European Review for Medical and Pharmacological Sciences 2019; 23: 2092-2102

CtIP contributes to non-homologous end joining formation through interacting with ligase IV and promotion of TMZ resistance in glioma cells

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Abstract. – OBJECTIVE: C-terminal-binding protein interacting protein (CtIP) participates in a variety of DNA metabolisms and DNA double strand break repair (DSBR). The role of CtIP has been proven in facilitating end resection in homologous recombination (HR). This study aimed to investigate the role of CtIP in non-homologous end joining (NHEJ) formation.

MATERIALS AND METHODS: In this study CtIP deficient U87 cell line was generated by using CRISPR/Cas9 method. HR and NHEJ reporter assay were conducted in U87 cells. The cell viability of U87 cells was evaluated by using Sulforhodamine B (SRB) assay. Ionizing radiation assay and clonogenic survival assay were also conducted in this study. Bacteria expressed CtIP and ligase IV proteins were collected and purified. Affinity capture assay was conducted to observe the interactions between proteins.

RESULTS: Both of the temozolomide (TMZ)-resistant and CtIP deficient glioma cell lines were successfully generated. The results indicated that CtIP participated in NHEJ formation through interacting with ligase IV in glioma cells. CtIP significantly improved the NHEJ efficiency in glioma cells. The CtIP deficient glioma cells were sensitive to the treatment of DNA damaging drug (TMZ). Meanwhile, the CtIP deficiency significantly enhanced the sensitivity of glioma cells to the treatment of TMZ.

CONCLUSIONS: The present study indicated that CtIP contributed to NHEJ formation through interacting with IV and promotion of TMZ resistance in glioma cells via promoting DSBR efficiency.

Key Words:
C-terminal-binding protein interacting protein, DNA double strand break repair, Non-homologous end joining, Glioma cells.

Introduction

DNA double strand break (DSB) is the most toxic DNA lesion, which is inevitable because it arises from normal DNA metabolism as well as irradiation, ultraviolet light (UV) and chemical agents[1,2]. DSB repair (DSBR) is critical to maintain the genome integrity; however, the failed or inaccurate DSBR always generates the mutations, translocations and causes the cell deaths[3-5]. There are two major DSBR pathways in mammalian cells, including homologous recombination (HR) and non-homologous end joining (NHEJ). HR, as a template-dependent DSBR pathway, is usually activated in the S and G2 phase and could repair DSB accurately[6-8]. The essential step for HR mechanism is a DNA end resection process generating 3'-overhang, which invades and locates the sequence in the sister chromatid[9-11]. NHEJ is free from the limitation of sister chromatid as the template. Therefore, the flexibility of NHEJ allows cells to incorporate this mechanism throughout the cell cycle, especially during the G0, G1 and early-S phase[12,13]. The essential proteins required to repair DSBs for the classical

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NHEJ mainly include Ku70/80 heterodimer (Ku), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), X-ray repair cross-complementing 4 (XRCC4)-ligase IV complex, and XRCC4-like factor protein (XLF, also known as Cernunnos)[14-19]. NHEJ is template-independent, which is also considered to be an error-prone pathway exhibiting the potential to generate insertions and deletions[20,21]. However, instead of repairing DSBs by end-to-end joining, NHEJ requires DNA end processing to expose ligatable DNA ends[22-27]. In addition to the different stages of cell cycle, DNA ends structure also determines the different DSBR pathways between HR and NHEJ[5]. NHEJ is more efficient to blunt DNA ends whereas HR requires 3'-overhangs. Therefore, DNA end processing factors play important roles in pathway choice. C-terminal-binding protein interacting protein (CtIP) is a 5' to 3' endo-nuclease, the role of which in generating the end resection is conserved in budding yeast, plants, chicken DT-40 cells and mammalian cells[28-31]. Unlike the other typical DNA nucleases and metabolizing enzymes, CtIP homologs are more unstructured and have the similarity to the carboxyl terminus. Therefore, the CtIP could promote the dimerization and oligomerization, both of which are required for their recruitment to repair DSBs[32-34]. CtIP is phosphorylated by the checkpoint protein kinase ataxia telangiectasia mutated (ATM) when the DSBs occur[35]. The role of CtIP in HR, regulating the 5' to 3' end resection of Mre11/Rad50/Nbs1 (MRN) complex, has been proven for two decades[36]. CtIP also participates in microhomology mediated end joining (MMEJ) to facilitate the formation of limited resections and then to generate short and complementary sequences between the DSBs[37]. However, the role of CtIP in classical NHEJ remains unclear. Our group observed that CtIP can interact with ligase IV and CtIP deficiency impairs the NHEJ formation in the human glioma cells.

Glioma is detected in 6.6 per 100,000 individuals in the USA and has become the most common primary tumor in central nervous system[38]. About 50% patients present with WHO grade IV disease (glioblastoma), which is the most common malignant primary brain tumor in adults with 12 to 16 month survival duration post the diagnosis[39]. In clinical, glioblastoma patients are usually treated with surgery, irradiation and temozolomide (TMZ). Unfortunately, TMZ resistance has been detected in recurrent tumors and the alternative chemotherapeutic drugs are absent, which bring an urgent need for novel regimen for glioblastoma. Since TMZ is a DNA alkylating agent and its mechanism of action relies on DSBs to generate cell death. DNA repair in cancer cells might be associated with the mechanism of TMZ resistance. The present research showed that depletion of CtIP contributed to both HR and NHEJ and sensitized the human glioma cells to TMZ treatment.

Materials and Methods

Cell Lines and Reagents

U87 cells (Cat. No. HTB-14, American Type Culture Collection, Manassas, VA, USA) and CtIP deficient derivatives were cultured at 37°C in 5% CO_2 atmosphere in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL. Co. Ltd., Grand Island, NJ, USA) supplementing with 10% fetal bovine serum (FBS, Gibco BRL. Co. Ltd) for less than 6 months. TMZ-resistant U87 cells were generated by incubating with 10 μM to 150 μM TMZ for 3 month. pICE-HA-CtIP (Cat. No. #82030, Addgene, Cambridge, MA, USA) was transfected into the glioma cells by using Lipofectamine 3000 (Cat. No. #3000008, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. Small interfere XLF (siXLF) and siXRCC1 (Sigma-Aldrich, St. Louis, MO, USA) were transfected into U87 cells by using Lipofectamine RNAiMAX (Cat. No. #13778075, Thermo Fisher Scientific, Waltham, MA, USA). Glutathione S-transferase (GST)-tagged human CtIP was cloned from pICE-HA-CtIP into the pET-GST vector (Cat. No. #42049, Addgene, Cambridge, MA, USA). The present study has been approved by the Ethics Committee of Jilin University (Changchun, China).

Western Blot Analysis

The protein samples were re-suspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime Biotech. Shanghai, China) sample buffer, and boiled for 5 min at 95°C. The samples of ligase IV and CtIP proteins were loaded and separated on an 8% polyacrylamide gel (29:1, Beyotime Biotech. Shanghai, China) at 120 V for 1.5 h by using an electrophoresis apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The separated samples were transferred onto the nitrocellulose membrane at 100 V and 4°C in cold room for 1 h. Membranes were then blocked by using 3% bovine serum albumin

(BSA) diluted in phosphate-buffered saline (PBS) with 0.1% Tween-20. Next, the membranes were incubated by using the relevant antibodies and the horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Finally, the protein signals were developed by using SuperSignal™ West FemtoMaximum Sensitivity Substrate (Cat. No. #34096, Thermo Fisher Scientific, Waltham, MA, USA) and detected with ChemiDoc™ (Bio-Rad Laboratories, Hercules, CA, USA).

Generation of CtIP Deficient U87 Cell Line by Using CRISPR/Cas9

Cas9 along with CtIP guide RNA plasmid was constructed by ligating oligonucleotide duplexes, which targeted exon1 of CtIP, into BbsI cut pX330-U6-Chimeric_BB-CBh-hSpCas9 (Cat. No. #42230, Addgene, Cambridge, MA, USA). The plasmid was transfected into U87 cell line along with pcDNA3.1.puro (Invitrogen/Life Technologies, Carlsbad, CA, USA) by using lipofectamine 2000 (Invitrogen/Life Technologies, Carlsbad, CA, USA). Successfully transfected cells were selected by media containing 100 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA) for 48 h. Cells were harvested and seeded onto 96-well plates (Corning, Corning, NY, USA) at density of 500 cells/ml and incubated for 2-3 weeks. Individual clones were passaged, expanded and screened for the CtIP expression.

HR and NHEJ Reporter Assay

Both HR and NHEJ reporters have been reported according to the previously published study[40]. Briefly, 10 µg of NHEJ or HR reporter plasmid was linearized with 50 U of NheI (TaKaRa Biotech., Dalian, China) in 50 µl for 6 h in 37°C water bath. The linearized DNAs were purified by using Qiagen gel extraction kit (Qiagen, Hilden, Germany) and 0.5 µg was transfected into U87 cells by using Lipofectamine 3000 according to the manufacturer's instruction. Cells with chromosomally integrated reporter constructs were selected by incubating in media with 1 mg/ml geneticin (Invitrogen/Life Technologies, Carlsbad, CA, USA) at 24 h post the transfection and then cultured for 2 weeks. Plasmid integrated cells were seeded at density of 3×10^5 cells/ml in a 6-well plate and cultured for 24 h to allow adhere. A total of 2 µg/well of I-SceI was transfected into cells with Lipofectamine 3000 and incubated for 48 h. Cells were harvested by using trypsin (Beyotime Biotech. Shanghai, China) and re-suspended in PBS by pipetting 10 times. GFP positive cells were counted by using flow cytometry (Beckman Coulter Inc., Brea, CA, USA).

Cell Viability Assay

Cells were seeded at the density of 4×10^3 cells/well and cultured overnight. The wide-type or ectopic CtIP expressed U87 cells were incubated with drug for 72 h. Then, the cell viabilities were detected by using Sulforhodamine B (SRB) assay. Briefly, the cells were fixed with 10% trichloroacetic acid at density of 100 µl/well at 4°C for 1 h. Plates were washed 4 times with slow running tap water and air dried for 1 h at room temperature (RT) or 20 min in fume hood. Cells were stained by using 0.02% SRB in 1% acetate acid at density of 100 µl/well for 1 h at room temperature (RT). Plates were washed for 3 times with 1% acetate acid at density of 200 µl/well and airdried. A total of 200 µl/well of 10 mM tris-HCl (pH 10.5) was added into each well to extract SRB with 1 h shaking on an orbital shaker. The absorbance was measured at 510 nm by using micro-plate reader (Bio-Tek Inc., Winooski, VT, USA).

Ionizing Radiation and Clonogenic Survival Assay

The wide-type or ectopic CtIP expressed U87 cells were re-suspended in 15 ml cell culture and exposed to ionizing radiation by using Gammator 50 ^{137}Cs source irradiator. Exposed cells were seeded at density of 500 cells/well in 6-well plates and incubated for 2 weeks or till colonies were visible. Colonies were stained with crystal violet solution for 20 min at room temperature. Plates were washed with slow running tap water till colonies were visible and the backgrounds were clean. Colonies were counted by using Quantity One 1-D analysis software (Bio-Rad Laboratories, Hercules, CA, USA).

Protein Expression and Purification

Bacteria expressed CtIP and ligase IV proteins were collected and purified by using the following methods. BL21 (DE3) *E. coli* cells (NEB) (Cat. No. C25271, New England Biolabs Ltd., Hitchin, English, UK) were transformed by heat shot method at 42°C with the plasmid, recovered at 37°C and expanded in Luria Broth (LB, Gibco BRL. Co. Ltd., Grand Island, NJ, USA) at 37°C, until the culture reached OD-600 of 1.0. A total of 1 mM isopropyl b-D-thiogalactopyranoside (IPTG) was added to induce protein expression at 18°C for 16 h. Induced E. coli was

harvested by centrifuging at 6000 × g and 4°C for 30 min. The medium was removed and the cell pellet was re-suspended in lysis buffer (1 × protease inhibitor cocktail, 10 mM eathylene diamine tetraacetic acid (EDTA), 20 mM Tris pH 7.9, 600 mM NaCl, 2 mM dithiothreitol (DTT), 2 mg/ml lysozyme, all were purchased from Beyotime Biotechnology, Shanghai, China). Lysates were then sonicated by using Branson Sonifier for 10 times (10 s per time) to shear DNA. The clarified supernatants were collected by centrifuging for 15 min at 20000 × g and 4°C. Tagged proteins were bound to 0.5 ml of Glutathione beads (GST-tagged protein) or Ni-NTA (Qiagen, Hilden, Germany) (his-tagged protein) in 50 ml tube by mixing at 4°C for 2 h. The beads were packed onto a column and washed with wash buffer (1× protease inhibitor cocktail, 10 mM elhylene diamine tetraacetic acid (EDTA), 250 mM NaCl, and 0.5 mM dithiothreitol (DTT, Beyotime Biotechnology Shanghai, China)). The his-tagged protein was eluted from the beads by using elution buffer (5 mM imidazole in wash buffer with 5% glycerol (Beyotime Biotechnology, Shanghai, China)). The GST-tagged proteins were eluted from the beads by using elution buffer (10 mM glutathione in wash buffer with 5% glycerol). Proteins were concentrated by using Pierce™ Protein Concentrator PES, 30K MWCO (Cat. No. #88521, Thermo Fisher Scientific, Hudson, NH, USA) with dialyzing buffer (50 mM Tris pH 7.9, 50 mM NaCl, 1 mM EDTA, 2 mM DTT, and 10% glycerol) to lower the salt concentration. The purified proteins were frozen by using liquid nitrogen and stored at -80°C. Protein concentrations were determined by using Bradford assay.

Affinity Capture Assay

A total of 50 µg GST-CtIP adhered to the 50 µl of glutathione beads (50% slurry) in 400 µl GST buffer (25 mM Tris pH 7.9, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) at 4°C with gentle mix for 1 h. The resin was collected by centrifuging at 1000 ×g and 4°C for 1 min. Then, the resin was washed with 0.5 ml binding buffer for 3 times. The resin was dried by centrifuging at 1000 × g for 1 min at 4°C, and incubated with 50 µg of his-tagged protein in a final volume of 500 µl at 4°C with gentle mix for 2 h. The resin was washed with binding buffer for 3 times and the supernatant was removed by centrifuging at 1000 × g for 1 min. Bound protein was eluted from the resin by incubating the resin with 50 µl of wash buffer containing 10 mM glutathione for 15 min at 4°C. The mix was centrifuged at 1000 × g for 1 min and the supernatant was collected. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added to the sample and boiled for 5 min. Then, the samples were loaded and separated on a SDS-PAGE gel and transferred onto the nitrocellulose membrane. Finally, the his-tagged protein was observed by using Western blot assay.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD) and the statistical analysis was conducted by using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). All of the experiments were performed at least six repeats. The Student's t-test was utilized for the statistical analysis between two groups. The Tukey's post-hoc test was used to validate the analysis of variance (ANOVA) for comparing data among the multiple groups. Two-sided p-value less than 0.05 was considered to be statistically significant.

Results

Generation of TMZ Resistant Glioma Cell Lines

TMZ was originally approved by USA Food and Drug Administration (FDA) in treatment of refractory anaplastic astrocytoma in adults in 1999[41]. In addition, TMZ has been used for the treatment of newly diagnosed adult glioblastoma since 2005[42-44]. However, plenty of TMZ treated patients have been developed the TMZ resistance in clinical. Therefore, it is urgent to clarify the mechanism of TMZ resistance and generate the new drugs and novel therapeutic strategy. In order to elucidate the mechanism of TMZ resistance, we treated wild type (WT) U87 cells with TMZ from lower concentration (10 µM) to higher concentration (150 µM). The results showed that the IC50 of TMZ resistant U87 cell line (U87 TR) was increased 3 folds as compared to that in WT cell line (Figure 1A). To determine whether CtIP expression was up-regulated in TMZ resistant cell line, we detected CtIP protein and mRNA expression by using western blot assay and real-time PCR assay, respectively. The Western blot results revealed that CtIP expression was increased by 3.1 folds in U87 TR cells as compared to that in U87 WT cells (Figure 1B). Similar to the protein expression, cells treated with TMZ for 1 month, 2 months, and 3 months had 1.4 folds, 2.5 folds

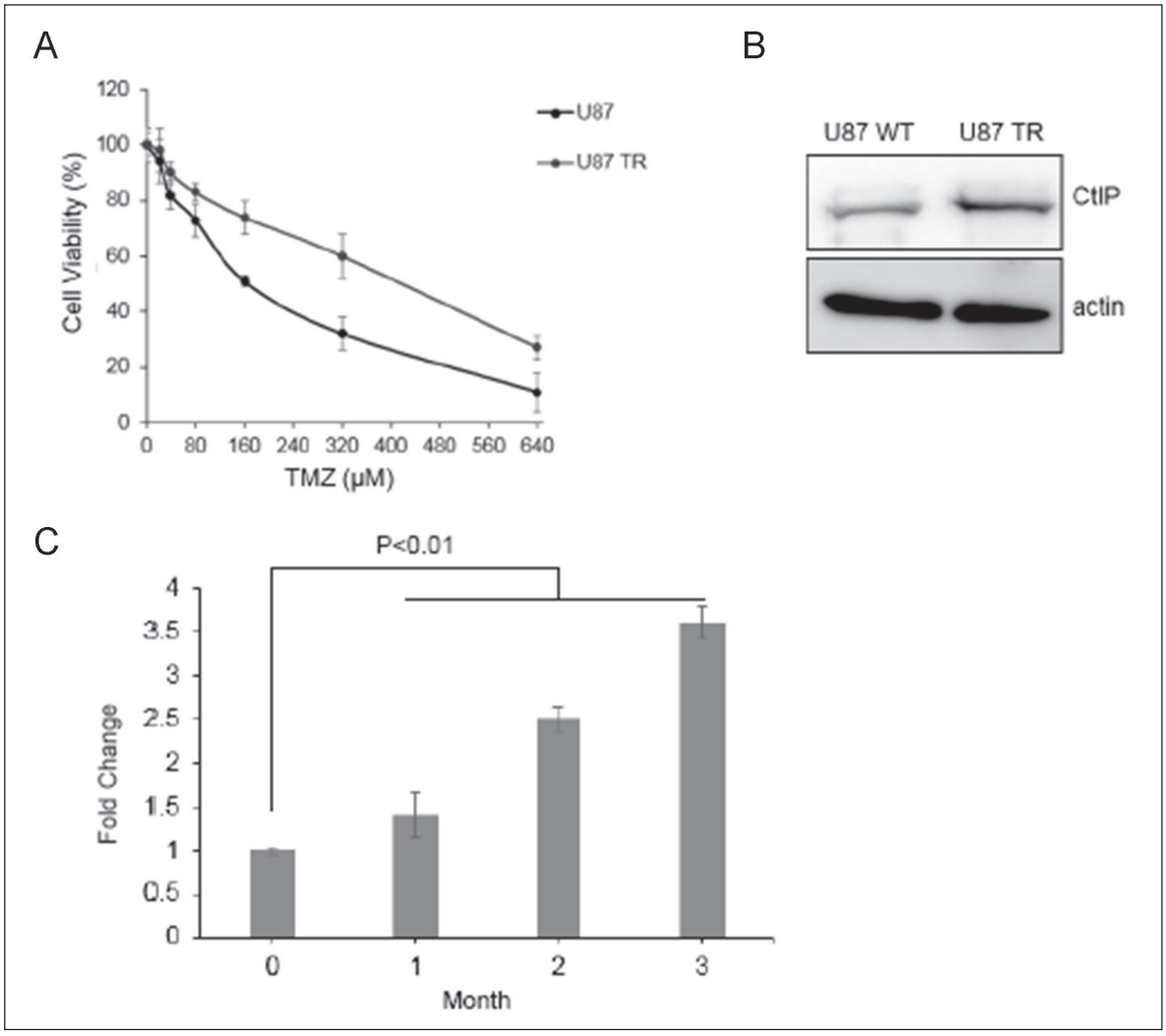

Figure 1. Generation of TMZ resistant glioma cell line. ***A.*** U87 cell line that is resistant to TMZ is generated. U87 TR: U87 cells that are resistant to TMZ. TMZ concentrations are 0 μM, 20 μM, 40 μM, 80 μM, 160 μM, 320 μM, and 640 μM. ***B.*** Western blot of CtIP expression in U87 WT and U87 TR cell lines. ***C.*** Real-time PCR showed increased CtIP mRNA level in U87 cells and U87 cells treated with TMZ for 1 month, 2 months or 3 months. Statistical significant difference ($p < 0.01$) in fold change was determined.

and 3.6 folds of CtIP mRNA increase (Figure 1C), respectively. The above findings suggest that CtIP participates in the TMZ resistance in glioma cells and DSBR pathway contributes to the DNA repair in cells treated with TMZ.

Establishment of CtIP Deficient Cell Line by Using CRISPR/Cas9 Method

Since the CtIP expression was increased in U87 TR cells, we hypothesized that CtIP depletion might optimize the sensitivity of U87 TR cells to TMZ treatment. In addition, to determine whether CtIP contributes to classical NHEJ (the most frequent used DSBR in cells), we used CRISPR/Cas9 genetic engineering method to generate CtIP deficient U87 cells. We used the "crispr.mit.edu" tool online to design the guide RNA that targets exon 1 of the CtIP gene. We harvested 7 individual CtIP clones and picked 3 clones that demonstrated the best knockout quality for further experiments (Figure 2A). Due to the well-established role of CtIP in HR, we used HR plasmid reporter assay as well as cell viability assay to further investigate the expression of CtIP. The results demonstrate that the CtIP knockout cells were indeed CtIP deficient. The HR reporter cassette contains a GFP gene with a 3 kb intron from Pem1 gene, which is engineered

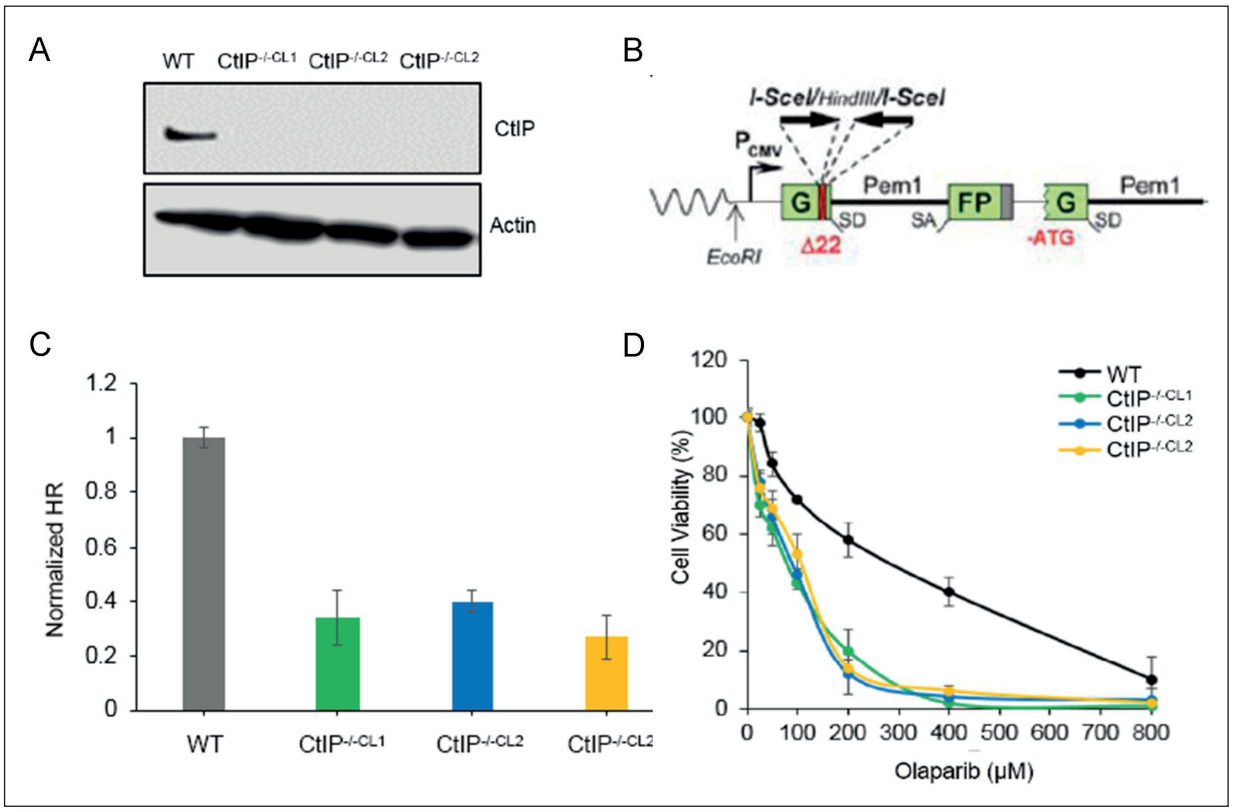

Figure 2. Establishment of CtIP deficient cell line by using CRISPR/Cas9 method. ***A***. Western blot of endogenous CtIP expression in U87 WT cell line and three clones of U87 CtIP deficient cell lines (CtIP$^{-/-}$CL1, CtIP$^{-/-}$CL2, and CtIP$^{-/-}$CL3) generated by CRISPR/Cas9. ***B.*** Homologous recombination (HR) reporter assay diagram. Adapted from Seluanov2010. Reporter plasmid contains GFP gene that is separated by Pem1 intron. DSBs are generated by introducing I-SceI. The inversed I-SceI orientation and the 22 nucleotides deletion between I-SceI sites ensure that the GFP restoration is generated by HR. ***C.*** Quantification of GFP events generated by HR IN U87 WT and three U87 CtIP deficient cell lines. The GFP events were normalized to that in U87 WT cells. Each result represents 3 independent experiments. $p < 0.01$. ***D.*** Cell viability of U87 and three U87 CtIP deficient cell lines to Olaparib. Each data point represents 3 independent experiments. $p < 0.001$.

with a 22 bp deletion combined with 3 restriction sites in the order of I-SceI/HindIII/I-SceI (Figure 2B). Since NHEJ does not use template to repair DSBs, the deletion ensures the GFP reconstitution can't be conducted by NHEJ. In addition, the 2 I-SceI sites generated after I-Scel plasmid transfection resulted in the inverted orientation. Since the second copy of GFP gene lacks the start codon and promoter, the GFP expression can't be generated by crossing over or single-strand annealing. Therefore, this reporter is exclusively used for quantification of HR pathway[40]. We also observed that three CtIP knockout cells showed significant decrease of HR efficiency (Figure 2C). It is also well proven that HR-deficient cells are extremely sensitive to the PARP inhibitor[45]. As an enzyme that participates in HR, we expected that CtIP knockout cells showed increased sensitivity to the poly adenosine diphosphate-ribose polymerase (PARP) inhibitor, Olaparib. The cell viability assay showed that all three knockout cell lines had significant increased sensitivity to the Olaparib (Figure 2D).

CtIP Interacted with Ligase IV and Improved NHEJ Efficiency

Ionizing radiation (IR) generated DSB is mainly repaired by the NHEJ[46-48]. To determine whether CtIP contributes to repairing DSBs by NHEJ, we evaluated the cell survival after a gradient of IR by using colony formation assay. We found that as compare to U87 WT cells, CtIP deficient cells have 52% decrease of cell viability. Whereas XLF knockdown cells, which are NHEJ deficient, showed only 5% cell viability decrease (Figure 3A). The above result suggests that CtIP contributes to NHEJ efficiency in glioma cells, however the CtIP is not essential for eliminating of NHEJ.

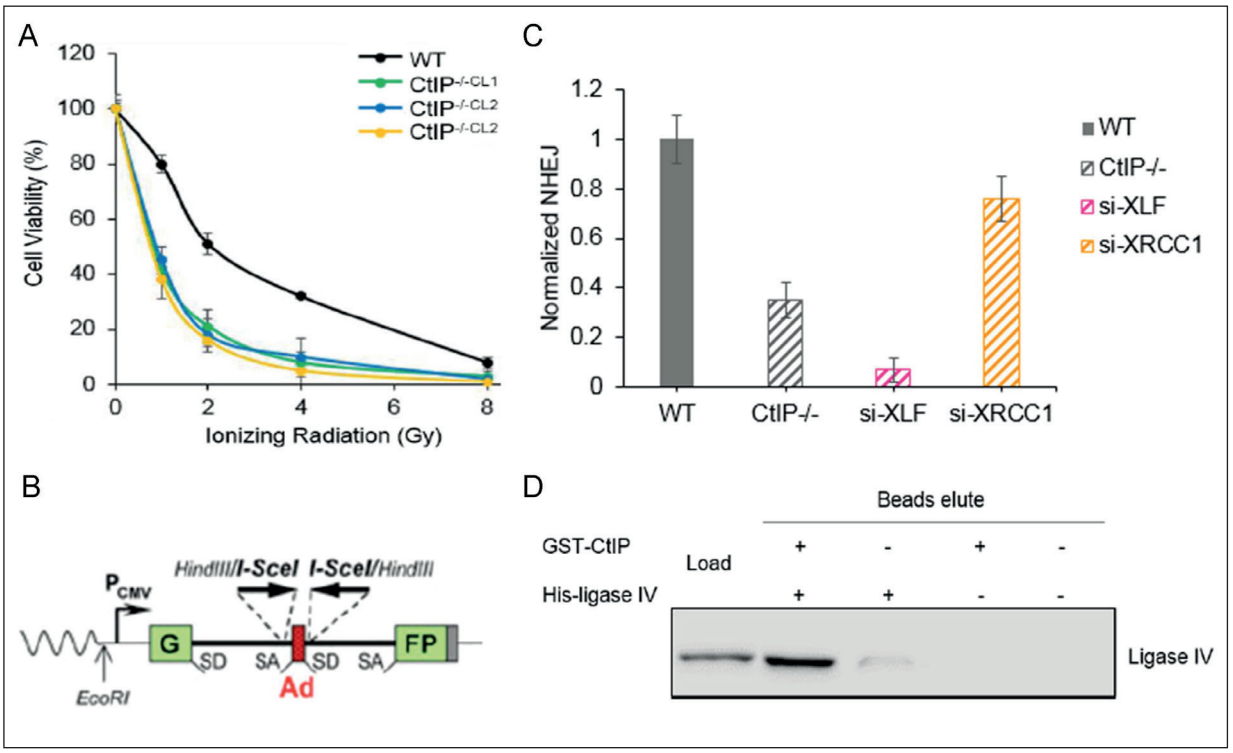

Figure 3. CtIP interacts with ligase IV and improves NHEJ efficiency. ***A.*** Quantification of clonogenic survival assay on U87 WT and CtIP deficient cell lines after IR treatment. IR dose: 0 Gy, 1 Gy, 2 Gy, 4 Gy, and 8 Gy. ***B.*** Non-homologous end joining (HHEJ) reporter assay diagram. Adapted from Seluanov 2010. Reporter plasmid contains GFP gene that is separated by Pem1 intron. DSBs are generated by introducing I-SceI. Successful repair of the DSBs by NHEJ can restore GFP expression. ***C.*** Quantification of GFP events generated by NHEJ in U87 WT, U87 CtIP deficient cell line, XLF knockout cells and XRCC1 knockout cells. The GFP events were normalized to that in U87 WT cell line. Each result represents 3 independent experiments. $p < 0.01$. ***D.*** Western blot of affinity capture assay. GST-CtIP was used to capture his-ligase IV on glutathione resin. Load: protein input of his-ligase IV. Samples were separated by SDS-PAGE, Western transferred and his-ligase IV was detected by using anti-ligase IV antibodies.

We also used NHEJ reporter assay to measure the NHEJ efficiency in CtIP deficient cell line. Similar to the HR reporter assay, the NHEJ reporter also used GFP to evaluate repair efficiency. Briefly, DSBs were generated by I-SceI restriction enzyme that flanking the Pem1 intron. NHEJ can restore GFP expression post successfully join the DSBs. The number of fluorescent cells was counted by using flow cytometry assay (Figure 3B). We observed that, CtIP deficient cells showed 35% NHEJ efficiency as compared to WT cells. Because the NHEJ efficiency measured by this assay includes classical NHEJ events and MMEJ events, we used XLF knockdown and XRCC1 knockdown cells to evaluate the events generated by these two pathways. XLF knockdown and XRCC1 knockdown cells lack the classical NHEJ and MMEJ, respectively[49]. We found that XLF knockdown cells only have 7% GFP events as compared to that in WT cells. Whereas XRCC1 knockdown cells showed 76% of NHEJ efficiency. This result suggests that CtIP contributes to the classical NHEJ in human glioma cells (Figure 3C). To understand how does CtIP participating in the classical NHEJ, we incorporated affinity capture assay to detect protein-protein interactions between purified GST-tagged CtIP (GST-CtIP) and his-tagged NHEJ factors. The results indicated that CtIP interacted with ligase IV (Figure 3D) in vitro. We also used DNA nuclease during the incubation for protein-protein interaction to eliminate DNA contamination. The results indicated that CtIP participated in NHEJ in glioma cells via interacting with ligase IV.

CtIP Deficiency Increased TMZ Sensitize of Glioma Cells

TMZ could generate the DNA damage and eventually induce the cell death. Therefore, CtIP, participating in HR, classical NHEJ and MMEJ

in glioma cells, may be a drug resistant mechanism during the TMZ treatment. In addition, since we also observed that CtIP was up-regulated in TMZ resistant glioma cells, we hypothesized that CtIP deficiency might re-sensitize the U87 TR cells to TMZ again. We carried out the cell viability assay and discovered that CtIP could re-sensitize the U87 TR cells to TMZ. Interestingly, CtIP knockdown in U87 WT cells also showed increased sensitivity to TMZ (Figure 4, Table I). In order to confirm that the increased sensitivity to TMZ of CtIP deficient cells is not off target effect of CRISPR/Cas9 knockout method, we ectopicly expressed the WT CtIP. The result showed that the resistance of cells to TMZ was restored. These data suggest that CtIP contributes to TMZ resistance in both primary and recurred glioma therapy.

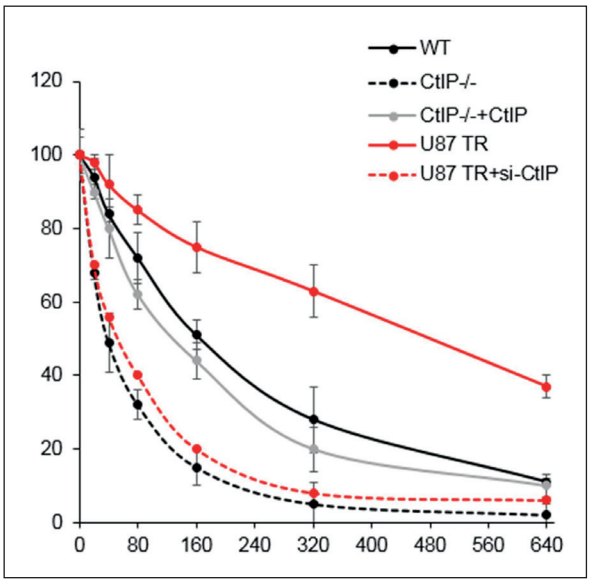

Figure 4. CtIP deficiency increase TMZ sensitize of glioma cells. Cell Survival assay of U87 WT, U87 TR, U87 CtIP$^{-/-}$, U87 CtIP$^{-/-}$+CtIP, and U87 TR + si-CtIP to TMZ. TMZ concentration: 0 μM, 20 μM, 40 μM, 80 μM, 160 μM, 320 μM, and 640 μM.6.

Discussion

CtIP has been well established as a DNA end-processing enzyme and proven to participate in the HR and MMEJ. We identified that CtIP was required for fully function of classical NHEJ, which might due to the interaction with classical NHEJ requires factor ligase IV. However, whether CtIP processes the DBSs for NHEJ and how does CtIP share stage with other DNA end processing enzymes in glioma cells have not been fully clarified. One of the major chemotherapy strategies is using small molecules to target DNA. DNA damage, including single strand DNA damage and double strand DNA damage, could induce cell death after a variety of DNA metabolism and cell response. However, cancer cells can take advantage of DNA repair pathway to overcome the therapy by recovering the damage. Therefore, DNA repair is believed to be responsible for a significant portion of anti-tumor drug resistance. TMZ, an alkylating agent, can generate DNA modifications by methylating DNA base to form N^3-methyladenine, N^7-methylguanine and O^6-methylguanine[50]. Therefore, directly blocking the O^6-methylguanine methyltransferase (MGMT) can reverse the methylation generated by MGMT, which is believed to be the most promising way to improve the TMZ resistance [[51]. However, MGMT activity is not correlated with the response to TMZ in 7 human glial tumor cell lines[52]. Moreover, some TMZ resistant glioma cells even do not express the MGMT protein, such as CCF-STTG1 cells[53]. Enzymes involved in multiple major DNA repair pathways and cell checkpoint regulations, such as CtIP. Therefore, the enzymes have the potential ability to generate synergistic effects with DNA damaging agents. TMZ can generate single strand-associated double strand breaks and replication-associated double strand breaks[54,55]. Therefore, compromising DNA repair pathways by CtIP deficiency indeed showed synergy with TMZ in glioma cells. Although this study received some interesting results, there were also a few limitations. Firstly, this study has not conducted the rescue

Table I. IC50 of TMZ in U87 WT, U87 TR, U87 CtIP$^{-/-}$, U87 CtIP$^{-/-}$+CtIP and U87 TR + si-CtIP cell lines.

	WT (U87)	CtIP$^{-/-}$	CtIP$^{-/-}$+CtIP	U87 TR	U87 TR + siCtIP
IC50 (μM)	157.23	38.35	138.92	544.16	44.56

experiment, which might disrupt the interaction to analyze whether CtIP still has an effect on the cell proliferation. Secondarily, over-expression of CtIP in wide-type U87 cells has not been investigated. In the following study, we would observe whether the similar phenotypes of CtIP-mediated drug-resistant occur in the wide-type U87 cells.

Conclusions

We indicated that CtIP contributed to NHEJ formation through interacting with IV and promotion of TMZ resistance in glioma cells via promoting DSBR efficiency.

Conflict of Interest

Authors declare no competing financial or commercial interests in this manuscript..

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