β-elemene enhances both radiosensitivity and chemosensitivity of glioblastoma cells through the inhibition of the ATM signaling pathway

SIWEI LIU[1], LEI ZHOU[1], YONGSHUN ZHAO[2*] and YUHUI YUAN[1*]

[1]Institute of Cancer Stem Cell, Dalian Medical University Cancer Center; The First Affiliated Hospital,
Dalian Medical University Cancer Center; [2]Department of Neurosurgery, The First Affiliated Hospital,
Dalian Medical University, Dalian, Liaoning, P.R. China

Abstract. Glioblastoma multiforme (GBM), a tumor associated with poor prognosis, is known to be resistant to radiotherapy and alkylating agents such as temozolomide (TMZ). β-elemene, a monomer found in Chinese traditional herbs extracted from *Curcuma wenyujin*, is currently being used as an antitumor drug for different types of tumors including GBM. In the present study, we investigated the roles of β-elemene in the radiosensitivity and chemosensitivity of GBM cells. Human GBM cell lines U87-MG, T98G, U251, LN229 and rat C6 cells were treated with β-elemene combined with radiation or TMZ. We used MTT and colony forming assays to evaluate the proliferation and survival of the cells, and the comet assay to observe DNA damage. Expression of proteins was analyzed by immunoblotting. In the present study, we found that β-elemene inhibited the proliferation and survival of different GBM cell lines when combined with radiotherapy or TMZ via inhibition of DNA damage repair. Treatment of GBM cells with β-elemene decreased the phosphorylation of ataxia telangiectasia mutated (ATM), AKT and ERK following radiotherapy or chemotherapy. These results revealed that β-elemene could significantly increase the radiosensitivity and chemosensitivity of GBM. β-elemene may be used as a potential drug in combination with the radiotherapy and chemotherapy of GBM.

Introduction

Glioblastoma multiforme (GBM) is the most frequently occurring and the most aggressive malignant type of primary brain tumor in the central nervous system (CNS). GBM accounts for 15.6% of all primary brain tumors and 45.2% of primary malignant brain tumors. More than 10,000 new patients are diagnosed every year in the United States (1-4). Current standard therapy for GBM includes surgery of maximal safe resection followed by radiotherapy in combination with temozolomide (TMZ) for newly diagnosed GBM (2,3). However, the relative survival estimates for GBM are very poor; less than 5% of patients survive 5 years post diagnosis (4). The reasons for the reduced survival include the early invasion of GBM into the central nervous system, making a surgical cure nearly impossible, and also the resistance to radiotherapy and TMZ (5).

Radiation induces a variety of DNA damage, including oxidized base damage, abasic sites, single-stand breaks (SSBs) and double-stand breaks (DSBs). TMZ as the first-line drug to treat GBM exerts its effects mainly via the mutagenic product O^6-methylguanine, a cytotoxic DNA lesion (6). TMZ can disrupt gene transcription and induce DSBs as well (7). It has been known that the activity of DNA repair processes, particularly DSB repair after radiotherapy and chemotherapy can regulate the response of tumor cells to treatment (8). The DSBs trigger the DNA damage response (DDR) mediated by ATM and ATR (the ATM and Rad3-related) protein kinases. ATM kinase, a member of the phosphatidylinositol 3-kinase (PI3K)-like kinase family, is activated by DSBs and then activates more than 700 proteins involved in cell cycle checkpoints, DNA repair and modulation of chromatin structure. ATM-dependent H2AX phosphorylation is one of the earliest signs of DNA damage and is necessary for efficient DSB repair (9).

Except for ATM and ATR, other signaling transduction pathways such as PI3K-AKT, nuclear factor-κB (NF-κB), ERK and transforming growth factor-β (TGFβ) pathways can also

Correspondence to: Dr Yuhui Yuan, Institute of Cancer Stem Cell, Dalian Medical University Cancer Center, The First Affiliated Hospital, No. 9 West Segment of South Lvshun Road, Dalian, Liaoning 116044, P.R. China
E-mail: yuhuiyuan@hotmail.com; yuhuiyuan@dlmedu.edu.cn

Dr Yongshun Zhao, Department of Neurosurgery, The First Affiliated Hospital of Dalian Medical University, 222 Zhong Shan Road, Dalian, Liaoning 116011, P.R. China
E-mail: zhaoyongshun_2005@aliyun.com

*Contributed equally

mediate radioresistance and chemoresistance by decreasing apoptosis or increasing DNA damage repair. In addition, ~40% of patients with GBM have an amplified EGFR gene which results in constitutive activation of AKT and ERK (10). AKT and ERK signaling pathways play vital roles in regulating many fundamental cellular processes and they are associated with resistance to treatment in GBM cells (11,12).

β-elemene, a Chinese antitumor medicine extracted from the plant *Curcuma wenyujin*, has been isolated as a monomeric drug and has a broad-spectrum antitumor effect in various tumor cells, such as lung (13), breast carcinoma (14), leukemia (15), ovarian cancer (16) and GBM (17). β-elemene can reverse the resistance to other drugs such as cisplatin (13). β-elemene with low toxicity has been well accepted by patients and is currently being used in clinical therapy for tumors, cancerous hydrothorax and ascites via intravenous injection, cavity or peritoneal perfusion in China.

In the present study, we investigated the roles of β-elemene in the radiosensitivity and chemosensitivity of GBM cells. We found that β-elemene significantly inhibited the repair of DNA damage of GBM cells combined with radiation or TMZ via interfering with the ATM, AKT and ERK signaling pathways.

Materials and methods

Cell lines and cell culture. The glioblastoma cell lines U87-MG, U251, T98G and LN229 from the human and C6 from the rat were used in the experiments. The GBM cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin, and grown at 37˚C in a humidified atmosphere with 5% CO_2.

Radiation with X-rays was performed using X-320ix (Precision X-Ray, Inc., North Branford, CO, USA) at a dose rate of 3.38 Gy/min. The cells were treated at room temperature (25-26˚C). The doses used were as follows: 2, 4 and 6 Gy for colony forming, 4 Gy for comet assay and 10 Gy for immunoblotting.

Reagents. β-elemene which was obtained from the National Institutes for Food and Drug Control (NIFDC; Beijing, China) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at 20 mg/ml as a stock solution. Temozolomide (Sigma-Aldrich) was dissolved in DMSO at 100 mM as a stock solution. The stock solutions were diluted immediately before each experiment. For precise assays, DMSO was added to a control sample at the same concentration as the treated samples.

Cell proliferation assay. Cell viability was detected by methylthiazolyl tetrazolium (MTT) assay. The cells were cultured in a 96-well plate at a concentration of 5,000 cells/100 μl/well. After incubation for 24 h, the cells were treated with the indicated concentrations of different drugs for 48 h. Then 10 μl of 0.5 mg/ml MTT (Sigma-Aldrich) was added to each well, and the mixture was incubated at 37˚C for 4 h. The culture medium was replaced with 150 μl DMSO to dissolve the formazan crystals. The absorbance of each well was determined at 490 nm by a plate reader (Perkin-Elmer, Waltham, MA, USA). Three replicate wells were designed for each sample.

Drug synergy was determined by combination index (CI) from the MTT assay data. The ratio CI was calculated as follows: CI = survival (combination)/survival (ELE) x survival (TMZ). If CI was <0.8, the combination was considered synergistic, if it was between 0.8 and 1.2, the combination was considered additive, and if CI was >1.2 the combination was considered antagonistic (18).

Colony forming assay. The effects of elemene combined with radiation or TMZ on GBM cell survival were assessed by colony forming assay. For radiation, the cells were incubated with or without elemene (40 μg/ml) for 24 h, and then exposed to X-rays at doses of 0, 2, 4 and 6 Gy. After irradiation, the cells were trypsinized and seeded in a 6-well plate at the indicated numbers with fresh medium. For TMZ treatment, the cells were seeded in a 6-well plate at 500 cells/well for 24 h, and then treated with the indicated concentrations of drugs for 48 h; the medium was replaced with fresh medium and cultured for 14-20 days. After 14-20 days, colonies of >50 cells were scored as survivors. Three replicate dishes were used for each treatment.

Comet assay. After the indicated treatment, GBM cells were trypsinized and washed with ice-cold PBS. Next, the cells ($1.5x10^4$/10 μl) were embedded in 120 μl of low-melting point agarose (0.5% in PBS at 37˚C) onto an agarose-coated (1.5% in PBS) slide. Then the slides were submersed for at least 1 h in pre-cooled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 7.5) with 1% Triton X-100 and 10% DMSO. The slides were denatured for 20 min at 4˚C in pre-cooled electrophoresis buffer (300 mM NaOH, 1 mM EDTA) to allow unwinding of the DNA and run at 25 V, 300 mA for 20 min at 4˚C. The slides were coated with drops of neutralization buffer (0.4 M Tris-HCl, pH 7.5), and allowed to sit for 5 min for three times. The dried slides were stained with ethidium bromide (2 μg/ml) and images were captured using a Leica microscope. Comet Assay Software Project (CASP) was used to record the percentage of DNA in the tail for each cell. The assay was completed three separate times evaluating 50 cells/experiment.

Immunoblotting. Cells harvested after radiation and drug treatments were lysed on ice in RIPA buffer with PMSF. The buffer included 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS. Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples that included the same amounts of protein were boiled for 5 min in Laemmli's buffer and separated by 8-12% SDS-PAGE, followed by electroblotting on polyvinylidene fluoride membranes. The blots were incubated with the following antibodies under conditions recommended by the manufacturers. The primary antibodies against p-ERK, ERK, p-AKT, AKT, γH2AX, p-ATM and GAPDH (Cell Signaling Technology, Beverly, MA, USA) were used. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit (Cell Signaling Technology) was used as the secondary antibodies. Singles were detected using ChemiDoc™ XRS+ Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).
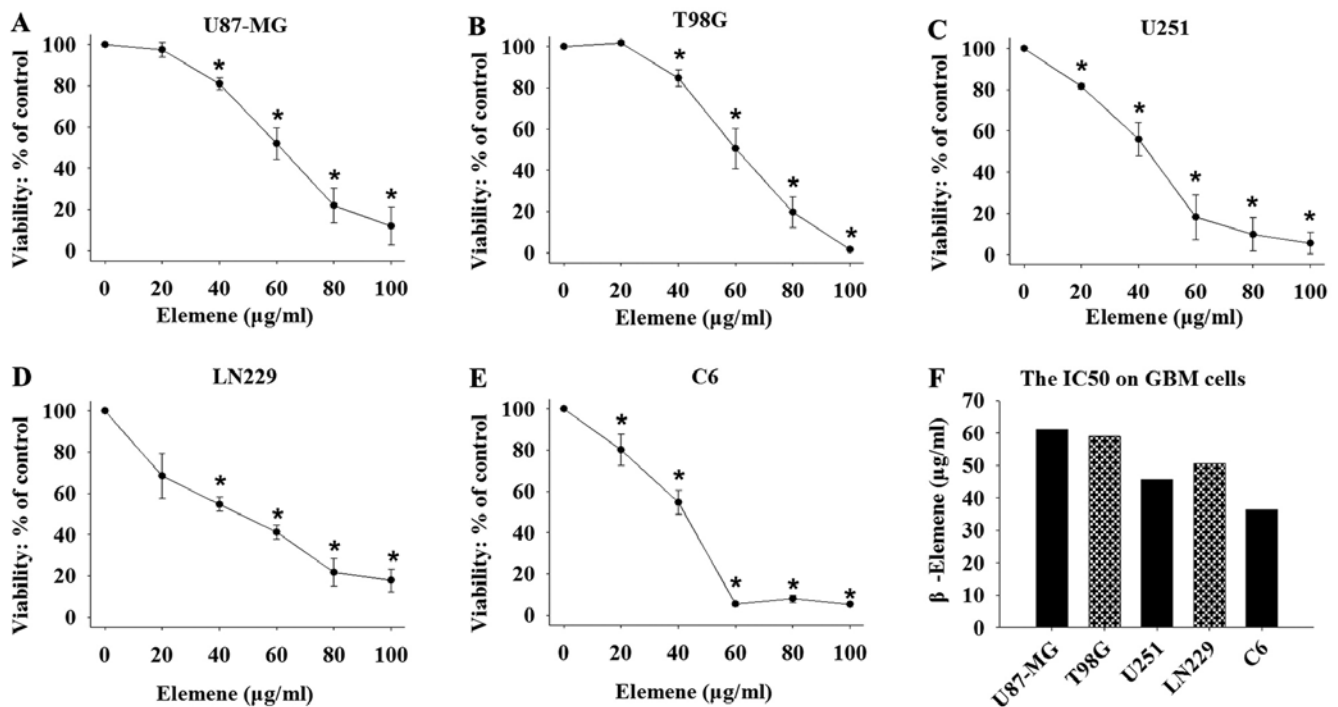
Figure 1. β-elemene inhibits the proliferation of GBM cells. Human GBM U87-MG, T98G, U251, LN229 cells and rat GBM C6 cells were seeded in 96-well plates at 5x10³/well and treated with different concentrations of β-elemene (0, 20, 40, 60, 80 and 100 μg/ml) for 24 h. (A-E) The ability of β-elemene to inhibit cell proliferation was determined by MTT assay as described in Materials and methods. The results are expressed as the mean ± SEM of at least three independent experiments. (F) The IC$_{50}$ of the different cell lines was defined as the concentration of β-elemene needed to achieve a half-maximal inhibitory effect. *P<0.05 vs. the untreated control.

Results

β-elemene inhibits the proliferation of GBM cells and enhances the radiosensitivity in different cell lines. We used MTT assay to evaluate the effects of β-elemene on the proliferation of GBM cells. The human GBM cell lines U87-MG, T98G, U251, LN229 and the rat GBM cell line C6 were used in the examination. Significant inhibition of cell proliferation was observed in each cell line following treatment with different concentrations of β-elemene at 0, 20, 40, 60, 80 and 100 μg/ml for 24 h (Fig. 1A-E). Cell viability was calculated as a percentage of the control, and the median inhibitory concentration (IC$_{50}$) was calculated from the growth inhibition curves fitted to the data. The results showed that β-elemene caused a dose-dependent inhibition with a half-maximal inhibitory effect on GBM cell growth at 61.02 μg/ml for U87-MG, 59 μg/ml for T98G, 55.66 μg/ml for U251, 55.95 μg/ml for LN229 and 36.4 μg/ml for C6 cells (Fig. 1F).

Then the human GBM cell lines U87-MG, T98G and U251 were used to study the effects of β-elemene on radiotherapy, and the surviving fractions (SFs) of the colony formation assays are shown in Fig. 2A-C. The cells were pretreated with β-elemene at 40 μg/ml for 24 h, exposed to radiation at 0, 2, 4 and 6 Gy, and then the cells were trypsinized and seeded in a 6-well plate and cultured for 14-20 days. The curves of the SFs were fitted by the multi-target single-hit model. For each cell line, radiosensitivity was significantly increased after cells were pretreated with β-elemene. Fig. 2D shows the representative images of the colony formation after treatment with radiation at 4 Gy alone or in combination with β-elemene.

Table I. Survival fractions at 2 Gy (SF2), quasi-threshold dose (Dq) and doses for 37% survival (D37).

Cell lines	Control	ELE	P-value
	SF2		
U87-MG	0.84±0.009	0.66±0.051	0.026[a]
T98G	0.89±0.017	0.67±0.010	<0.01[a]
U251	0.83±0.041	0.65±0.019	0.019[a]
	Dq		
	Control	ELE	P-value
U87-MG	2.55±0.023	1.95±0.164	0.022[a]
T98G	2.94±0.068	1.97±0.051	<0.01[a]
U251	2.60±0.226	1.67±0.131	0.024[a]
	D37		
	Control	ELE	DER
U87-MG	3.44±0.100	2.84±0.221	1.21±0.063
T98G	4.26±0.849	3.09±0.104	1.38±0.315
U251	3.98±0.409	3.41±0.192	1.17±0.101

Control, β-elemene(-); ELE, pretreated with 40 μg/ml β-elemene for 24 h. Date values: mean ± SEM for SF2 and Dq, mean ± 95% CI for D37. [a]P<0.05 group treated with β-elemene vs. untreated group.

In addition, we analyzed the difference between the groups treated with and without β-elemene following radiotherapy by
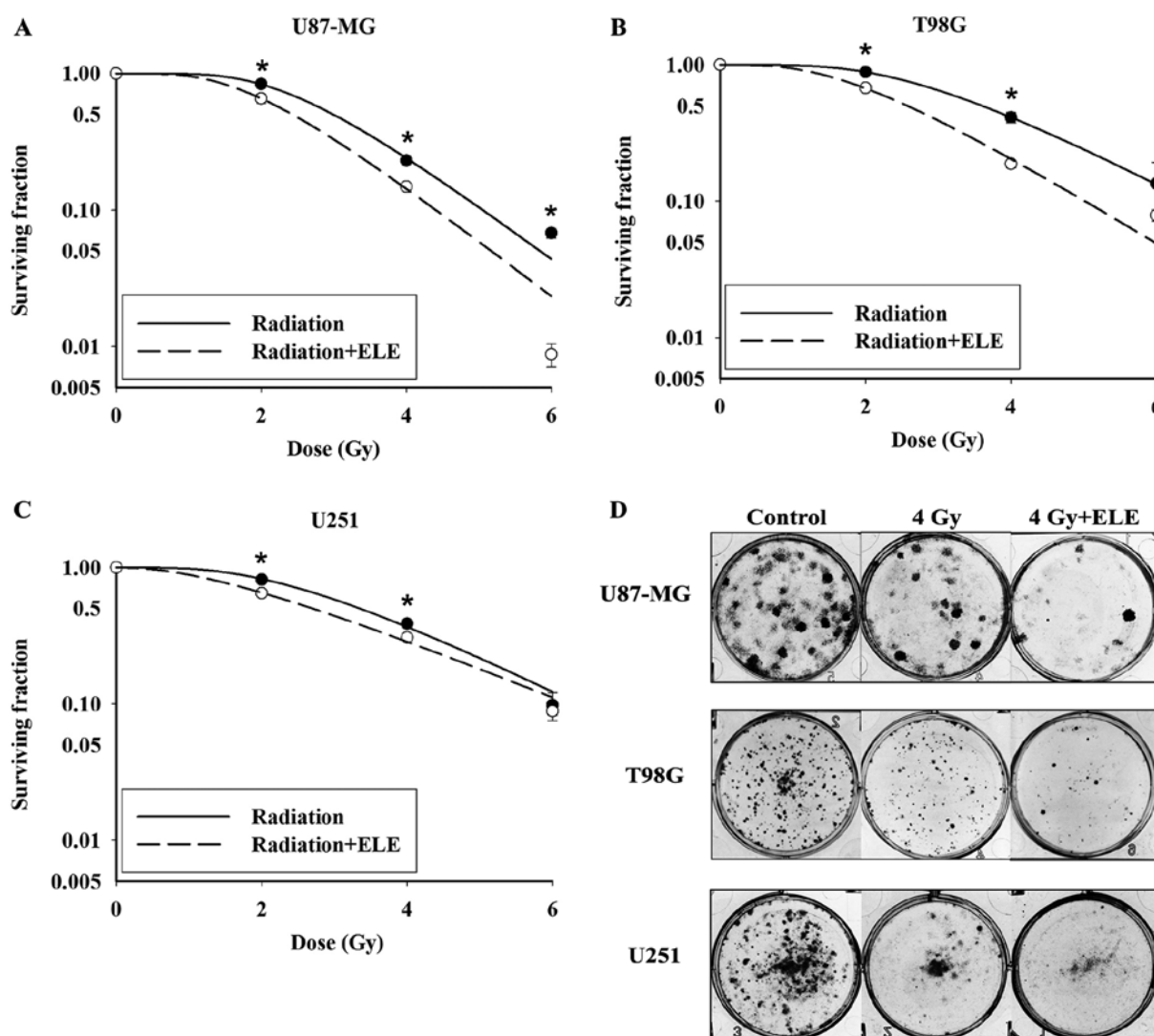
Figure 2. β-elemene enhances the radiosensitivity of GBM cells. Human GBM U87-MG, T98G, U251 cells were pretreated without or with β-elemene (40 μg/ml) for 24 h, and exposed to different doses of radiation at 0, 2, 4 and 6 Gy. Then the cells were seeded in a 6-well plate as 250, 250, 500 and 1,000/ well corresponding to 0, 2, 4 and 6 Gy (A-C) or 500/well (D). (A-C) The surviving fraction curves were fitted using the multi-target single-hit model. (D) The colony formation assay was carried out as described in Materials and methods. The results are expressed as the mean ± SEM of at least three independent experiments. *P<0.05, group treated with β-elemene vs. untreated group.

calculating the SFs at 2 Gy (SF2), quasi-threshold dose (Dq) and doses of radiation for 37% survival (D37) (Table I). The data showed that β-elemene was more effective in the T98G cells with a DER of 1.38±0.315. Taking all the factors into consideration, β-elemene significantly radiosensitized the GBM cells.

β-elemene enhances the radiation-induced DNA damage of GBM cells. We used comet assay to examine the effects of β-elemene combined with radiation on the DNA damage in GBM cells. The tail intensity (percent DNA in the tail) in the comet assay is a marker for the degree of DNA damage. The human GBM cell lines U87-MG and T98G were used in the examination. Cells were pretreated with β-elemene (40 μg/ml) for 24 h, exposed to radiation at 4 Gy and were collected immediately or after 4 and 24 h to study the repair of DNA damage. We found that the DNA damage of the GBM cells was almost repaired at 24 h after radiation, while the cells pretreated with

β-elemene still had obvious DNA damage at the same time-point (Fig. 3). The results showed that β-elemene inhibited the repair of the DNA damage caused by radiation.

β-elemene inhibits the phosphorylation of ATM, H2AX, AKT and ERK signaling pathways to reduce the repair of DNA damage after radiation in GBM cells. To understand the mechanism of the radiosensitivity caused by β-elemene in GBM, human GBM cell lines U87-MG, T98G and U251 were pretreated with β-elemene at 20 and 40 μg/ml for 24 h, exposed to radiation at 10 Gy and collected for immunoblotting after 5 min. Phosphorylated ATM and γH2AX levels are shown in Fig. 4A-C. Furthermore, we used U87-MG cells to examine the activation of AKT and ERK following the indicated treatments. The levels of total and phosphorylated AKT and ERK were analyzed and are shown in Fig. 4D. The results showed that β-elemene significantly inhibited the radiation-induced phosphorylation of ATM, AKT and ERK.
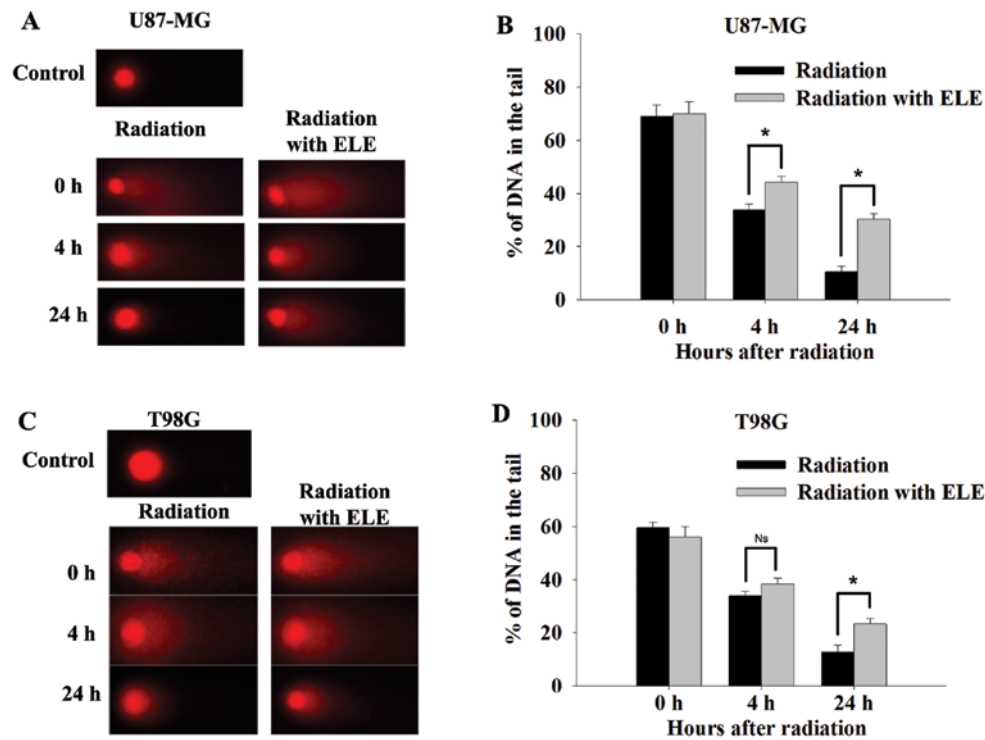
Figure 3. β-elemene enhances the radiation-induced DNA damage of GBM cells. Human GBM cell lines U87-MG (A and B) and T98G (C and D) were pretreated without or with β-elemene (40 µg/ml) for 24 h, exposed to radiation at 4 Gy and collected for comet assay as described in Materials and methods. The percentage of DNA in the tail was calculated for 50 random cells. The results are expressed as the mean ± SEM of at least three independent experiments. *P<0.05, group treated with β-elemene vs. untreated group.
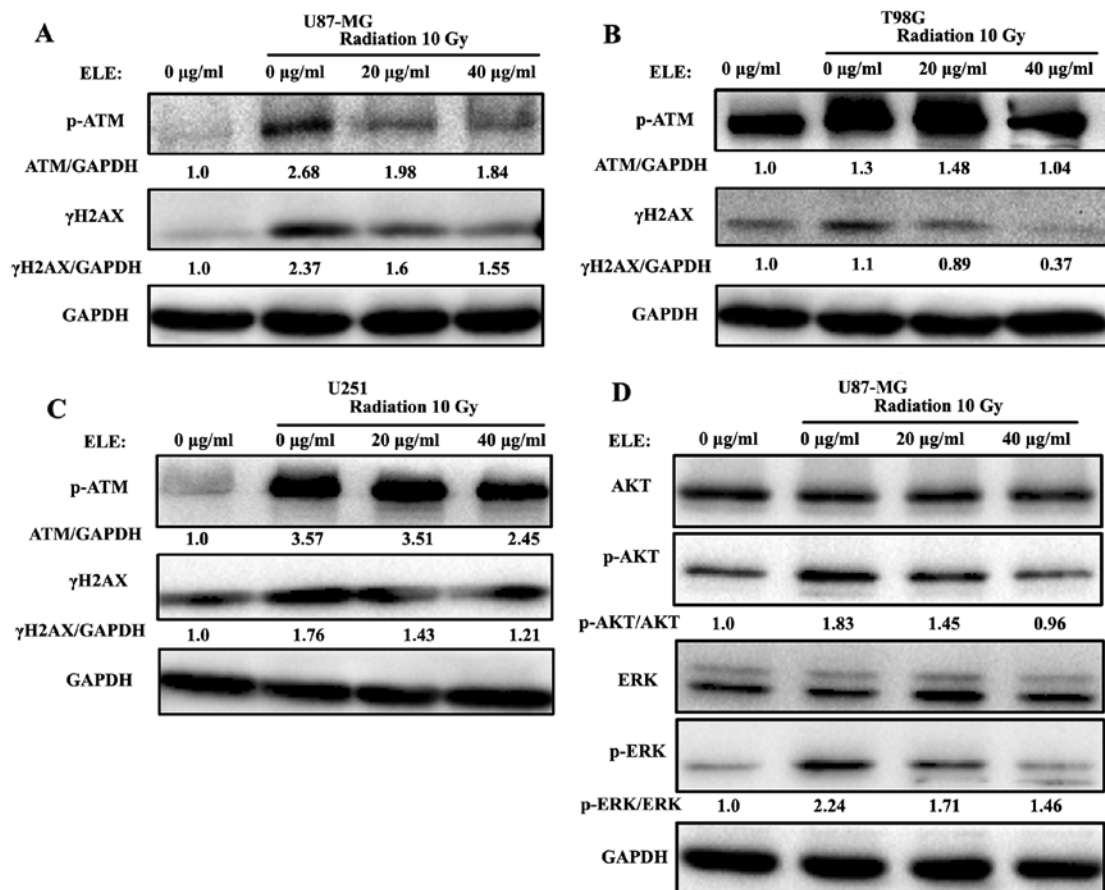

Figure 4. β-elemene inhibits the ATM, AKT and ERK signaling pathway in GBM cells when combined with radiation. Human GBM U87-MG, T98G and U251 cells were pretreated without or with β-elemene (20 and 40 µg/ml) for 24 h, exposed to radiation at 10 Gy and collected for immunoblotting after 5 min as described in Materials and methods. The levels of p-ATM, γH2AX (A-C) and AKT, p-AKT, ERK and p-ERK (D) were evaluated.
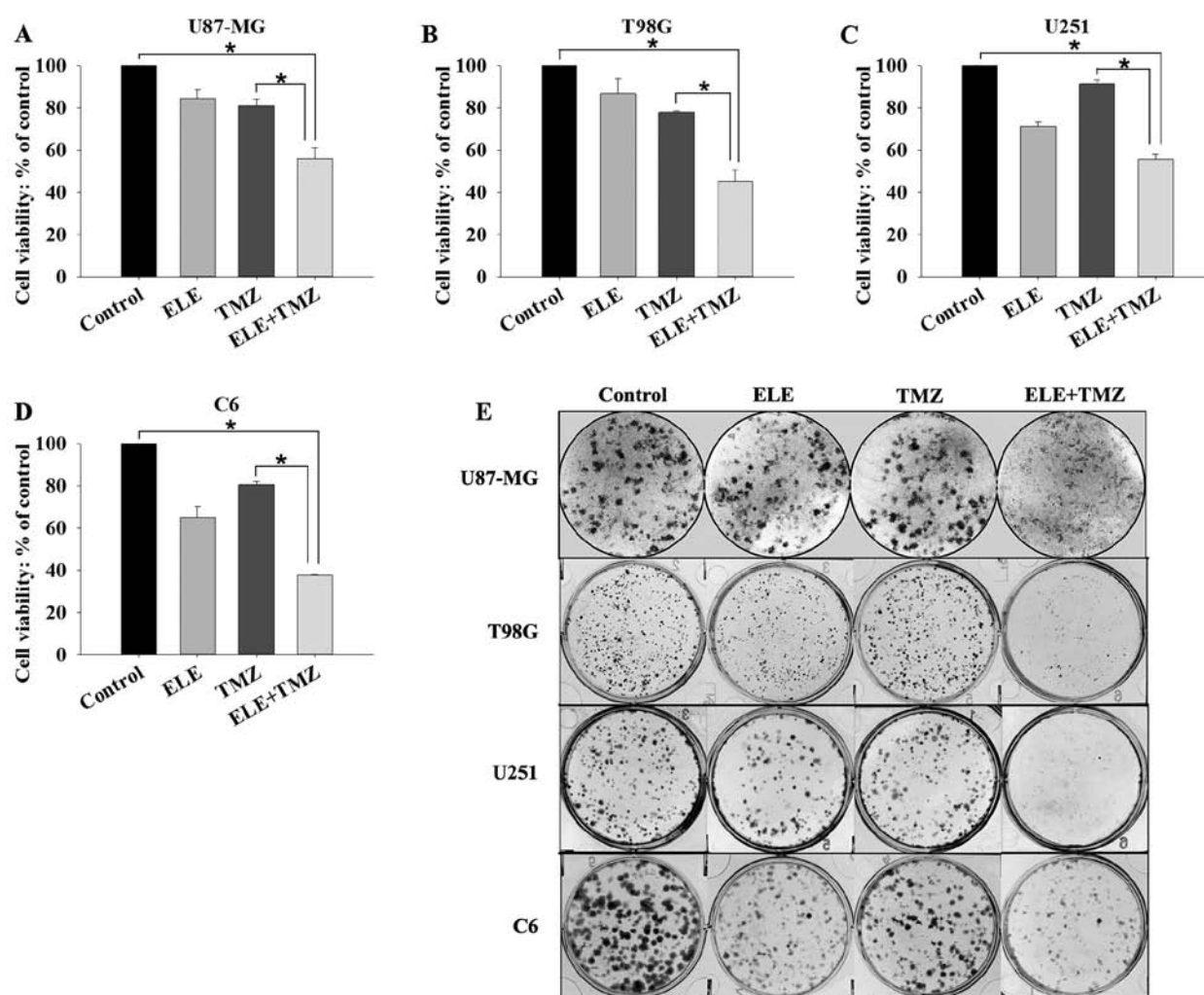
Figure 5. β-elemene increases the inhibitory effect of TMZ on proliferation of GBM cells. Human GBM U87-MG, T98G, U251 cells and rat GBM C6 cells were seeded in 96-well plates at 5×10^3/well and treated with β-elemene (40 µg/ml) alone, TMZ (500 µM) alone or both for 48 h. (A-D) The ability of β-elemene to increase the effect of TMZ was determined by MTT assays. (E) Colony formation for GBM cells. Cells were seeded in 6-well plates at 500/well and treated with β-elemene (30 µg/ml) alone, TMZ (50 µM for U87-MG, U251 and C6; 100 µM for T98G) alone or both for 48 h. Then the medium was replaced with fresh medium and cultured for 14-20 days. The results are expressed as the mean ± SEM of at least three independent experiments. *P<0.05 vs. untreated control.

β-elemene increases the inhibitory effects of TMZ on the proliferation and survival of GBM cells. In order to determine the combinatorial effects of β-elemene and TMZ in GBM, we used MTT and colony formation assays to examine the effects of β-elemene plus TMZ in GBM cell lines U87-MG, T98G, U251 and C6 cells. T98G has been reported to be resistant to TMZ (11). We examined the different effects of TMZ at 0, 100, 250, 500 and 1,000 µM on GBM cells for 48 h by MTT assay and selected 500 µM for the following combination experiments. We treated the cells with β-elemene alone (40 µg/ml), TMZ alone (500 µM) or both for 48 h, and then examined the effects using the MTT assay (Fig. 5A-D). We found that β-elemene markedly increased the TMZ-induced inhibition of cell proliferation in the four cell lines and the CI was calculated: 0.87 for U87-MG, 0.672 for T98G, 0.857 for U251, 0.699 for C6 cells. For the colony formation assay (Fig. 5E), the cells were seeded in 6-well plate for 24 h at 500/well, and treated with the indicated concentrations of β-elemene alone, TMZ alone or the combination for 48 h, and then cultured for 14-20 days. The numbers of the colonies were fewer and the

sizes were smaller in the combination treatment group when compared to the group treated with TMZ alone or the control. The data demonstrated that β-elemene strongly enhanced the inhibition of cell proliferation and survival induced by TMZ in the human and rat GBM cell lines and it was more effective in the TMZ-resistant cell line T98G.

β-elemene inhibits TMZ-induced activation of ATM, AKT and ERK signaling and enhances DNA damage of TMZ. We used the comet assay to examine the DNA damage of GBM cells treated with β-elemene and TMZ, and assessed the change in the p-ATM and total/phosphorylated levels of AKT and ERK proteins by immunoblotting at the same time. The human GBM cell line T98G was treated with β-elemene alone (40 µg/ ml), TMZ alone (500 µM) or both for 48 h and collected for comet assay and immunoblotting. The percentage of DNA in the tail was almost 50% in the combined group and was higher than that in the group treated with TMZ alone. We also found that TMZ enhanced the phosphorylation of ATM, AKT and ERK, whereas β-elemene reduced their phosphorylated
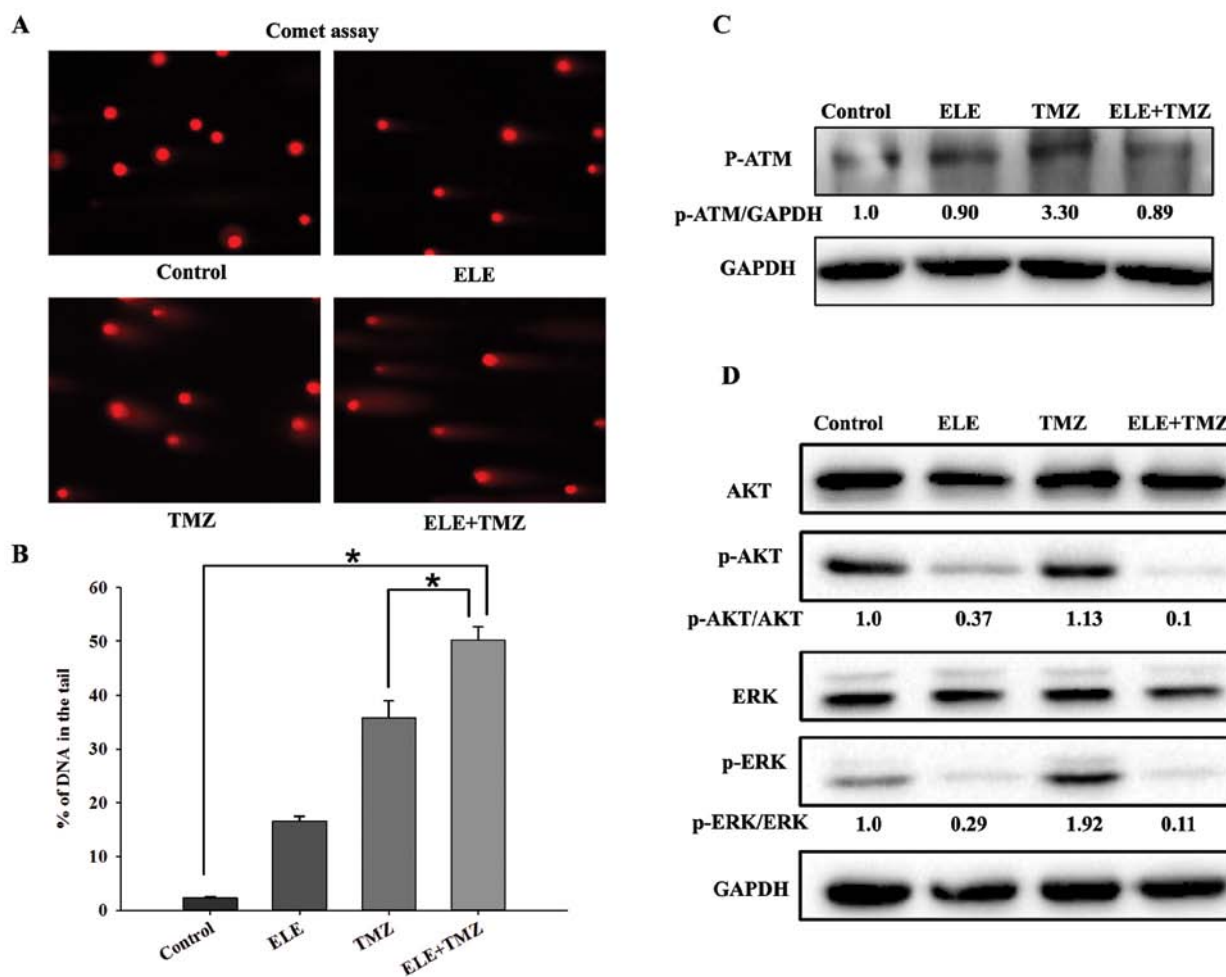
Figure 6. β-elemene inhibits the phosphorylation of ATM, AKT and ERK to enhance the DNA damage of GBM cells combined with TMZ. Human GBM T98G cells were treated with β-elemene (40 μg/ml) alone, TMZ (500 μM) alone or both for 48 h and then collected for (A and B) comet assay or (C and D) immunoblotting. The levels of p-ATM (C) and AKT, p-AKT, ERK, p-ERK (D) were evaluated. The results are expressed as the mean ± SEM of at least three independent experiments. *P<0.05, group treated with β-elemene vs. untreated group.

counterparts compared to the total protein levels which were not affected in T98G cells (Fig. 6). Thus, β-elemene decreased the phosphorylation of ATM, AKT and ERK and inhibited the DNA damage repair caused by TMZ.

Discussion

The resistance to radiotherapy and chemotherapy of glioblastoma multiforme is a critical issue associated with its poor prognosis and reduced patient survival. New treatments to reverse the resistance are urgently needed. β-elemene, a Chinese medicine, has demonstrated antitumor effects in many types of tumors (13-17) with low toxicity, and has been widely used in China. Some reports have shown that β-elemene has a sensitization effect on radiotherapy and cisplatin in lung cancer cells (19,20). We carried out clinical experiments of β-elemene on patients with GBM. We found that injection of β-elemene through the internal carotid artery had significant effects on reducing the size of tumors. The antitumor effect of β-elemene on GBM patients is definite, and β-elemene has few side-effects with some local vasculitis (21). It has been reported that β-elemene increased the ratio of Bax:Bcl-2, and activated caspase-9, -3 and -7 to

induce the apoptosis of GBM cells (22,23). β-elemene can also activate the p38 MAPK (17) and inactivate RAF/MEK/ERK signaling pathways to induce cell cycle arrest of GBM cells in the G0/G1 phase (23).

We found that β-elemene inhibited the proliferation of GMB cell lines and had a sensitization effect on radiotherapy. Our data showed that β-elemene co-treated with radiation increased the inhibition of DNA damage repair.

The ataxia telangiectasia mutated (ATM) kinase is triggered by DSB formation and initiates a series of signaling events by phosphorylating substrate proteins (8). ATM is a 350-kDa protein that exists as an inactive dimer and undergoes monomerization following DSB induction. Phosphorylated ATM regulates cell cycle checkpoints through p53 phosphorylation at Ser15 and activates checkpoint kinase 2 (CHK2) through phosphorylation at Thr68 (24,25). As ATM kinase is a potential therapeutic target for the therapy of GBM, the ATM inhibitor increases the death of GBM cells combined with radiation (9,26,27). Histone H2AX can be phosphorylated by p-ATM to γH2AX and takes parts in the repair of DNA damage directly, thus, we not only tested the level of p-ATM but also γH2AX to measure the DNA damage repair after radiotherapy.

In the present study, we showed that β-elemene inhibited the phosphorylation of ATM after radiation. The levels of p-ATM decreased depending on the concentrations of β-elemene. The downstream protein, γH2AX was also decreased by β-elemene. We also found that untreated T98G cells had a higher level of p-ATM, and this may be the reason why T98G is more resistant to radiotherapy. Thus, β-elemene enhances the sensitivity of GBM cells to radiation via inhibiting the activation of the ATM signaling pathway to reduce DNA damage repair.

The constitutive activation of AKT and ERK in GBM cells increases the ability of survival, proliferation, migration and invasion (10). The radiosensitivity of GBM cells can be affected by AKT and ERK signaling pathways through promoting the activation of the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (28). ERK and AKT signaling pathways have positive effects on DSB repair in an ATM-dependent manner (29). It has been discovered that ATM indirectly induces AKT and ERK phosphorylation, and inhibiting the ATM kinase with an ATM-specific inhibitor was found to reduce the phosphorylation of AKT and ERK (30,31). Meanwhile, the phosphorylated ERK promotes ATM-dependent foci formation, making ATM and ERK signaling under the control of a regulatory feedback loop (32). Our findings showed that the total AKT and ERK did not change significantly after treatment while the phosphorylation of AKT and ERK was inhibited by β-elemene with increased concentrations.

As TMZ is the first-line alkylating agent in clinical therapy and has an antitumor effect by inducing DNA damage, we examined the effect of β-elemene combined with TMZ and found that β-elemene had a chemosensitizing effect on TMZ. The phosphorylation of ATM, AKT and ERK induced by TMZ were reduced by β-elemene to inhibit the repair of DNA damage. In addition, the effect on the TMZ-resistant cell line T98G was more pronounced following the combination of β-elemene and TMZ which indicated that β-elemene could reverse the resistance of GBM cells to TMZ.

The present study demonstrated that β-elemene not only inhibited the proliferation but also enhanced the radiosensitivity and chemosensitivity of GBM cells. We found that β-elemene inhibited the DNA damage repair induced by both radiation and TMZ by decreasing the levels of phosphorylated ATM, AKT and ERK. Thus, β-elemene may be used as a potential novel drug in combination with the radiotherapy and chemotherapy of GBM.

Acknowledgements

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