

Knockdown IncRNA CRNDE enhances temozolomide chemosensitivity through autophagy inhibition by activating PI3K/Akt/mTOR pathway in glioblastoma

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Abstract

Background

Long non-coding RNA (lncRNA) CRNDE is highly expressed in glioma acting as an oncogene in gliomas. However, the regulatory roles of CRNDE in Temozolomide (TMZ) chemoresistance to glioblastoma multiforme (GBM) still are poorly understood. Therefore, we intended to explore the role and possible mechanism of CRNDE in TMZ-induced chemoresistance in GBM.

Methods

Firstly, we tested the expression level of CRNDE in glioma cell lines and in 30 cases of normal brain tissues and 58 cases of glioma tissue specimens by qRT-PCR. Meanwhile, the correlation between CRNDE level and the clinicopathological characteristics and survival time of patients were analyzed. Then, the CRNDE expression in various glioma cell lines was detected, and CRNDE knockdown cell models were constructed. Subsequently, to explore the effect of CRNDE on chemosensitivity to TMZ, cell viability was detected by the CCK-8 assay and IC_{50} values, and cell proliferation was detected by cell clone assay and EdU assay, as well as cell survival was detected by apoptosis with flow cytometry under TMZ treatment. Further, the expression of drug-resistance protein ABCG2, autophagic related proteins and PI3K/AKT pathway related proteins were determined by western blot or qRT-PCR in TMZ-treated glioma cells. Finally, the tumor volume and weight were measured and ABCG2 expression was detected by immunohistochemistry assay in mouse tumor xenograft model.

Results

Our integrated approach demonstrated lncRNA CRNDE was a poor prognosis factor for GBM patient, which was up-regulated in patients who are resistant to TMZ, and closely associated with chemotherapeutic response status to TMZ treatment. Further, functional assays revealed that knockdown of CRNDE could notably reduce glioma cell viability and proliferation, and elevate apoptosis to enhance the chemosensitivity to TMZ *in vitro* and *in vivo*. Mechanistically, the depression of CRNDE down-regulated LC3 II/I, Beclin1 and Atg5 expression levels and increased expression of p62 to inhibit autophagy due to the activation of PI3K/Akt/mTOR pathway as well as highly correlated with ABCG2 expression.

Conclusions

Overall, the study provided a preclinical basis in developing lncRNA CRNDE as a reliable clinical predictor of outcome and prognosis of GBM patient, a potential biomarker for predicting TMZ treatment response

and a new therapeutic target for enhancing TMZ sensitivity in GBM by modulating the autophagy through PI3K/Akt/mTOR pathway.

Background

Glioma is one of the most common primary brain tumors with a median survival of 14.6 months and less than 3% of the 5-year survival rate in patients and glioblastoma multiforme (GBM) accounted to about 70% of gliomas in adults is the most malignant and aggressive brain tumor resulted in very poor clinical outcomes and prognosis[1, 2]. The temozolomide (TMZ)-based chemotherapy is regarded as the first-line chemotherapeutic agent for GBM treatment[3, 4], however glioma cells eventually become metastatic and chemoresistance as an obvious block to the TMZ-therapeutic efficacy to GBM[5]. Therefore, elucidating the molecular mechanisms underlying and suppressing chemoresistance to TMZ can provide and develop potential novel therapeutic targets.

Long non-coding RNAs (lncRNAs) are a diverse set of noncoding RNA transcripts with the length of more than 200 nucleotides involved in a major part of critical biological processes and influence on regulation of various of cancer progress including chemoresistance[6, 7]. The lncRNA Colorectal neoplasia differentially expressed (CRNDE) is transcribed from chromosome 16, sharing a bi-directional promoter with the adjacent IRX5 gene[8]. There is an increasing interest in the function of CRNDE in brain tumors, especially in glioma[9–11]. However, the report about the function of CRNDE in chemotherapy resistance was rare. The regulation of chemoresistance of CRNDE was only confirmed in colorectal cancer[12, 13] and liver cancer[14] as so far. Therefore, the function and possible mechanism of chemoresistance to TMZ of CRNDE in glioma, particularly in GBM, have not yet been elucidated. Autophagy has been obviously documented to play a critical role in tumor biological behaviors including chemotherapy resistance, and TMZ-induced autophagy is an important participator in the drug resistance of glioma[15, 16].

In the present study, the clinicopathological features of CRNDE were assessed and the role of CRNDE in glioma cell viability, colony formation, proliferation, apoptosis and autophagy induced by TMZ were performed *in vitro* and *in vivo*. Moreover, the correlation between CRNDE and PI3K/Akt/mTOR pathway was explored to identify the mechanism in regulating TMZ-resistance in glioma, which might provide a new therapeutic target for GBM treatment.

Methods

Clinical specimens and follow-up

All of 58 cases of glioma and corresponding 30 cases of normal brain tissues were obtained from Xiangya Hospital, Central South University under informed consent from May 2015 to February 2016 with the approval of the Ethic Committee of the Xiangya Hospital (approval no. 201907855). Primary tumor and normal brain tissues were frozen in liquid nitrogen and stored until total RNAs were extracted. The

clinical data were obtained from the medical records simultaneously (Table 1). The relative factors of over-survival of patients were analyzed (Table 2). According to the Response Evaluation Criteria in Solid Tumors version 1.0 criteria, the tumor response status to TMZ treatment were evaluated and the patients were assigned with complete/partial response (CR/PR) and stable/progressive disease (SD/PD) in tumor measurements confirmed by repeat studies performed no less than four weeks after the criteria for response was first met.

Table 1
Correlation of the CRNDE expression with clinical and pathological characteristics in tissue samples from glioma patients

Characteristics		CRNDE expression		Total (n = 58)	χ^2	P value
		Low (n = 23)	High (n = 35)			
Age (years)	< 55	12	18	30	0.003	0.584
	\geq 55	11	17	28		
Gender	Male	13	21	34	0.069	0.502
	Female	10	14	24		
Location	Supratentorial	16	26	42	0.155	0.459
	Subtentorial	7	9	16		
Tumor size (mm)	< 50	15	13	28	4.381	0.034*
	\geq 50	8	22	30		
Peritumoral edema range (mm)	< 10	10	6	16	4.819	0.030*
	\geq 10	13	29	42		
Histological grade	Grade I-II	17	5	22	20.960	< 0.001*
	Grade III-IV	6	30	36		
Postoperative recurrence	Yes	8	23	31	5.337	0.020*
	No	15	12	27		

*P < 0.05 was considered to be statically significant

Table 2

Univariate and multivariate Cox proportional hazards regression model analysis of factors related to overall survival for patients

Characteristics		Univariate analysis			Multivariate analysis		
		HR	95% CI	<i>P</i> value	HR	95% CI	<i>P</i> value
Age (years)	< 55 vs. ≥55	0.947	0.628–1.298	0.683	-	-	-
Gender	Male vs. Female	0.752	0.451–1.364	0.464	-	-	-
Location	Supratentorial vs. Subtentorial	1.134	0.967–1.602	0.301	-	-	-
Tumor size (mm)	< 50 vs. ≥50	0.828	0.754–8.976	0.832	-	-	-
Peritumoral edema range (mm)	< 10 vs. ≥10	1.876	0.667–3.546	0.795	-	-	-
Histological grade	I + II vs. III + IV	0.392	0.265–0.769	0.024*	0.492	0.328–0.893	0.040*
Postoperative recurrence	No vs. Yes	0.487	0.291–0.896	0.039*	0.537	0.314–0.915	0.043*
CRNDE expression	High vs. Low	2.468	1.109–4.654	0.002*	1.897	1.132–2.387	0.006*

**P* < 0.05 was considered to be statically significant

Cell culture and transfection

The human glioma cell lines (U87 and U251) were obtained from the Cell Bank of Shanghai Institutes of Biochemistry and Cell Biology, Chinese Academy of Sciences, and normal human astrocyte cells (NHA) were purchased from BNBIO (Beijing, China). All the cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C with 5% CO₂.

Three small interference RNAs (siRNAs) targeting CRNDE (target sequence in Table S1) were synthesized (RiboBio, Guangzhou, China), as well as negative control (si-NC), for knockdown groups *in vitro*. And sh-CRNDE plasmids and non-targeting control (sh-NC) were synthesized (Life technology, MA, USA) *in vivo* (Table S1). The cells were transfected with plasmids or siRNAs by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions, and stable cell lines were established and selected.

Primary glioblastoma cell culture

All the primary glioma samples collected had the informed consent of the patients. The samples were minced by GentleMACS Dissociator (Miltenyi Biotec, Gladbach, Germany) and digested in 0.25% trypsin at 37°C for 30 min. Then digestion was stopped by adding trypsin inhibitor and cells were passed through a 40 µm nylon cell strainer (Corning, 352340) to obtain single-cell suspensions. Next, the cells were cultured in DMEM/F12 containing 10% FBS. The primary glioblastoma cells were tested with GFAP staining.

RNA isolation and qRT-PCR

Total RNA from the cells and tissues was harvested with Trizol reagent (Invitrogen, CA, USA). The SYBR Green RT-PCR Kit (QIAGEN, Germantown, USA) was used to perform the reverse transcription and qRT-PCR reactions according to the instruction. Parameters of thermal cycle were: 95 °C for 10 s, 45 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 10 s, followed by extension at 72 °C for 5 min. Each reaction was repeated in triplicates followed manufacturer protocol. The relative expression levels of the mRNA were calculated by the $2^{-\Delta\Delta C_t}$ method and normalized to the housekeeping gene GAPDH as a control. The primers sequences of CDNDE and GAPDH were supplied in Table S1.

CCK-8 cell viability assay and colony formation assays

According to the manufacturer instructions, 3,000 cells/150 µL of medium were seeded into 96-well plate per well and cultured for 1–3 days. Then 15 µL of CCK-8 (Dojido, Kumamoto, Japan) was added to each well, and cells were further incubated for 3 hours. Absorbance was then measured at 450 nm using a microplate reader. For colony formation assay, the transfected cells were placed in 6-well plates in a triplicate manner in a concentration of 1,000 cells/well. A total of 2 weeks later, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and stained with 0.01% crystal violet dye at room temperature for 15 min. The colonies were captured and counted.

EdU cell proliferation assay

According to the manufacturer instructions, 2,000 cells/150 µL of medium were seeded into the 96-well plate per well and cultured for 4 hours. The medium was replaced by 10 µM EdU DNA Cell Proliferation kit ((RiboBio, Guangzhou, China) and cells were cultured for an additional 12 hours. Thereafter, the cells were fixed with 4% formaldehyde for 30 min. Next, the cells were exposed to 100 µL per well of Apollo staining for 30 min and incubated with 5 µg/mL of DAPI to cell nuclear staining for 15 min. Finally, the cells were counted using a fluorescent microscope (Olympus, Tokyo, Japan).

Flow cytometric analysis

Apoptosis was assessed by annexin-V and propidium iodide (PI) staining as previously described[17]. The annexin V-FITC/PI apoptosis detection kit (BD PharMingen, CA, USA) was performed to label the harvested cells according to the instruction of manufacture. The FACSCalibur (BD Biosciences, CA, USA) flow cytometer was used to quantify the apoptotic and necrotic cells. The cells of early apoptotic (Annexin V-positive and PI-negative) or late apoptotic (Annexin V-positive and PI-positive) phases were included in cell death determinations.

Western blot analysis

Total proteins from the cells were extracted using RIPA buffer with protease inhibitors (Beyotime Institute of Biotechnology, Shanghai, China) and concentrations were estimated. The equal amounts of protein samples were subjected to 10% SDS-PAGE and transferred to PVDF membranes. After blocking with BSA, the membranes were incubated with primary antibodies at 4°C overnight and the appropriate correlated HRP-conjugated secondary antibody at room temperature for 2 hours orderly. The proteins were defined with chemiluminescence detection kit (Aidlab Biotechnology, Beijing, China) and quantified by Image Lab 4.0 software. The relative integrated density values were calculated based on GAPDH as an internal control. The primary antibodies included Phospho-PI3K/PI3K, Phospho-AKT/AKT, Phospho-mTOR/mTOR, LC3I/II, Beclin1, Atg5, p62, ABCG2 and GAPDH (Cell Signaling Technology, MA, USA).

Mouse tumor xenograft model and immunohistochemistry (IHC)

This procedure was carried out as previously described[17]. The nude mice were purchased from Laboratory Animal Center of Xiangya School of Medicine, Central South University. The cells were suspended at a concentration of 1×10^7 /ml in PBS, respectively. Inoculated subcutaneously 200 μ l cancer cell suspension into dorsal flanks subcutaneously of (male, 4-6w) each nude mouse (six in each group). Once tumors were palpable (about 50 mm³), these mice were treated with 5 μ g/g TMZ in 25% DMSO saline solution by intraperitoneal injection (5 days/week \times 4 weeks). The necropsies were performed after 28 days from the first TMZ treatment. Tumor growth was monitored by caliper measurement every 4 days. Tumor volume was calculated as follows: $V = L \times l^2 / 2$, where L and l represent the larger and the smaller tumor diameters, respectively. The animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal experiments were approved by the Animal Experimental Committee of the same school. The euthanasia was conducted by barbiturate of 150 mg/kg with intravenous injection. The tumor tissues were paraffin embedded and stained of ABCG2 (Cell Signaling Technology, MA, USA) according to standard IHC protocols as previously described [17]. The image analysis and total gray value were estimated by GSM-2000P pathology image analysis system (Heima, Zhuhai, China).

Statistical analysis

All results were presented as mean \pm standard deviation (SD) from 3 independent experiments and SPSS 21.0 software (SPSS Inc., IL, USA) was used for statistical analysis. Overall survival was plotted according to the Kaplan-Meier method, and chi-square test or *t* test was used to analyze the relationship of CRNDE with the clinicopathological characteristics of glioma patients. The t-test was performed to appropriate data comparisons between the two groups and ANOVA test was used to compare the difference between multiple groups. $P < 0.05$ was considered statistically significant.

Results

LncRNA CRNDE expression is up-regulated in glioma and correlates to poor prognosis and TMZ resistance

To analyze the expression of CRNDE in glioma, we tested CRNDE mRNA level in 58 glioma specimens compared with 30 normal brain tissues. The CRNDE expression was found to be significantly up-regulated in glioma tissues (Fig. 1a, $P < 0.001$), and the expression level was positively correlated with pathological grading of WHO stage (Fig. 1b, $P < 0.05$). Meanwhile, CRNDE expression was relatively higher in 21 patients with negative response (SD/PD) to TMZ compared with 15 cases with positive response (CR/PR) in high histological grade glioma (Grade III-IV) (Fig. 1c, $P < 0.05$). Further, we analyzed the correlation of CRNDE expression with the clinicopathological characteristics of glioma patients. The expression of CRNDE in glioma patients were measured and recorded to calculate the average expression of CRNDE in glioma patients. According to the average expression level of CRNDE, the patients were classified into low expression group ($n = 23$) and high expression group ($n = 35$) (Table 1). The clinicopathologic analysis revealed that CRNDE expression significantly correlated with tumor size ($P = 0.034$), peritumoral edema range ($P = 0.030$), histological grade ($P < 0.001$) and postoperative recurrence ($P = 0.020$) (Table 1). Patients with high CRNDE expression was more correlated with larger size, worse peritumoral edema, higher histological grade and easier recurrence than those with low CRNDE expression group. Additionally, the factors related to overall survival for patients were analyzed using univariate and multivariate Cox proportional hazards regression model analysis. And the survival analysis confirmed that histological grade (95% CI, 0.328–0.893, $P = 0.040$), postoperative recurrence (95% CI, 0.314–0.915, $P = 0.043$) and CRNDE expression (95% CI, 1.132–2.387, $P = 0.006$) resulting in different survival time (Table 2). Moreover, the prognosis of these 58 glioma patients was followed up for 60 months. The median survival of patients with high CRNDE expression group was 19.2 months while that with low CRNDE expression group was 32.5 months ($t = 2.412, P = 0.012$). Kaplan-Meier survival analysis revealed that glioma patients with higher CRNDE level exhibited shorter overall survival time and worse prognosis than patients with low expression of CRNDE (Fig. 1d, $P < 0.001$). Furthermore, to determinate CRNDE function in glioma much closer and more precise to clinical effect, we isolated and cultured primary GBM cells (referred to as PGC) derived from GBM specimens. These PGCs were identified by immunofluorescence staining with GFAP antibody known as a glioma molecular maker (Figure S1). Then, the expression level of CRNDE was detected in U251, U87 and PGC lines respectively by qTR-PCR, confirming that CRNDE was significantly upregulated in all glioma cell lines compared with that in NHA cells, especially much higher in PGC line (Fig. 1e, $P < 0.05$). These data suggested that lncRNA CRNDE was up-regulated and correlated to poor prognosis and TMZ resistance in glioma.

Knockdown CRNDE enhances chemosensitivity to TMZ, inhibits cell viability, cell proliferation, facilitates apoptosis and reduces ABCG2 expression in glioma cells

To assess the biological role of CRNDE to chemosensitivity in glioma, CRNDE knockdown was first analyzed by transfecting si-CRNDEs or si-NC into three cell lines. Since only the si-CRNDE-2 could notably reduce CRNDE expression in all lines (Figure S2), si-CRNDE-2 was used in the subsequent experiments. The cell viability was monitored by CCK-8 assay and the results showed that compared with the control

group, knockdown of CRNDE dramatically restrained cell viability with relatively lower IC_{50} values exposed to TMZ in a dose-dependent manner for 72 h in all cell lines respectively (Fig. 2a, $P < 0.05$). Meanwhile, based on the IC_{50} value of si-CRNDE group in three cell lines (98.3 μ M, 102.3 μ M and 78.9 μ M, respectively, Fig. 2a), we chosen the TMZ concentration of 100 μ M for subsequent experiments. Then, the effect of CRNDE expression on cell proliferation was assessed by clone formation assays in three cell lines treated with or without the treatment of TMZ at 100 μ M for 72h. The results revealed that the number of colonies was reduced in silence CRNDE group compared with control group without TMZ treatment (Fig. 2b, $P < 0.05$). Furthermore, the number of colonies was much more decreased in si-CRNDE groups than si-NC groups after TMZ exposure in three cell lines (Fig. 2b, $P < 0.05$). Simultaneously, EdU assay was applied to further assess the influence of CRNDE expression on cell proliferation. The percentage of EdU-positive cells was significantly decreased in si-CRNDE groups when compared with that of si-NC groups, which were more evident after TMZ treatment (Fig. 2c, $P < 0.05$). Additionally, to further evaluate the CRNDE expression on cell survival, cell apoptosis analysis was implemented in glioma cells with ditto treatment. The results showed that both the rate of apoptosis was increased in si-CRNDE groups when compared with si-NC groups without or with TMZ treatment, which were more significant with TMZ treatment (Fig. 2d, $P < 0.05$). Besides, considering the multidrug resistance (MDR) that occurs in cancer cells is a major obstacle to efficient chemotherapy for tumors[18], the expression of certain ATP-binding cassette (ABC) transporters which are mainly influenced the multidrug resistance (MDR) in chemotherapy included ABCB1, ABCC1 and ABCG2 were assessed in these cell lines by qRT-PCR with the TMZ at 100 μ M for 72h. We found that only ABCG2 mRNA expression was significantly decreased in CRNDE silenced groups in all three cell lines (Figure S3). Western blot analysis was further examined, showing that silencing of CRNDE significantly downregulated expression of ABCG2 compared with control group in protein level with the TMZ treatment (Fig. 2e, $P < 0.01$). Taken together, these data indicated that knockdown of lncRNA CRNDE could enhance the sensitivity to TMZ by inhibiting cell viability and cell proliferation, elevating apoptosis and decreasing ABCG2 expression in glioma cells.

Knockdown CRNDE suppresses the TMZ-induced autophagy in glioma cells

Previous studies pointed that TMZ could induce autophagy and TMZ-induced autophagy inhibition could improve the efficacy of TMZ therapy in glioblastomas [16, 19] implying a potential role in chemoresistance of glioma. To this end, we explored the effect of CRNDE on the autophagy related pathway in TMZ-treated glioma cells. We initially evaluated the effect of TMZ treatment in inducing autophagy in U87 and PGC cell lines with the treatment of TMZ at 100 μ M for 72h. The common markers of autophagic flux as LC3, Beclin1 and p62 were detected by Western blot analysis. The activation of autophagy was found in the increased transition from LC3 I to LC3 II (rate of LC3 II/I), expression of Beclin 1 and decreased protein levels of p62 (Fig. 3a, $P < 0.05$). Furthermore, to evaluate the CRNDE expression on the influence of TMZ-induced autophagy, si-CRNDE/si-NC groups in U87 and PGC lines with ditto treatment were examined by Western blot analysis. The results showed that with the silence of CRNDE, the transition from LC3 I to LC3 II, protein levels of Beclin 1 and Atg5 were notably diminished with the levels of p62 ascent in autophagic flux in both cell lines respectively, compared with control

groups (Fig. 3b, $P < 0.05$). Collectively, these data suggested that TMZ could activate the autophagy and down-regulation expression of CRNDE might suppress the TMZ-induced autophagy in glioma cells.

CRNDE regulates TMZ-induced autophagy through PI3K/Akt/mTOR pathway in glioma cells

The PI3K/Akt signaling pathway was well known to participate in the regulation of autophagy in anticancer therapy[20]. To elucidate the potential mechanism underlying autophagy exerting its effects on glioma cells, we explored whether CRNDE impacted on the PI3K/Akt pathway then subsequently modulated TMZ-induced autophagy. We firstly monitored both of the key marker expressions in PI3K/Akt/mTOR pathway and autophagic flux on protein level within the CRNDE knockdown in PGC cells with ditto treatment. We found that the depletion of CRNDE significantly up-regulated the level of p-PI3K/PI3K, p-Akt/Akt and p-mTOR/mTOR to activate the signaling pathway and decreased the rate of LC3 II/I, level of Beclin 1 with increased level of p62 to suppress the autophagic flux simultaneously, compared with the control groups in PGC cells (Fig. 4A). To further confirm these results, LY294002, a synthetic compound that was designed as a specific inhibitor for PI3K[21], was applied in si-CRNDE cells (si-CRNDE + LY294002 group). The data verified that the level of p-PI3K/PI3K, p-Akt/Akt and p-mTOR/mTOR were reduced with the increased rate of LC3 II/I and level of Beclin 1 and decreased level of p62 compared with the si-CRNDE group (Fig. 4a, $P < 0.05$) which indicated that LY294002 could restore the activation of PI3K/Akt/mTOR pathway and downregulation of autophagy synchronously. Moreover, CCK-8 assay assessed the cell viability in PGC cells with the treatment of TMZ at 100 μ M for different hours. The results revealed that the cell viability was increased in si-CRNDE + LY294002 group when compared with the si-CRNDE group, suggesting that LY294002 could reverse the cell viability which was suppressed by down-expression of CRNDE (Fig. 4b, $P < 0.05$). Additionally, EdU assay and cell apoptosis analysis were further confirmed that LY294002 could significantly reverse the decreased cell proliferation and increased apoptosis by knockdown of CRNDE respectively in PGC cells with the treatment of TMZ at 100 μ M for 72 h (Fig. 4c-d, $P < 0.05$). Together, all of these above data demonstrated that the altered expression of CRNDE could in some way regulate the TMZ-induced autophagy through the activation of PI3K/Akt/mTOR pathway in glioma cells.

Knockdown CRNDE enhances sensitivity to TMZ and reduces ABCG2 expression in vivo

To further determine the effect of altered CRNDE on the sensitivity to TMZ *in vivo*, PGC cells with knockdown expression of CRNDE by transfecting with sh-CRNDE or appropriate control cells by transfecting with sh-NC, were inoculated into nude mice subcutaneously. After 4 weeks of inoculation, the mice injected with sh-CRNDE PGC cells showed a significantly shrunken tumor volume (Fig. 5a-b, $P < 0.05$) and decreased weight (Fig. 5c, $P < 0.05$) compared to empty control mice with TMZ treatment. More deeply, the IHC analysis of the tumors from the mice demonstrated that down-regulated CRNDE reduced the ABCG2 expression notably in sh-CRNDE groups when compared with the sh-NC groups with TMZ treatment (Fig. 5d-e, $P < 0.05$). Overall, these data *in vivo* further verified that the altered CRNDE expression played a crucial biological role in chemosensitivity to TMZ in glioma and potentially related with the regulation of ABCG2.

Discussion

Glioma is known as the most common and intractable type of intracranial tumor in adults with a badly poor prognosis due to the greatly invasive growth pattern and frequent chemoradiotherapy resistance[22]. As the most malignant histopathological type, GBM is almost invariably fatal with an overall survival of just only approximate one year[2]. TMZ-based chemotherapy, as the first-line chemotherapeutic agent, is the routine and crucial therapy against GBM followed up the regular surgical excision[3, 4]. However, the eventual occurrence of chemoresistance is still the main choke point to influence the GBM treatment efficacy[5]. Therefore, the underlying mechanism of chemoresistance is urgently sought to be investigated.

LncRNAs are a diverse set of noncoding RNA transcripts which have been revealed that playing fundamental roles in the pathological processes related to tumorigenesis, invasion, metastasis and chemoresistance involved in glioma[23–26]. LncRNA CRNDE has been validated to be dysregulated in several cancers and functioned as a promoter in glioma. Overexpression of CRNDE could promote glioma cell growth and invasion[9] and positively correlates with EGFR activation[10]. Meanwhile, through attenuating miR-384/PIWIL4/STAT3 axis, CRNDE promotes malignant progression in glioma cells. Moreover, CRNDE could act as a ceRNA to promote glioma malignancy by preventing miR-136-5p-mediated downregulation of Bcl-2 and Wnt2[11]. Nevertheless, the report about the function of CRNDE in chemotherapy resistance was rare. The regulation chemoresistance of CRNDE was only reported in colorectal cancer[12, 13] and liver cancer[14] as so far.

In this study, we confirmed that CRNDE was up-regulated in human glioma tissues compared with normal brain tissues. Meanwhile, the CRNDE expression was positively correlated with the histopathological stage of glioma. Moreover, the CRNDE expression was significantly higher in glioma tissues showing no response to chemotherapy of TMZ than those showing response in the patients with high grade stage of glioma. Additionally, we separated and cultured primary cell line (PGC) derived from GBM patients applied in experiments, which could demonstrate the pathophysiological alteration of GBM more precisely and the clinical effect more objectively during the study. Then, we tested the CRNDE expression in U251, U87 and PGC cell lines and found the similar results as in glioma tissues. Further, we verified that the glioma patients with high level expression of CRNDE potentially had a relatively high risk of larger size, worse peritumoral edema range, higher histological grade and easier recurrence in comparison with the lower expression level group. Importantly, we found that patients with higher expression of CRNDE received worse outcomes and shorter survival time compared to those who had a lower expression. Collectively, all these data suggested that the expression of CRNDE was as a poor prognostic factor independently for glioma patients and potentially associated with TMZ-based chemoresistance in glioma.

Although previous study had indicated that overexpression of CRNDE could promote glioma cell growth and invasion[9], the function of CRNDE in chemotherapy resistance in glioma was unclear. Therefore, we conducted the CCK-8 assay, colony formation assay, EdU assay, and cell apoptosis analysis to detect the

glioma cell functions affected by CRNDE through altering the expression of CRNDE with or without the TMZ treatment. We found that depression of CRNDE could suppress cell viability and proliferation while promoting cell apoptosis without the exposure of TMZ, while these trends were more significant in response to TMZ treatment *in vitro*. Meanwhile, silence of CRNDE was also confirmed to reduce tumorigenesis with TMZ treatment *in vivo*. Additionally, knockdown of CRNDE dramatically lower IC₅₀ values exposed to TMZ in a dose-dependent manner. Consistent with these results, we considered that knockdown CRNDE could enhance the sensitivity to TMZ treatment in glioma. Similar with our findings, downregulation of CRNDE could suppress drug resistance to Adriamycin in liver cancer cells[14]. While, the CRNDE was demonstrated as a ceRNA to promote oxaliplatin resistance in colorectal cancer[12].

The multidrug resistance (MDR) that occurs in cancer cells is a major obstacle to efficient chemotherapy for tumors[18]. The protein ABCG2 as a main member of ABC transporters, utilizing ATP to efflux endogenous small molecules and exogenous cytotoxic drugs, potentially participated in the drug resistance mechanisms in glioma[18]. ABCG2 could determine the response of GBM to TMZ and inhibiting ABCG2 could suppress cell growth in TMZ-resistant glioma[27, 28]. In our study, ABCG2 was verified to be positively regulated by the altered expression of CRNDE in the functional experiments both *in vitro* and *in vivo* which reminded us that the ABCG2 might be involved in the regulation of CRNDE in the TMZ-based chemoresistance. However, the specific molecular mechanism of the regulation of ABCG2 by the expression of CRNDE was needed to be further explored in the future. At least, these results could demonstrate that CRNDE was potentially related with the TMZ-resistant in GBM cells and the altered CRNDE expression might improve sensitivity to TMZ in glioma.

Autophagy has been known to promote cellular survival during nutrient depletion and is essential for maintaining cellular hemostasis by degrading damaged organelles and proteins[29]. Previous studies pointed that TMZ could induce autophagy and TMZ-induced autophagy inhibition could improve the efficacy of TMZ therapy in glioblastomas[16, 19] implying a potential role in drug resistance of glioma. However, the role of autophagy in TMZ cytotoxicity is not consistent[30]. A previous study found promoted autophagy could enhance the cell to TMZ chemosensitivity in glioblastoma[31, 32]. Inconsequently, the protective roles of autophagy in glioma cell during TMZ-based chemotherapy have been confirmed[30, 33]. Therefore, our further mechanism analysis firstly identified that the autophagy could be evoked by exposure to TMZ with the increased the conversion from LC3 I to II, Beclin 1 and suppressed accumulation of p62. Meanwhile, autophagy-relevant in drug-resistance has been featured in chemotherapy recently in literature[33]. In consequence, the influence of the CRNDE expression on autophagic flux related pathway in TMZ-treated glioma cells was detected and the results showed that depression of CRNDE could impair the TMZ-induced autophagy in GBM cells through the reduced the rate of LC3 II/I, protein levels of Beclin 1, Atg5 and raised the level of p62. Considered with our findings in the current study, treatment with TMZ increased autophagy in GBM cells and the results substantiated that down-expression of CRNDE antagonized the activation of TMZ-induced autophagy in GBM cells.

The PI3K/Akt/mTOR signaling pathway is well known to participate in the regulation of proliferation, migration, invasion, mitochondrial dysfunction and inducing autophagy in glioma[20, 34, 35] and

anticancer therapy related to autophagy in cancers[36]. We next deciphered the potential molecular mechanism underlying CRNDE mediated depletion in TMZ-induced autophagy. And our findings revealed that depression of CRNDE contributed to the induced autophagy in glioma cells through the up-regulation of PI3K/Akt/mTOR pathway with the suppression of cell viability, proliferation and apoptosis under the treatment of TMZ. Moreover, we found that induced autophagy and cellular effects could be restored by the PI3K inhibitor of LY294002. The recent researches demonstrated that CRNDE promoted cell proliferation through PI3K/Akt signaling in hepatocellular carcinoma and non-small cell lung carcinoma[37, 38]. Here, we blocked the PI3K by LY294002 resulted in not only PI3K/Akt/mTOR pathway inactivation but also the reactivation of autophagy, indicating that CRNDE might enhance TMZ-induced autophagy activation in a PI3K-dependent manner. More intriguingly, suppressing PI3K also reversed the increased chemosensitivity by the depression of CRNDE to TMZ *in vitro*. These findings indicated that CRNDE could regulate the activation of PI3K/Akt/mTOR signaling to reduce the autophagy to enhance the sensitivity to TMZ in GBM cells, while the further detailed mechanisms remain to be further illustrated.

Conclusions

In the study, our integrated approach firstly demonstrated lncRNA CRNDE was a poor prognosis factor for GBM patient, which was up-regulated in patients who are resistant to TMZ, and closely associated with chemotherapeutic response status to TMZ treatment. Meanwhile, we identified the mechanism underlying CRNDE regulated TMZ chemoresistance in GBM *in vitro* and *in vivo*. The depression of CRNDE enhanced the sensitivity to TMZ in GBM cells by reducing autophagy and highly correlated with drug-resistance protein ABCG2. The reduction of autophagy was due to the activation of PI3K/Akt/mTOR signaling pathway, even though more detailed mechanisms for CRNDE in glioma need to be revealed. Our findings provided a preclinical basis in developing CRNDE as a reliable clinical predictor of outcome and prognosis of GBM patients, a potential biomarker for predicting TMZ treatment response and a new therapeutic target for enhancing TMZ sensitivity in GBM.

Abbreviations

GBM: glioblastoma multiforme; TMZ: temozolomide; lncRNA: long non-coding RNA; CRNDE: Colorectal neoplasia differentially expressed; CR/PR: complete/partial response; SD/PD: stable/progressive disease; PGC: primary GBM cells; ABC: ATP-binding cassette; MDR: multidrug resistance.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. The studies involving human participants were reviewed and approved by Ethics Committee of Xiangya Hospital, Central South University (approval no. 201907855). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of Xiangya

School of Medicine, Central South University. The animal experiments were strictly implemented according to institutional guidelines and were performed in a humanistic way.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZZJ and LQ conceived the ideas and designed the experiments. ZZJ and LMM carried out the samples collection and performed the experiments. LWY, YJ and LHY analyzed the data. ZC and TGD and provided critical materials. ZZJ and LMM wrote the manuscript. JWX, YXR, WMH and LQ supervised the study. All authors reviewed submitted version and approved the final version of the manuscript.

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Figures

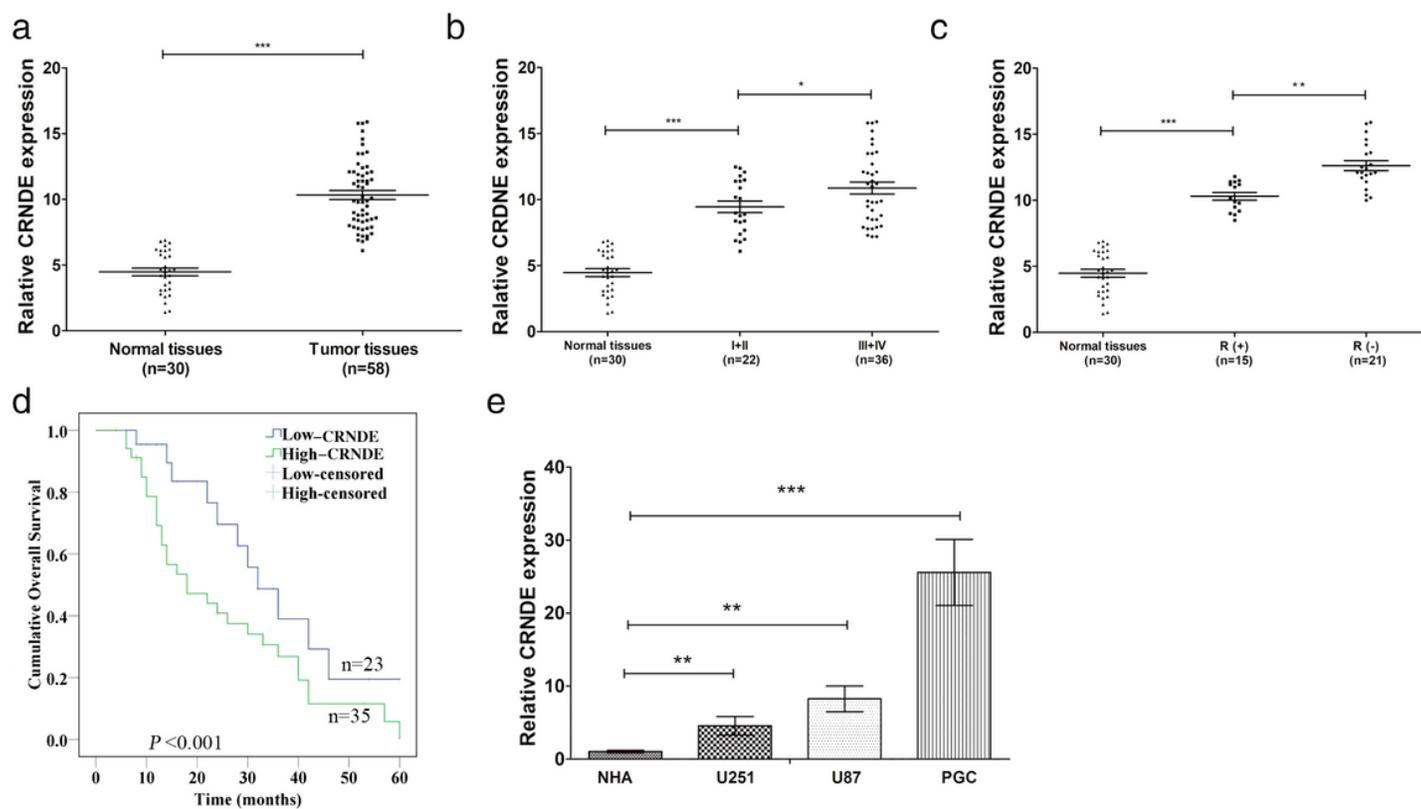


Figure 1

CRNDE expression was upregulated in glioma and correlated to poor prognosis and TMZ resistance of patients with glioma. a CRNDE expression in normal brain tissues and glioma tissues was determined by

qRT-PCR. ***P < 0.001. b CRNDE expression was measured in normal brain tissues and glioma tissues at different pathological stage by qRT-PCR. *P < 0.05, ***P < 0.001. c CRNDE expression was detected in glioma tissues showing response (R+) or no response (R-) to TMZ in high-grade stage compared to normal tissues. **P < 0.01, ***P < 0.001. d Kaplan-Meier plot of overall survival of patients was stratified by CRNDE expression. e CRNDE expression was determined in normal cell line HNA, glioma cell lines (U251 and U87) and PGC line by qRT-PCR. **P < 0.01, ***P < 0.001. Data are presented as mean ± SD from three independent experiments.

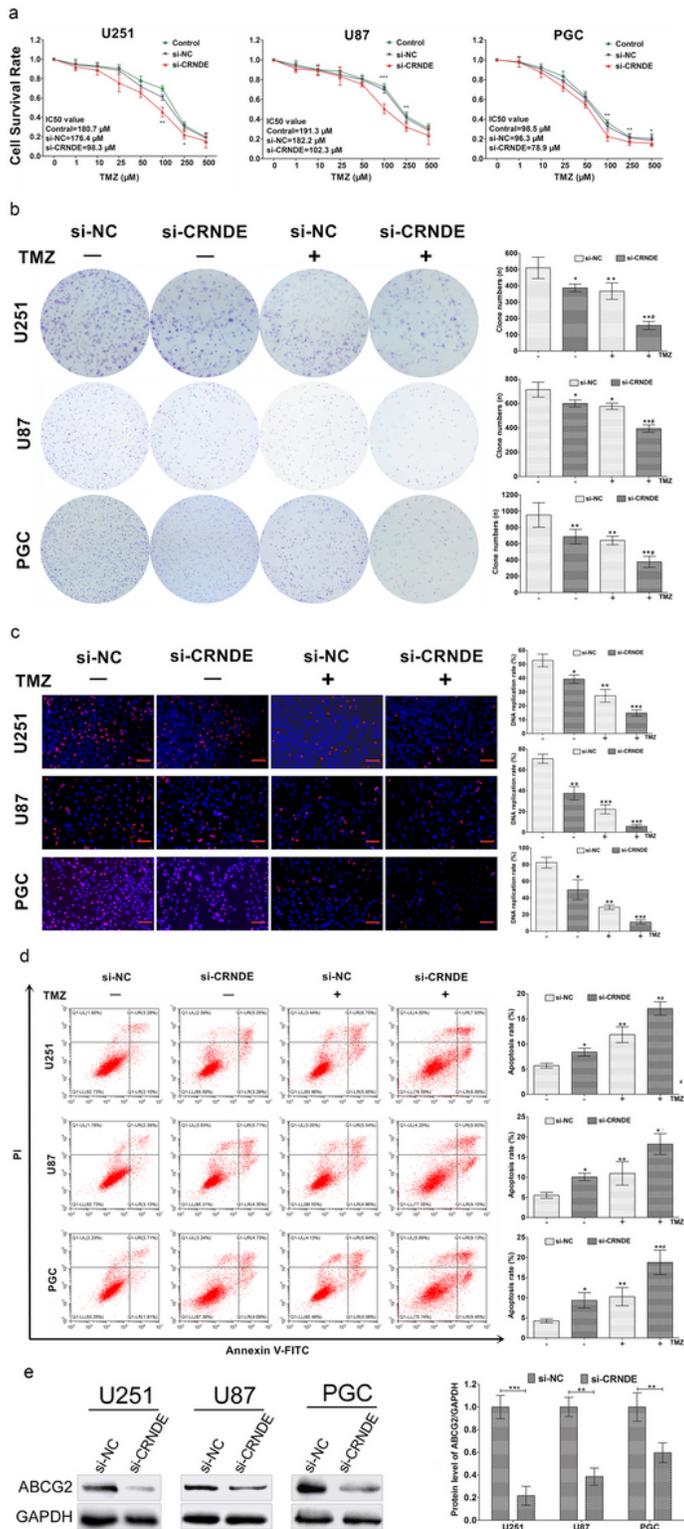


Figure 2

Knockdown CRNDE enhanced chemosensitivity to TMZ and reduced ABCG2 expression in vitro. a CCK-8 assays revealed cell viability with the exposure of TMZ depending on different doses for 72 h and IC50 values after si-NC or si-CRNDE transfection in three cell lines, respectively. *P < 0.05, **P < 0.01 and ***P < 0.001 represent si-CRNDE groups compared with si-NC groups, respectively. Colony formation assays (b) and EdU assays (c) showed cell proliferation ability of si-NC or si-CRNDE cells with or without exposure of TMZ at 100 μ M for 72 h in three cell lines, respectively. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with si-NC cells without TMZ treatment, *#P < 0.05, **#P < 0.01 compared with si-NC group cells with TMZ treatment. d Cell apoptosis analysis revealed cell apoptosis ability of si-NC or si-CRNDE cells with or without exposure of TMZ at 100 μ M for 72 h in three cell lines, respectively. *P < 0.05, **P < 0.01 compared with si-NC cells without TMZ treatment, *#P < 0.05, **#P < 0.01 compared with si-NC group cells with TMZ treatment. Scale bars = 100 μ M. e Western blot detected the ABCG2 protein expression after si-NC or si-CRNDE transfection in three cell lines treated with TMZ at 100 μ M for 72 h, respectively. Data represent mean \pm SD from 3 independent experiments.

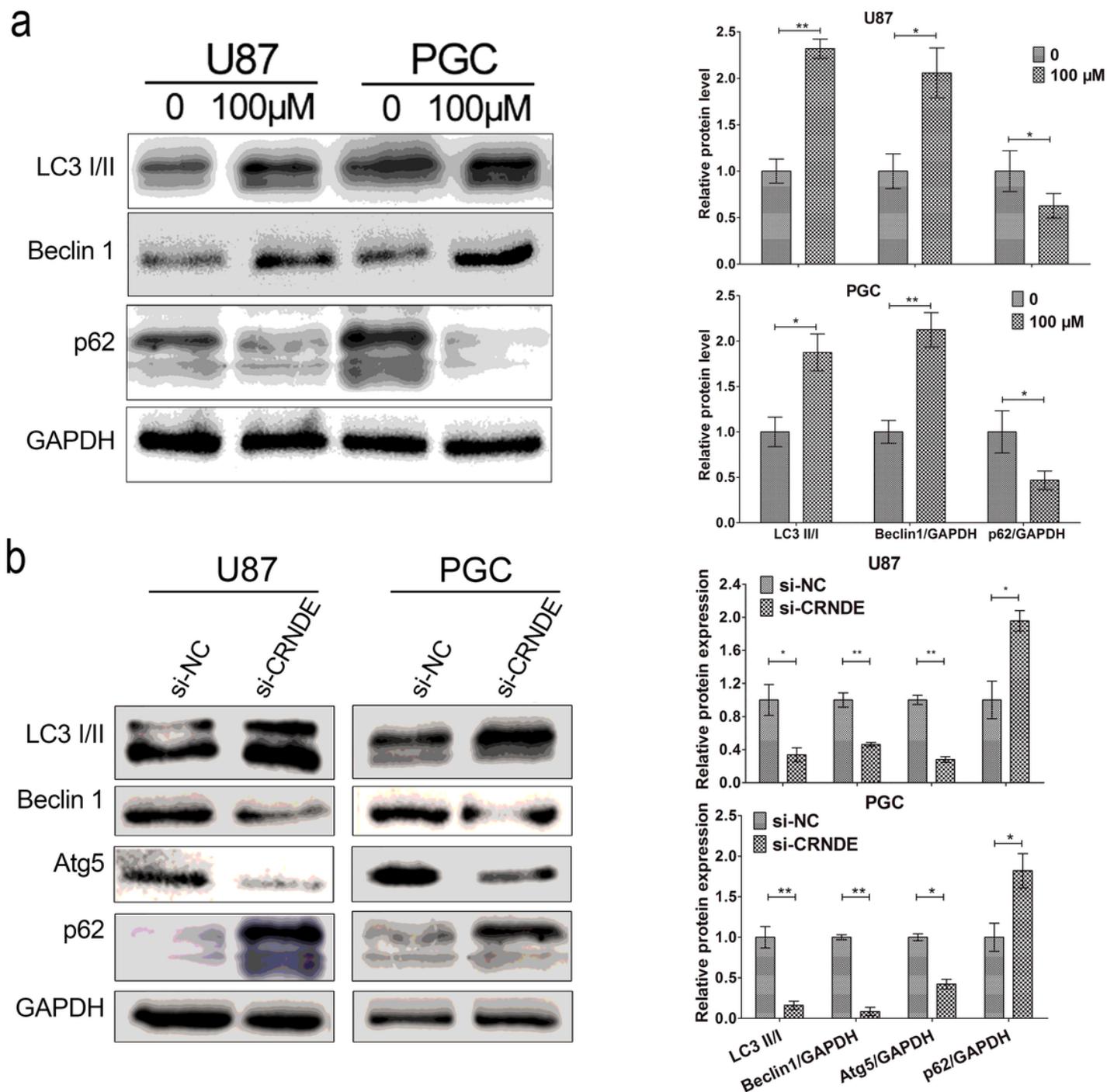


Figure 3

Knockdown CRNDE suppressed the TMZ-induced autophagy in glioma cells. a Western blot revealed the rate of protein LC3 II/I, protein levels of Beclin 1 and p62 in autophagic flux after treated with TMZ at 100 μ M for 72 h in U87 and PGC lines. * $P < 0.05$, ** $P < 0.01$. b Western blot showed the rate of protein LC3 II/I, protein levels of Beclin 1, Atg5 and p62 after CRNDE knockdown with the same treatment in U87 and PGC lines. * $P < 0.05$, ** $P < 0.01$. Data are presented as mean \pm SD from three independent experiments.

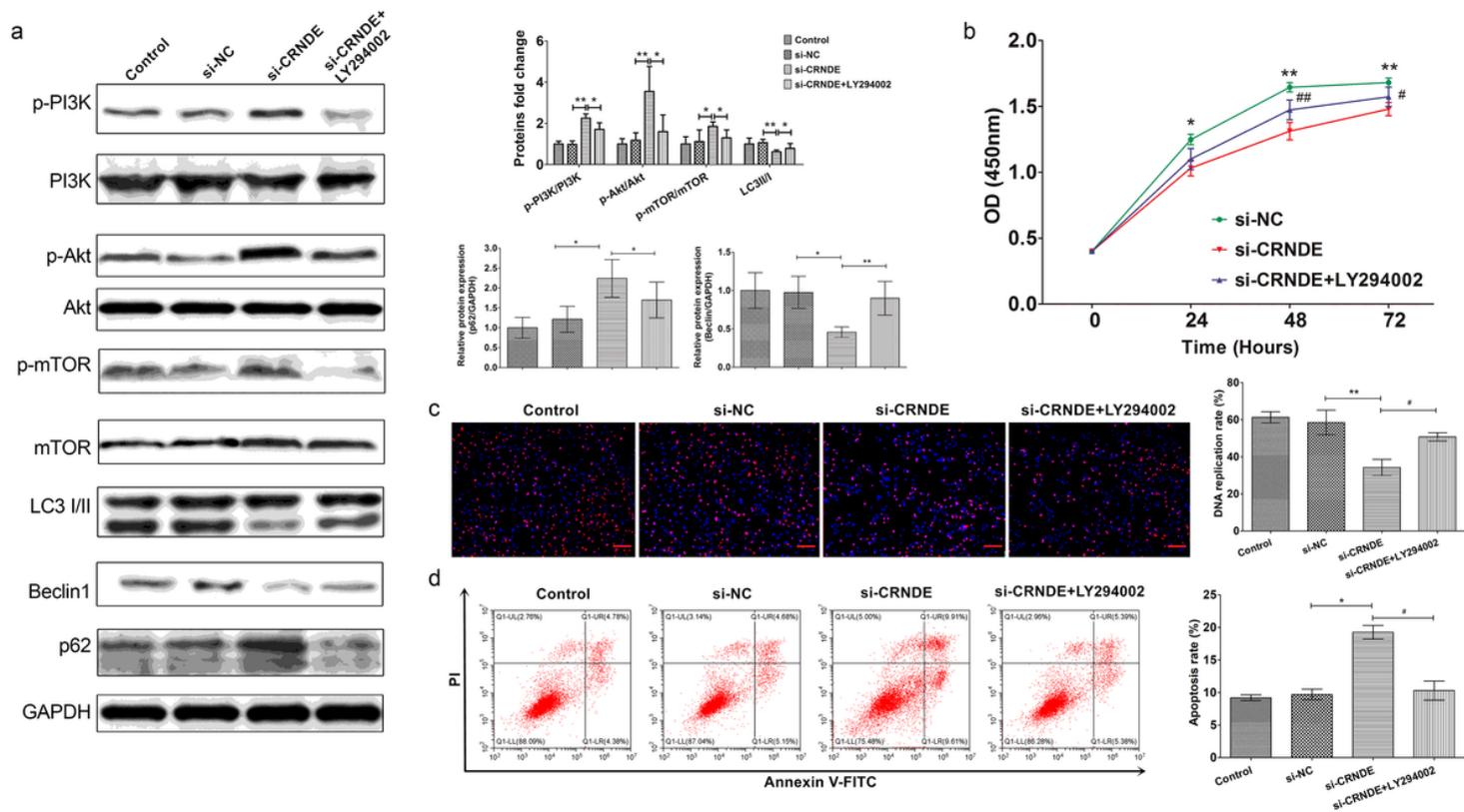


Figure 4

CRNDE regulated TMZ-induced autophagy through PI3K/Akt/mTOR pathway in glioma cells. a Western blot revealed the protein levels of p-PI3K/PI3K, p-Akt/Akt and p-mTOR/ mTOR in PI3K/Akt/mTOR pathway and LC3 II/I, Beclin 1 and p62 in autophagy after CRNDE knockdown and addition with LY294002 with exposure of TMZ at 100 μ M for 72 h in PGC line. * $P < 0.05$, ** $P < 0.01$. b CCK-8 assays the cell viability after CRNDE knockdown and addition with LY294002 with exposure of TMZ at 100 μ M for different hours in PGC line. * $P < 0.05$, ** $P < 0.01$ represent si-CRNDE groups were compared with si-NC groups, respectively. # $P < 0.05$, ## $P < 0.01$ represent si-CRNDE+LY294002 groups were compared with si-CRNDE groups, respectively. c EdU assays detected the cell proliferation after CRNDE knockdown and addition with LY294002 with exposure of TMZ at 100 μ M for 72 h in PGC line. ** $P < 0.01$ represent si-CRNDE group was compared with si-NC group. # $P < 0.05$ represent si-CRNDE+LY294002 groups was compared with si-CRNDE group. Scale bars = 100 μ M. d Cell apoptosis analysis revealed cell apoptosis ability after CRNDE knockdown and addition with LY294002 with exposure of TMZ at 100 μ M for 72 h in PGC line. * $P < 0.05$ represent si-CRNDE group was compared with si-NC group. # $P < 0.05$ represent si-CRNDE+LY294002 groups was compared with si-CRNDE group. Data represent mean \pm SD from 3 independent experiments.

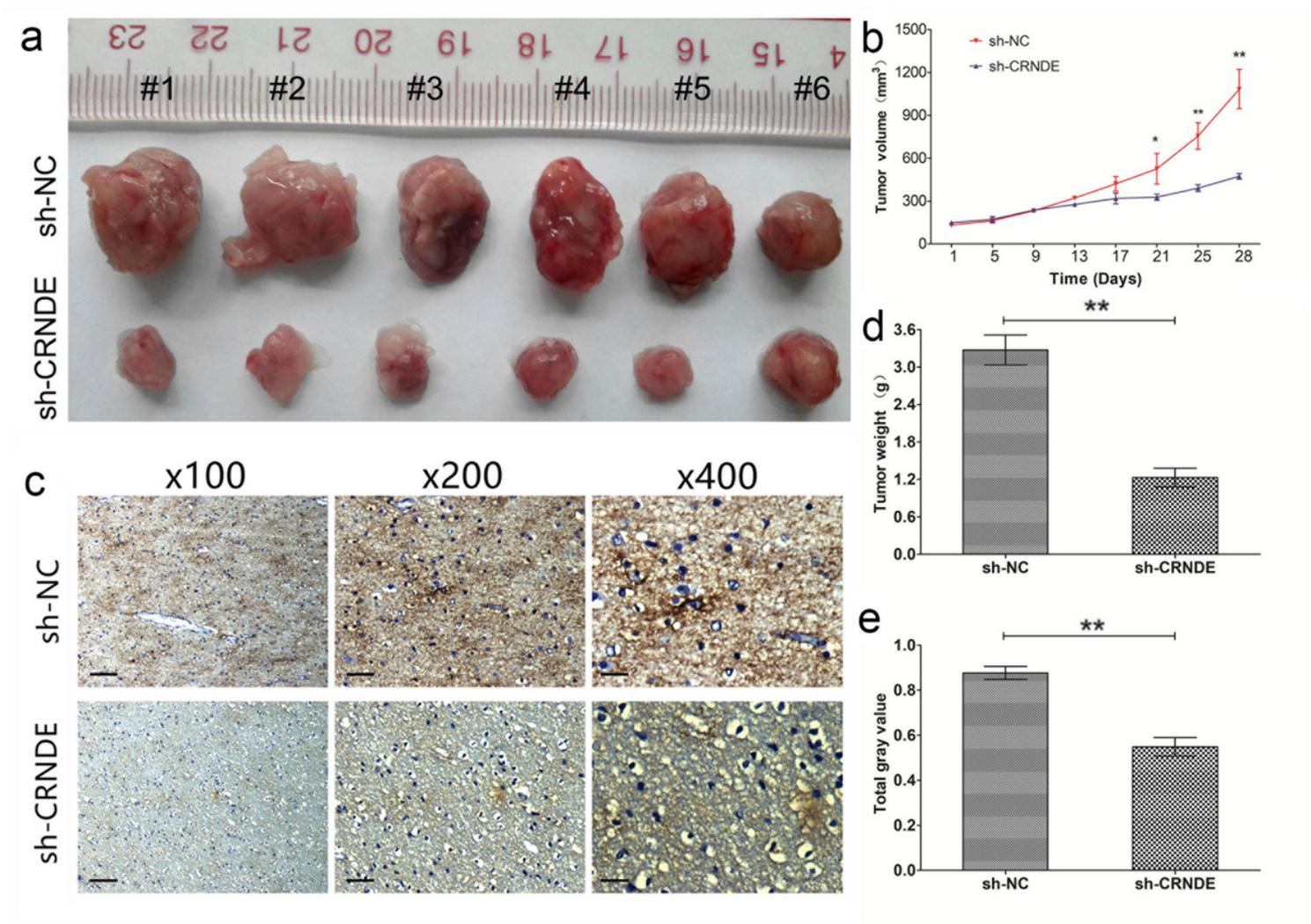


Figure 5

Knockdown CRNDE enhanced sensitivity to TMZ and reduced ABCG2 expression in vivo. a Representative images of xenograft tumors produced by CRNDE silenced or control cells in nude mice with TMZ treatment. b Tumor growth volume curve of the CRNDE knockdown subcutaneous PGC xenografts. *P < 0.05, **P < 0.01. c Tumor growth weights of the CRNDE knockdown subcutaneous PGC xenografts. **P < 0.01. d Representative IHC images of ABCG2 expression in subcutaneous xenograft tumor tissue. Original magnification X100 (Scale bars = 100 μ M), X200 (Scale bars = 50 μ M) and X400 (Scale bars = 25 μ M). e The analysis of total gray value. **P < 0.01. Data are presented as mean \pm SD from three independent experiments. **P < 0.01

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