knockdown CCND1 can effectively prevent tumor formation *in vivo* after temozolomide treatment.

Fig. 8. CCND1 *in vivo* tumor formation from U251 cells in nude mice after TMZ treatment. Stable shCCND1 transfected and empty vector U251 cells were implanted subcutaneously in the left flank of nude mice. (A) After 42 days, mice were sacrificed and tumors were excised. (B) Effect of CCND1 on tumor volume in a nude mice model. Apoptosis and Ki67 expression were detected by (C) TUNEL and (D) immunohistochemistry assays. (E) RT-PCR analysis the mRNA levels of CCND1, MDR1 (p-gp), MRP1, MMP-2, MMP-9, Caspase-3, Bcl-2, p53 and FasL in U251 and SHG-44 cells. (F) Western blot analysis the protein levels of CCND1, MDR1 (P-gp), MRP1, Bcl-2 CASPASE-3 and CLEAVED-CASPASE-3. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ by unpaired two-tailed Student's t-test.

Discussion

Studies have clearly proved that CCND1 overexpression in some tumors would lead to chemoresistance [13-19]. CCND1 suppression has some apoptosis-inducing effects in pancreatic cancer cells, and it makes tumor cells more susceptible to cisplatin in cisplatin-resistant ovarian and prostate cancer cells [19]. CCND1 expresses more significantly in grade IV astrocytomas than in grades II and III astrocytomas, and overexpression is correlated with dismal survival [9, 10, 12]. Results from our Genechip analysis (unpublished data) and previous study [11] suggest that overexpression of CCND1 might be related to chemoresistance in glioblastoma cells.

To unravel the role of CCND1 in affecting temozolomide sensitivity in glioblastoma cells, we stably constructed cell lines overexpressing CCND1, or expressing a CCND1-specific shRNA that lead to stable CCND1 underexpression in two glioblastoma cell lines in this research. In this study, we explored the expression of CCND1 in two glioblastoma cell lines, and found that CCND1 expression level in these two glioblastoma cell lines was also associated with their temozolomide IC₅₀ values. Further cell proliferation assay and colony survival assay confirmed that combined CCND1 knockdown using shRNA and temozolomide treatment inhibited cell growth and colony formation *in vitro* more significantly than the single treatments. Thus, our results suggested that overexpression of CCND1 in glioblastoma cells induced the resistant to temozolomide treatment.

Patients with malignant glioma, especially for the glioblastoma multiforme, have a very poor prognosis. Treatment with alkylating agents, specifically TMZ and chloroethylating drugs carmustine and nimustine remains to be standard therapy today. TMZ and presumably some other methylating drugs will become more dominant than chloroethylating agents in future due to the less severe side effects [5]. Although several different drug resistant

mechanisms of temozolomide have been identified, few people focus on the relationship between overexpression of CCND1 and resistance to temozolomide in glioblastoma cells.

In the present research, we found that overexpression of CCND1 correlated with temozolomide resistance in glioblastoma cell lines and CCND1 inhibition potentiate glioblastoma cells more susceptible to the cytotoxic effects of temozolomide. Our data suggested that this susceptibility resulted from both the increased apoptotic rate and decreased p-gp expression in CCND1-inhibited glioblastoma cells, which proved the chemoresistance role of CCND1 in glioblastoma cells.

As an ATP-dependent membrane transporter which pumped many cytotoxic drugs out of the cells, the MDR1 gene codes for P-glycoprotein could confer resistance to chemotherapy when overexpressed [6]. Our data demonstrated that increased temozolomide IC50 in CCND1-overexpression cells was related to the upregulated level of p-gp and MDR1 knockdown could decreased temozolomide IC50 in U251-CCND1 cells. Schaich et al. revealed that MDR1 efflux pump targeted the temozolomide and MDR1 involved in the resistance of tumor cells to temozolomide, suggesting that the increased MDR1 observed in CCND1-overexpressing U251 cells might account for the increased temozolomide IC50 value [6]. Further studies were needed to clear this CCND1-dependent mechanism of MDR1 expression.

A great obstacle to the successful treatment of human cancer lies in the intrinsic or acquired resistance to cytotoxic agents, and a variety of mechanisms of drug resistance have been identified in human tumors [16]. For many years, it has been assumed that the interaction between a drug and its molecular target would yield a lethal lesion, and that determinants of intrinsic drug resistance should therefore be explored either at the target level (quantitative changes or/and mutations)[25] or upstream of this interaction. Now it's cleared that independent of the aforementioned factors, downstream cellular responses to a given DNA lesion can determine the outcome of therapy [26]

We also examined the role of MDR1 in CCND1-overexpression associated chemoresistance to explore if this chemoresistance was a direct consequence of upregulation of MDR1 [18]. We found that although MDR1 inhibitor Tariquidar effectively suppressed the expression of MDR1, temozolomide IC50 decreased in MDR1-depleted cells. Based on our observations, CCND1-related chemoresistance was partially conferred by activation of MDR1.

Conclusion

We proved that suppression of CCND1 expression in gliomas cell lines resulted in the increased sensitivity to temozolomide, whereas overexpression of CCND1 protein conferred resistance to the cytotoxic effects of temozolomide. The enhanced chemoresistance of CCND1-overexpression cells might be mechanistically resulted from the dual roles of CCND1 in upregulating MDR1 expression and in inhibiting drug-induced apoptosis. Collectively, these data implicated CCND1 was an important factor in the chemoresistance of glioblastoma cells. Finally, combined CCND1 knockdown using small-interfering RNA and temozolomide treatment inhibited cell growth *in vitro* significantly more effectively than any of these single treatments. Therefore, CCND1 could be potentially used as a marker for treatment stratification and as a molecular target to improve the treatment of temozolomide resistant tumors.

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Disclosure Statement

The authors declare that they have no competing interests.

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