Tetrandrine Exerts a Radiosensitization Effect on Human Glioma through Inhibiting Proliferation by Attenuating ERK Phosphorylation

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Abstract

Tetrandrine (Tet), a bisbenzylisoquinoline alkaloid, has been reported to have a radiosensitization effect on tumors. However, its effects on human glioma and the specific molecular mechanisms of these effects remain unknown. In this study, we demonstrated that Tet has a radiosensitization effect on human glioma cells. It has been hypothesized that Tet has a radiosensitization effect on glioma cells by affecting the glioma cell cycle and DNA repair mechanism and that ERK mediates these activities. Therefore, we conducted detailed analyses of the effects of Tet on the cell cycle by performing flow cytometric analysis and on DNA repair by detecting the expression of phosphorylated H2AX by immunofluorescence. We used western blot analysis to investigate the role of ERK in the effect of Tet on the cell cycle and DNA repair. The results revealed that Tet exerts its radiosensitization effect on glioma cells by inhibiting proliferation and decreasing the expression of phosphorylated ERK and its downstream proteins. In summary, our data indicate that ERK is involved in Tet-induced radiosensitization of glioma cells via inhibition of glioma cell proliferation or of the cell cycle at G0/G1 phase.

Key Words: Tetrandrine, Glioma, Radiosensitization, Proliferation, ERK

INTRODUCTION

The most common primary brain tumor is glioma, which constitutes approximately 80% of all malignant brain tumors (Ostrom *et al*., 2015). The 2-year survival rate of patients with grade III and IV gliomas is only 58.2% and 33.9%, respectively (Huang *et al*., 2011). The treatment methods for glioma include surgery, radiotherapy, chemotherapy and molecularly targeted therapy (Ostrom *et al*., 2015). Notably, regular chemotherapy and sole radiotherapy are always unsatisfactory for glioma treatment. At present, increasing numbers of chemotherapy drugs, such as celecoxib and tamoxifen, have been verified to have a radiosensitization effect on glioma (Kuipers *et al*., 2007; Michalski *et al*., 2010). Thus, drugs that can be used to enhance the effect of glioma radiotherapy to meet the patients' needs must be explored.

Tetrandrine (Tet) is a bisbenzylisoquinoline alkaloid extracted from the root of the Chinese traditional medicine *Stephania tetrandra* S. Moore (Ferrante *et al*., 1990). The molecular mechanisms of the anti-tumor activity of Tet, including promo-

tion of apoptosis and inhibition of angiogenesis and proliferation, have been well studied (Liu *et al*., 2015; Wu *et al*., 2015). An increasing number of studies have focused on the properties of radiosensitization (Sun *et al*., 2007a). Radiotherapy can induce cancer cells to arrest in G2/M phase; however, it also allows cells to repair their damaged DNA to survive (Bases *et al*., 1994). Tet causes G0/G1 phase arrest, thereby altering radiation-induced G2/M phase arrest in nasopharyngeal carcinoma (Yu *et al*., 2011). Tet has also been shown to inhibit glioma cell proliferation by blocking the cell cycle at G0/G1 phase (Ma *et al*., 2015), the specific molecular mechanism that regulates radiosensitization requires further investigation.

Extracellular signal-regulated kinases (ERKs) belong to the Ras/Raf/MEK/ERK pathway, which plays an important role in the survival of cells, including cancer cells (Sun *et al*., 2015). ERKs promote cell proliferation and cell survival via upstream activation of epidermal growth factor receptor (EGFR) and Ras small guanosine triphosphatases (GTPases) and are stimulated by radiation (Asati *et al*., 2016). ERK1/2 are serine/threonine kinases whose activities are positively regulated by

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phosphorylation and mediated by MEK1 and MEK2 (McCubrey *et al*., 2012b). After phosphorylation, ERK1/2 translocate to the nucleus, resulting in changes in gene expression and regulation of a variety of proteins involved in cell proliferation and survival (McCubrey *et al*., 2012a). These proteins include Cyclin D1 (CCND1), Bcl-2, and Bcl-xL (Li *et al*., 2014; Du *et al*., 2015). Increasing numbers of radiosensitive drugs, such as sorafenib, have been verified to induce radiosensitization by inhibiting ERK phosphorylation (Dai *et al*., 2013). Whether Tet exerts a radiosensitization effect through down-regulating phosphorylated ERK (p-ERK) in glioma cells requires further studies.

In this study, we investigated the radiosensitive effects of Tet on glioma and further examined the effect of ERK on Tet-induced radiosensitization.

MATERIALS AND METHODS

Cell lines and cell culture

The human glioma cell lines U251 and U87 were obtained from Shanghai Institute of Cell Biology, the Chinese Academy of Sciences, Shanghai, China. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Paisley, Scotland, UK) with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 units/ml penicillin (Biyuntian Biotechnology, Suzhou, China). All cell lines were maintained in a humidified incubator (Thermo Fisher Scientific, MA, USA) at 37°C and 5% CO_2.

Cell viability assay

Cells were plated onto 96-well plates in triplicate at a density of 3×10^3 cells per well and allowed to adhere overnight in DMEM medium. Cells were incubated with Tet (Sigma-Aldrich Bio, CA, USA) for 24 h. After this incubation period, 20 μl/well of MTT solution (5 mg/ml phosphate-buffered saline [PBS]) was added, and the cells were incubated for 5 h. Then, the medium was aspirated and replaced with 150 μl/well of acidic isopropanol (0.04 N HCl in isopropanol) to dissolve the formazan salt that had formed. The absorbance (OD) of the formazan solution, which reflects the cell growth conditions, was measured at 570 nm using a microplate spectrophotometer (ELx800 BioTek Instruments, LA, USA). The results are represented as the OD ratio of the 20% inhibitory concentration (IC_{20}) of Tet at 24 h, which was calculated and chosen for the following experiments (Ma *et al*., 2015).

Western blot analysis

The cells were lysed in SDS buffer containing protease inhibitors and phosphorylated protease inhibitors (F. Hoffmann-La Roche Ltd., Basel, Switzerland). The obtained protein samples were subjected to 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes with a pore size of 0.22 μm, blocked in 5% fat-free milk and incubated overnight at 4°C with antibodies directed against phosphorylated ERK (p-ERK), total ERK (t-ERK), CCND1, PCNA and GAPDH (Cell Signaling Technology, NY, USA). All of these antibodies were diluted 1:1000. Protein expression was detected by horseradish peroxidase-conjugated secondary antibodies (Pierce, Rockford, IL, USA, diluted 1:10,000). Immunoreactive bands were visualized using an enhanced chemiluminescence as-

say (Pierce).

Inhibition of p-ERK expression

Cells were cultured in DMEM complete medium for 24 h and then treated with 20 μM U0126 (Cell Signaling Technology) for 24 h.

Radiation treatment

Cells were exposed to radiation at a dose rate of 1.21 Gy/min with 160 kV photons at room temperature using a RS2000 Biological Research Irradiator that contained a linear accelerator (Rad Source Technologies Asia Limited, LA, USA).

Immunofluorescence assay and phospho-H2AX foci quantification

Glioma cells were cultured on cover glasses slips and treated with or without Tet at IC_{20} for 24 h. Then, the cells were exposed to radiotherapy, fixed in 4% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in PBS, and incubated with 5% bovine serum albumin (BSA) and rabbit anti-human monoclonal phosphorylated H2AX (p-H2AX, 1:400; Cell Signaling Technology) for 60 min at room temperature and overnight at 4°C, respectively. Cultured cell slides were then incubated with Alexa Fluor 594 goat anti-rabbit IgG secondary antibody (1:1000; Cell Signaling Technology) for 1 h and with 5 μg/ml of DAPI for 15 min at 37°C. The slides were then washed with PBS and imaged under a fluorescence microscope (Olympus IX51, Fukushima, Japan). The number of p-H2AX foci per cell was determined using stored images of 50 cells (Xiao *et al*., 2012).

Clonogenic assay

U87 and U251 cells (2×10^6 cells/flask) cultured in flasks (Corning, USA) were treated with Tet at the respective IC_{20} concentrations or with the same volume of DMSO (control) for 24 h. Then, $100 \sim 1 \times 10^5$ cells were seeded in T75 flasks. After 8 h, the control and Tet-treated cells were exposed to radiation (0, 2, 4, 6, or 8 Gy) and then cultured with medium for 14-20 days. When colonies had formed, the cells were fixed in 4% formaldehyde and stained with gentian violet. The numbers of clones were then scored manually. Specifically, a colony was defined as more than 50 cells in a group. Survival fractions (SFs) were calculated as follows: SF=[the mean plating efficiency of radiation (± Tet)-treated cells divided by the mean plating efficiency of control (± Tet) cells], expressed as a %. SFs of combination-treated cells were corrected for the cytotoxicity of Tet. Radiation treatment (RT) survival curves were fitted according to the linear-quadratic model using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA): Survival= $e^{-(\alpha D + \beta D^2)}$ (Xiao *et al*., 2012). According by the linear-quadratic model, we calculated the α value, β value, α/β value, the SF at 2 Gy (SF2) and sensitivity enhancement ratio (SER, in which the SF2 of the RT group is divided by the SF2 of the RT+Tet group).

Flow cytometric analysis

Cells were seeded in six-well plates, treated with Tet at IC_{20} for 24 h and then exposed to radiation. Before the cells were analyzed, 100 μl of binding buffer containing 1 μl of 100 μg/ml propidium iodide (PI) was added to these cells, and the cells were incubated for 30 min in the dark. Analyses were performed using a FACScan flow cytometer (Beckman Coul-

ter, CA, USA). Cell cycle distribution was calculated based on DNA plots using MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

Statistical analysis

All experiments were performed at least three times (n=3). The data are presented as $\bar{x} \pm$ SEM. The experimental data were statistically analyzed using SPSS 13.0 for Windows (Chicago, IL, USA). Two-way ANOVA was used to study the influences of RT dose and Tet treatment on cell survival as determined by clonogenic assay. A two-sample t test was used to compare the mean percentage of G0/G1, and G2/M phase cells and the mean p-H2AX foci number between specific two-treatment conditions. One-way ANOVA was used to compare the expression levels of proteins. A value of *p<0.05 or **p<0.01 was considered statistically significant.

RESULTS

Tet enhanced the radiosensitivity of glioma cell lines

As shown in Fig. 1A, cell viability significantly decreased with increased Tet concentrations in a dose-dependent manner. The IC_{20} values of Tet for U87 and U251 cells were 3.91 ± 1.09 and 4.36 ± 1.32, respectively. Thus, 4 μM Tet was chosen for the following experiments. We treated two glioma cell lines (U251 and U87) with 4 μM Tet for 24 h followed by exposure to different doses of radiotherapy. Next, we investigated the SFs of the two glioma cell lines by clonogenic assay. As shown in Fig. 1B and 1C, the SFs of Tet treatment groups decreased after received radiotherapy compared with control cell lines (U251, U87) at the same dose. Finally, we calculated the parameters for RT and RT+Tet. As shown in Table 1, the SF2 of RT+Tet was lower than that of RT alone, and the SERs of U251 and U87 cells were 1.716 and 2.884. Drugs are considered to cause radiation sensitivity when the SER is above 1 (Xiao *et al*., 2012). These data indicated that the glioma cell lines were more sensitive to radiotherapy when treated with Tet.

Tet enhanced radiosensitivity by blocking the cell cycle at G0/G1 phase and not by damaging DNA

Initially, we observed the cell cycle distribution of U251 and U251+Tet cells after radiotherapy by flow cytometry (FCM). As shown in Fig. 2A and 2B, the proportions of cells in G0/G1 phase in the Tet treatment group were higher than were those in the control group. We also noticed that the proportions of cells in G2/M phase increased gradually after radiotherapy until 12 h in both U251 and U251+Tet groups in Fig. 2C; however, we did not observe that Tet could increase the G2/M proportions after the cells received radiotherapy. Next, we detected the repair of radiation-induced DNA double-strand breaks
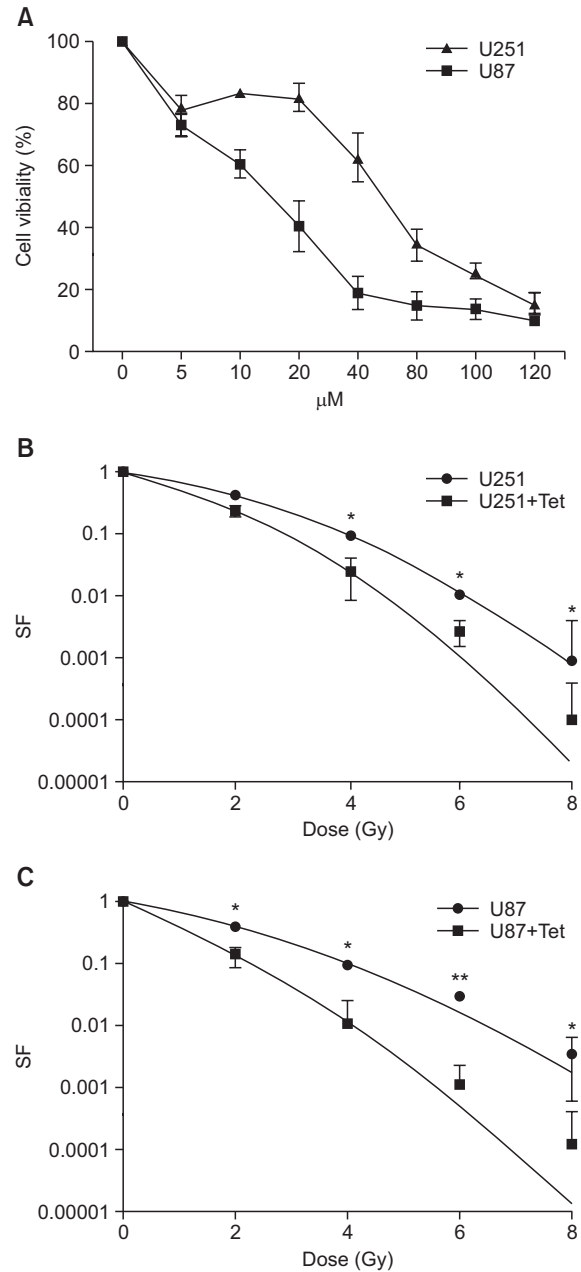

Fig. 1. Tet enhanced the radiosensitivity of glioma cell lines. (A) The cell viability of two glioma cell lines (U87 and U251) following treatment with increasing concentrations of Tet for 24 h (n=3). (B, C) The survival fractions of glioma cell lines (U251 and U87) following treatment with increasing doses of radiotherapy with or without 4 μM Tet. *p<0.05 and **p<0.01 indicate significant differences between the control and Tet groups (two-way ANOVA).

Table 1. Radiobiological parameters for radiation and Tetrandrine-radiation treatment

Cell line	Subgroup	α value (Gy-1)	β value (Gy-2)	α/β value	SF2	SER (ratio of SF2)
U251	RT	0.2841	0.07655	3.711	0.417	
	Tet+RT	0.4897	0.10890	4.497	0.243	1.716
U87	RT	0.3497	0.05529	6.325	0.398	
	Tet+RT	0.8524	0.06896	12.361	0.138	2.884

Abbreviations: SF2, survival fraction of 2 Gy; SER, sensitivity enhancement ratio
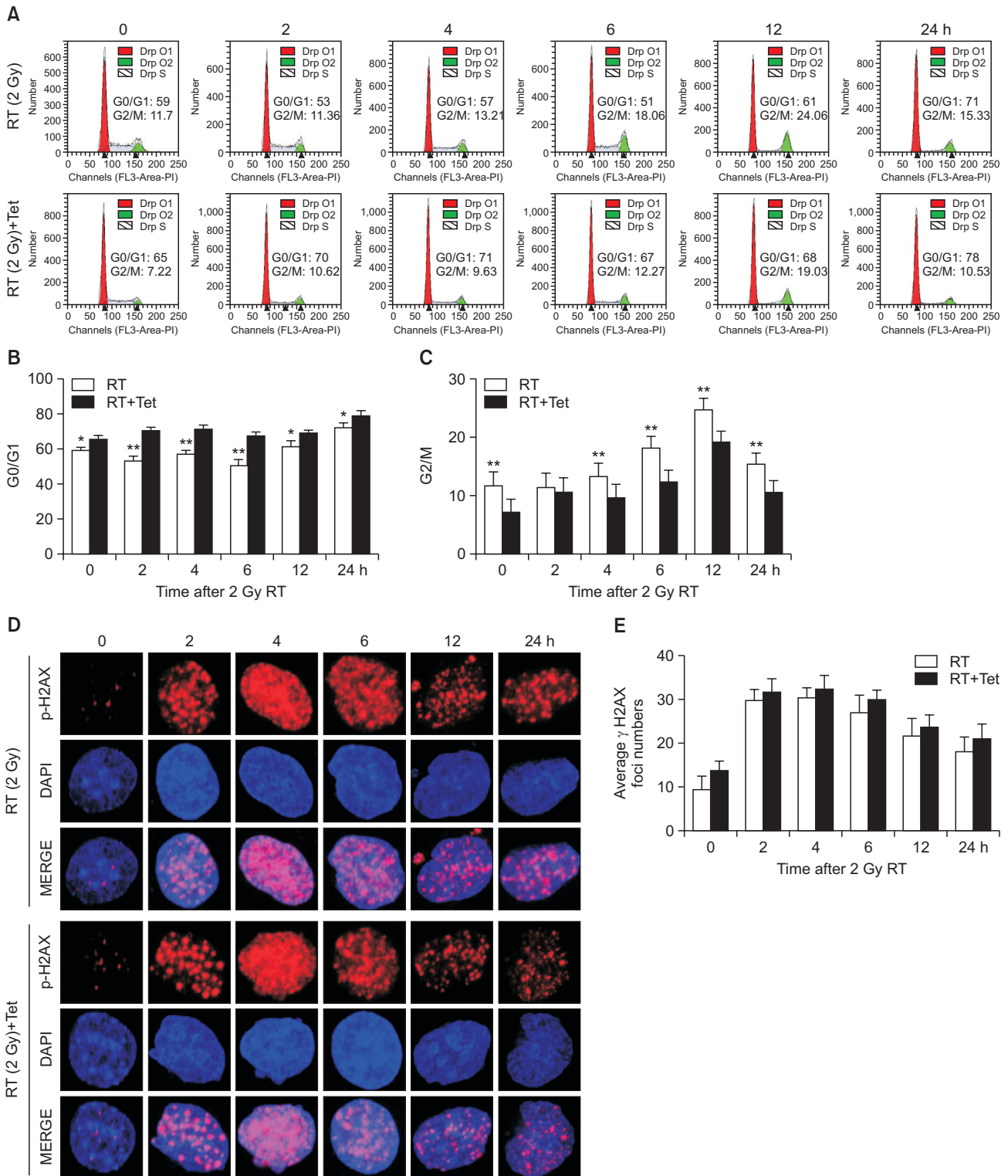
Fig. 2. Tet enhanced radiosensitivity by blocking the cell cycle at G0/G1, not by enhancing DNA damage. (A-C) Cell cycle progression of U251 glioma cells after the cells received 2 Gy radiotherapy with or without 4 μM Tet (n=3). (D) Glioma cells with immunofluorescence staining for p-H2AX after the cells received radiotherapy with or without 4 μM Tet. (E) Quantification of p-H2AX foci numbers in the RT and RT+Tet groups. Magnification ×40 in D. *$p<0.05$ and **$p<0.01$ indicate significant differences between the control and Tet groups (two-sample t test).
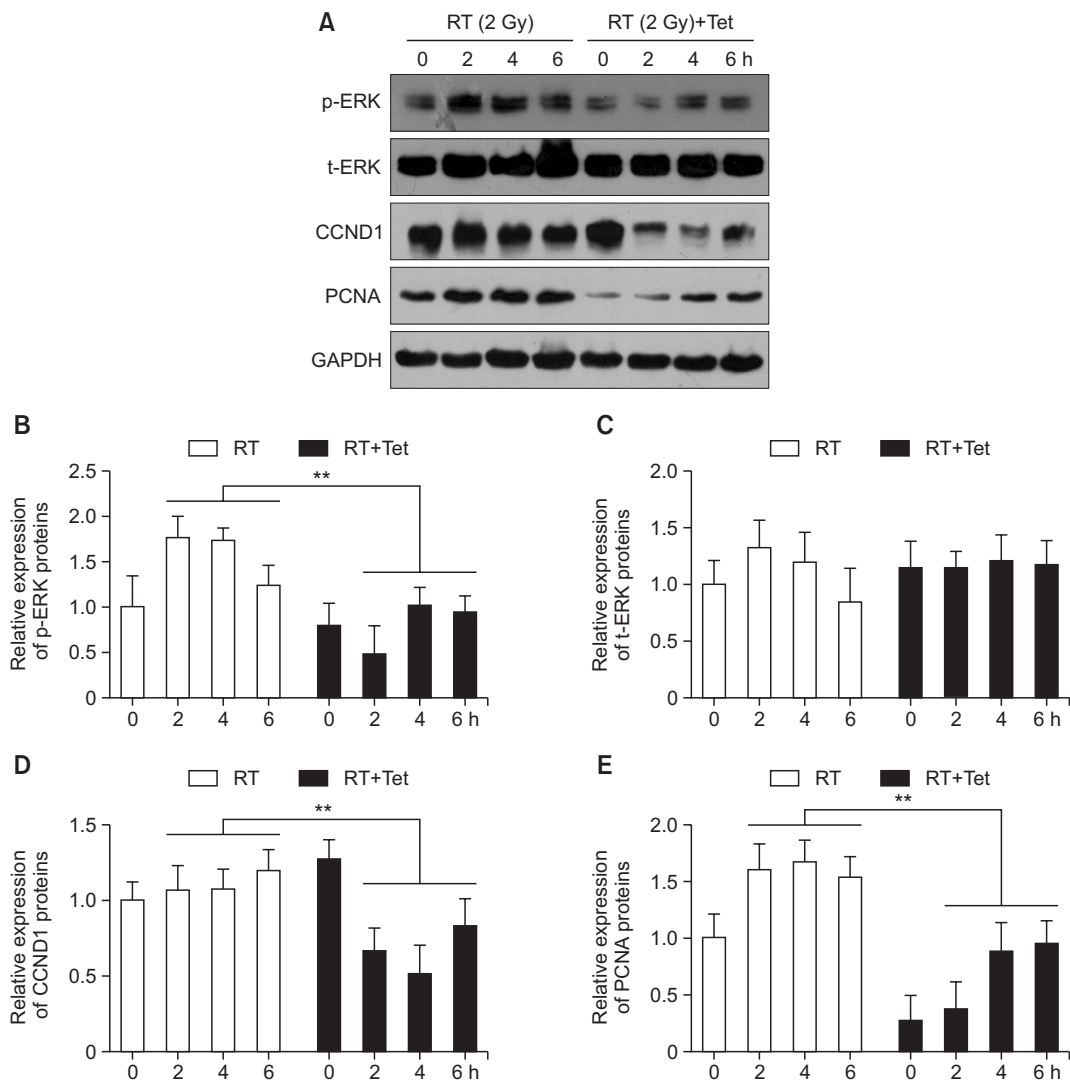
Fig. 3. Tet inhibited the expression of p-ERK and its downstream proliferation-related proteins after glioma cells received radiation treatment. (A) The expression levels of p-ERK, ERK, CCND1 and PCNA were measured in the RT and RT+Tet groups by western blot analysis. (B-E) The relative expression levels of these proteins are presented by graphs. **$p<0.01$ indicate significant differences between the RT and RT+Tet groups (one-way ANOVA).

(DSBs) by calculating the number of p-H2AX foci. As shown in Fig. 2D and 2E, the average p-H2AX foci. Numbers increased, reached peaks at 4 h after 2 Gy radiotherapy and then decreased gradually in both U251 and U251+Tet groups. More importantly, no differences in the p-H2AX foci numbers were observed between the U251 and U251+Tet groups. These data indicated that Tet exerted a radiosensitization effect on glioma through inhibiting proliferation and blocking cells at G0/G1 phase, not by enhancing DNA damage.

Tet decreased the expression of p-ERK and its downstream proliferation-related proteins after radiotherapy

As we observed that Tet could enhance radiotherapy by inhibiting proliferation, we detected the proliferation-related protein p-ERK and its downstream proteins. As shown in Fig. 3A, p-ERK expression increased at 4 and 6 h after radiotherapy. Simultaneously, p-ERK expression could not be stimulated when U251 cells were treated with Tet, even after receiving

radiotherapy at the same time point. We also investigated the expression of the proliferation-related proteins CCND1 and PCNA, which are also downstream proteins of p-ERK. The expression of CCND1 and PCNA decreased after the cells were treated with Tet. The relative expression levels of these proteins are shown in Fig. 3B-3E. These data indicated that Tet could inhibit p-ERK and its downstream proteins even after receiving radiotherapy.

Inhibition of p-ERK increased the radiosensitivity of glioma cells

We used U0126 to inhibit p-ERK expression (Stepanenko *et al.*, 2016). First, we assessed the SFs of glioma cells treated with or without the p-ERK inhibitor U0126 after RT by clonogenic assay. As shown in Fig. 4A, the SFs of glioma cells treated with U0126 were lower than were those of the control cell line at the same dose. Finally, we detected the expression of p-ERK, CCND1 and PCNA. As shown in Fig. 4B, the
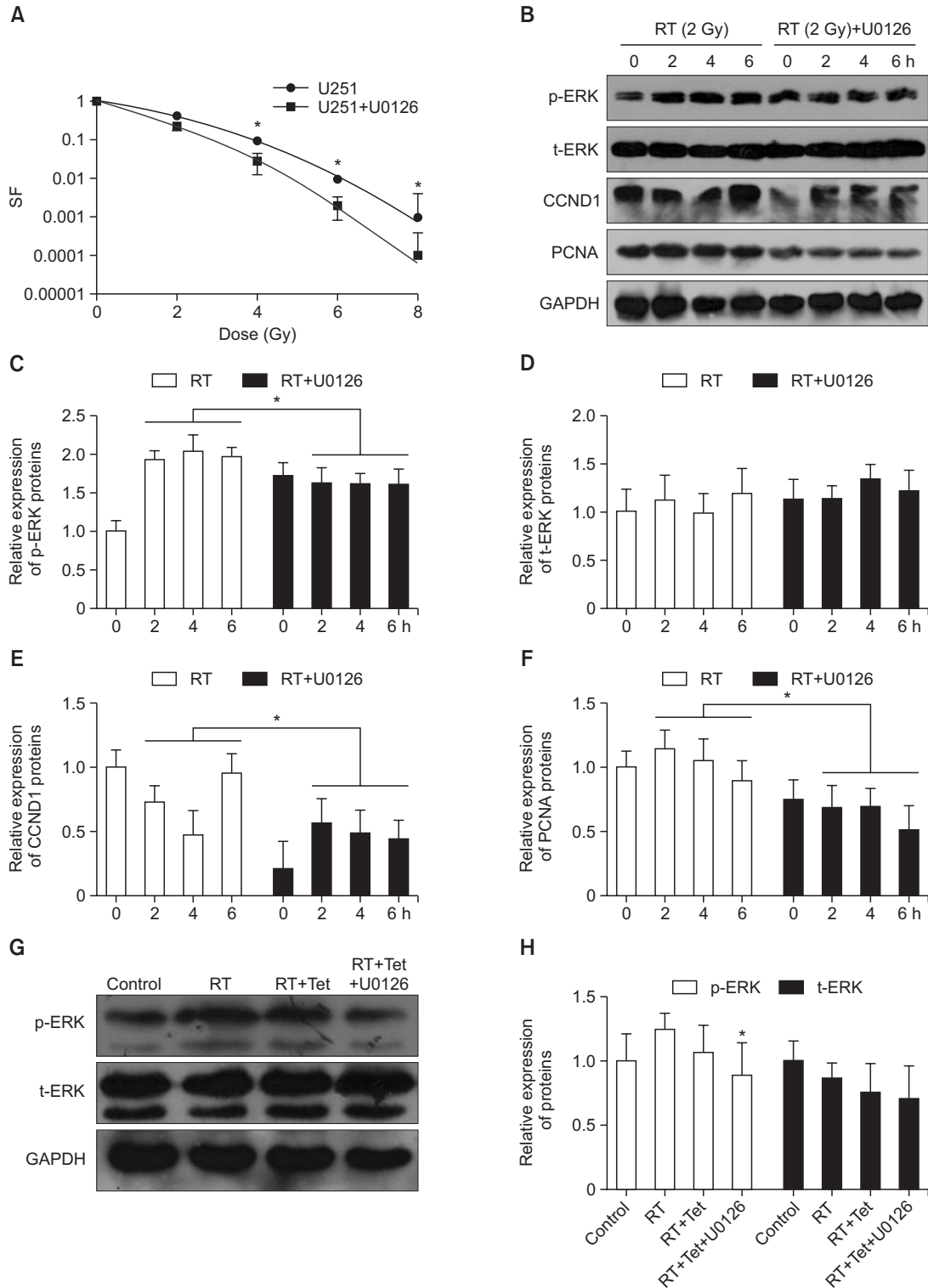
Fig. 4. Inhibition of p-ERK expression increased the radiosensitivity of glioma cells. (A) The survival fraction of U251 cells following treatment with increasing doses of radiotherapy with or without U0126. *$p<0.05$ indicates a significant difference between the RT and RT+U0126 groups (two-way ANOVA). (B) The expression levels of p-ERK, ERK, CCND1 and PCNA in cells treated with RT or RT+U0126. (C-F) The relative expression levels of these proteins are presented by graphs. *$p<0.05$ indicate significant differences between the RT and RT+U0126 groups (one-way ANOVA). G, H: The expression levels of p-ERK and ERK in cells treated with RT, RT+Tet or RT+Tet+U0126. *$p<0.05$ indicates a significant difference between the RT and RT+Tet+U0126 groups (one-way ANOVA).

U0126+RT group displayed decreased p-ERK expression compared with the group that received only RT. The expression of the p-ERK downstream proteins CCND1 and PCNA also decreased after the cells were treated with U0126. The relative expression levels of these proteins are shown in Fig. 4C-4F. We also detected p-ERK expression levels when glioma cells were treated with Tet+U0126 and then exposed to radiation. As shown in Fig. 4G and 4H, the expression level of p-ERK in the RT+Tet+U0126 clearly decreased compared with that of the RT only group. These data indicated that U0126 could enhance the radiosensitivity of glioma cells and inhibit the expression of proliferation-related proteins.

DISCUSSION

The anti-tumor mechanism of Tet, isolated from the root of *Stephania tetrandra* S. Moore, has been well studied. Tet inhibits the proliferation, survival and angiogenesis of glioma, breast cancer, colon cancer and non-small cell lung cancer (NSCL) (Gao *et al*., 2013; Ma *et al*., 2015; Lin *et al*., 2016). Tet has also been reported to cause radiosensitivity in esophagal carcinoma and breast cancer and to abrogate radiation-induced G2/M phage arrest to increase apoptosis in nasopharyngeal carcinoma cells (Sun *et al*., 2007a, 2007b; Yu *et al*., 2011). In our study, we confirmed that Tet could enhance the radiosensitivity of U251 and U87 glioma cells (Fig. 1, Table 1). Cell cycle arrest at G2/M phase upon exposure to radiation was reversed by Tet treatment; however, the apoptosis rates did not increase in the RT+Tet group compared to T*et al*one group (Supplementary Fig. 1), suggesting that apoptosis may not the major mechanism of Tet-induced radiosensitivity in glioma cells, especially when treated with low-dose (the IC_{20} dose) Tet. RT induces DNA damage; therefore, the homologous recombination or nonhomologous end-joining pathway may also mediate tumor cell radiosensitivity. However, our study showed that Tet could not induce more severe DNA damage at the baseline RT dose (Fig. 2D and 2E). Interestingly, Tet blocked the cell cycle at G0/G1 phase when glioma cells received radiation, resulting in the inhibition of cell proliferation. Hence, we hypothesize that Tet radiosensitizes glioma cells by inhibiting proliferation or arresting the cell cycle at G0/G1 phase.

The effects of Tet inhibition of tumor cell proliferation are variable. In hepatoma, Tet was shown to induce cell cycle arrest at G2/M phase (Ng *et al*., 2006). For the majority of tumor cells, including human lung carcinoma A549 cell and glioma cells, Tet induces cell cycle arrest at G0/G1 phase (Lee *et al*., 2002). Tet inhibited proliferation through the PI3K/AKT/GSK3β pathway by down-regulating CCND1 and up-regulating p27 (kip1) in the HT-29 colon cancer cell line; similar mechanisms were reported in mouse endothelial cells (EOMA cells), Moreover, intracellular accumulation of reactive oxygen species (ROS) and decreased phosphorylated Akt (p-Akt) protein levels play an important role in Tet-induced cell cycle arrest (Chen *et al*., 2008; Wu *et al*., 2010; Xiao *et al*., 2015). In contrast to p-Akt, increased p-ERK expression has also been shown to be involved in promoting tumor cell proliferation, and radiation-induced p-ERK expression has been shown to mediate radioresistance (Ahmed *et al*., 2009; Cho *et al*., 2009; Liang *et al*.; 2011, Park *et al*., 2015). In our study, we demonstrated that radiation could increase the expression of p-ERK, while Tet
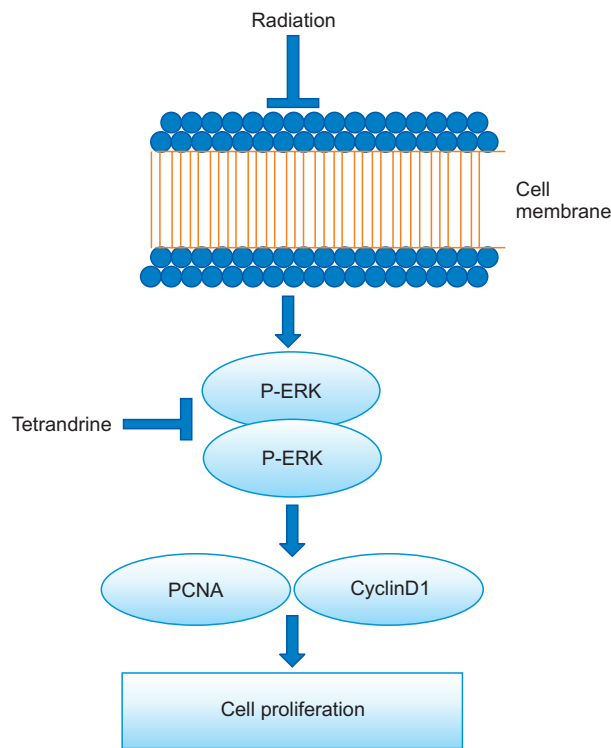

Fig. 5. A summary model depicting the possible mechanisms of Tet-enhanced radiosensitivity.

inhibited the expression of the proliferation-related proteins p-ERK, CCND1 and PCNA even when glioma cells received radiation. Similar to Tet, the MEK inhibitor U0126 also inhibited the expression of p-ERK and their downstream proteins.

In conclusion, we demonstrated that Tet exerts its radiosensitization effect on glioma cells by inhibiting proliferation, which is mediated by decreasing the expression of p-ERK and its downstream proteins (Fig. 5). In this context, Tet could potentially be used as a radiosensitizer when patients with glioma receive radiation.

ACKNOWLEDGMENTS

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