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Original Article

β-elemene promotes the senescence of glioma cells through regulating YAP-CDK6 signaling

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Abstract: Glioma is currently the most widespread and malignant primary intracranial tumor, which is characterized by high heterogeneity and high fatality rates. β-elemene, which is a bioactive compound extracted from a Chinese herb, Curcuma wenyujin, has been reported to reduce resistance of chemotherapeutic drugs and induce apoptosis in tumor cells. However, the role and mechanisms of β-elemene in glioma senescence remains unknown. In the present study, we found that a low concentration of β-elemene (10 μg/mL) induced senescence in glioma cells, including reduction of cell proliferation, hypertrophic morphology, increase of senescence-associated β-galactosidase (SA-β-Gal) activity, upregulation of several senescence-associated genes such as *p16*, *p53* and *NF-κB*, and down-regulation of *Lamin B1*. However, a high concentration of β-elemene induced apoptosis in glioma cells. Treatment with β-elemene caused a marked down-regulation of Yes-associated protein (YAP) expression in glioma cells, which is a key transcriptional co-activator in multiple cancers. Moreover, cyclin dependent kinase 6 (CDK6), which is a known downstream target of YAP, was decreased in glioma cells that treated with β-elemene. The overexpression of YAP and CDK6 significantly rescued β-elemene-induced senescence in glioma cells. Finally, β-elemene treatment also induced the senescence of glioma cells in glioma xenograft model through inactivation of YAP-CDK6 pathways, which might inhibit the glioma growth. Taken together, these results reveal a previously unknown role of β-elemene in glioma cell senescence *in vitro* and *in vivo* that is associated with YAP-CDK6 signaling pathway, which will enhance our understanding of glioma cell senescence, and provide novel strategies for the treatment of gliomas.

Keywords: β-elemene, senescence, glioma, YAP-CDK6 signaling, proliferation

Introduction

Gliomas are the most widespread intracranial tumors of the nervous system, accounting for 81% of malignant brain tumors [1]. Gliomas are characterized by diffuse infiltration into the brain and a pronounced genetic heterogeneity [2, 3]. Current therapies do not effectively relieve disease symptoms and patients have a poor prognosis, with a 5-year relative survival of ~5% [4-6]. To date, the development of new drugs and strategies to cure gliomas has been a critical issue and with huge challenges.

Increasing studies have revealed that the induction of senescence in cells, which prevents cell cycle progression by effectively blocking cell proliferation, could be an ideal therapeutic strategy to combat gliomas [7]. Senescence is essentially a state of cell cycle arrest, with limited cell proliferation, but metabolic and immune functions still occur. Senescent cells are larger and flatter, exhibit increased SA-β-Gal activity, and secrete factors of senescence-associated secretory phenotype (SASP), such as growth modulators and chemokines, proinflammatory cytokines, angiogenic factors, and matrix metalloproteinases (MMPs) [8]. p53/p16 are well-known key signaling components of cellular senescence [9]. In addition, although certain senescence-triggering compounds have received FDA approval,

including CDK4/6 inhibitors such as abemaciclib, palbociclib, and ribociclib [10, 11], a few traditional Chinese drugs have been developed to induce senescence of glioma.

β-elemene, which is a bioactive compound extracted from Curcuma wenyujin, can improve the clinical efficacy and survival rate of patients diagnosed with glioma [12, 13]. As a noncytotoxic Class II anti-tumor drug, β-elemene have been demonstrated to affect cell autophagy and apoptosis in various types of cancer including glioma [7, 14]. However, whether β-elemene can induce senescence in glioma cells and the underlying mechanisms remain unknown.

YAP is a key transcription co-activator of the Hippo pathway, which plays crucial roles in regulating organ sizes, cell proliferation, and tumor metastasis [15, 16]. YAP is also involved in the regulation of aging response in tissues and cells. In Werner syndrome (WS), loss of WRN protein activity triggers formation of a complex between YAP and PML, which participates in the activation of p53 and accelerates senescence of WS-derived fibroblasts [17]. In osteoarthritis, YAP plays a key role in maintaining the vitality of human mesenchymal stem cells (hMSCs) and knockout of YAP in hMSCs causes premature cellular senescence [18]. Knockdown of YAP inhibits cell proliferation and accelerates senescence in stem cells from apical papilla (SCAPs) [19]. In addition, cyclin dependent kinases, CDK4/6 are the key molecules responsible for regulating cell cycles that determine whether cells proliferate [20]. FDA-approved CDK4 and CDK6 inhibitor, palbociclib, reduces the phosphate-RB levels in adrenal small cell carcinoma cells, which in turn induces cell cycle arrest and aging [21]. Previous studies have revealed that YAP controls cellular senescence by transcriptionally regulating the expression of its downstream molecule, CDK6 in p16/p53-dependent manner [22, 23]. However, the involvement of YAP-CDK6 pathway in senescence of glioma cells remains unknown.

Through *in vitro* and *in vivo* experiments, the present study demonstrated that β-elemene inhibited cell proliferation, slowed down tumor growth, and caused cellular senescence in glioma cells by inactivation of the YAP-CDK6 signaling pathway, which provides novel insights into the application of traditional Chinese medicine such as β-elemene in the treatment of gliomas.

Materials and methods

cDNA constructs

YAP-Flag (https://www.addgene.org/66853/) and pcDNA3.1-CDK6 (http://www.addgene.org/75170/) constructs were purchased from Addgene. The constructs were verified by sequencing and used for over-expression and rescue experiments.

Cell culture and transfection

Glioma cell lines C6, DBTRG-05MG (DBTRG), and U87MG (U87) were supplied by Pro. Maojin Yao (Guangzhou Medical University, Guangzhou, China). C6 cells were grown in DMEM/F-12 (Gibco), supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco). U87MG cells were grown in DMEM (Gibco), supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco). DBTRG-05MG cells were grown in RPMI-1640 medium supplemented with 10% FBS (Gibco), and 1% penicillin/streptomycin (Gibco). All cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Appropriate plasmids (2 μg per 35-mm dish) were transfected into the cells using Lipofectamine™ 3000 transfection reagent (L3000-015, Invitrogen) according to the manufacturer's protocol. Cells were used for subsequent experiments 48-72 h after transfection.

Drugs

β-elemene (>98%) (#E4418) was purchased from Dalian Jingang Pharmaceuticals, Ltd. (Liaoning, China). A stock solution of 100 mg/mL was prepared in ethanol and stored at -4°C [14, 24].

SA-β-Gal staining

A SA-β-Gal staining kit (G1580, Solarbio, Beijing, China) was used to evaluate senescence of C6 or DBTRG cells according to the manufacturer's instructions as previously described [25, 26]. SA-β-Gal-positive cells displayed blue signals. The ratio of the number of SA-β-Gal-positive (blue) cells versus to the number of total cells was calculated as the a percentage of SA-β-Gal-positive cells [27].

Cell counting kit-8 (CCK-8) assay

Cell viability was measured using CCK-8 cell counting kit (A311-01/02, Vazyme Biotech, Nanjing, China) as previously described [28]. Cells were seeded into 96-well plates at a density of approximately 2,000 cells per well and cultured for 24-48 h. Subsequently, 10 μl CCK-8 solution was added to each well and incubated at 37°C for 2 h. The optical density of cells was measured at a wavelength of 450 nm using a microplate reader (Varioskan Flash, Thermo scientific, Waltham, MA, USA).

Western blotting

Western blotting was carried out as described previously [29]. C6, DBTRG or U87MG cells were lysed using ice-cold RIPA Buffer (P0013B, Beyotime, Shanghai, China) and incubated at 4°C for 30 min. After centrifugation at 12,000 × g for 30 min, proteins were extracted with 5 × loading buffer and boiled at 100°C for 8-10 min. The protein samples were subsequently separated using 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Life sciences, Piscataway, NJ, USA). After blocking in TBST containing 5% skimmed milk for 1 h, immunoblots were incubated with different primary antibodies overnight at 4°C. Primary antibodies included rabbit anti-caspase-3 [#13008, Cell Signaling Technology (CST), 1:1,000], rabbit anti-cleaved caspase-3 (#9579, CST, 1:1,000), rabbit anti-Lamin B1 (ab16048, Abcam, 1:1,000), rabbit anti-p53 (bs-2090R, Bioss, 1:1,000), rabbit anti-NF-κB (ab16502, Abcam, 1:1,000), rabbit anti-p-YAP (#13008, CST, 1:1,000), mouse anti-YAP (WH0010413M1, Sigma-Aldrich, 1:1,000), mouse anti-CDK6 (#3136T, CST, 1:1,000), mouse anti-β-actin (A5316, Sigma-Aldrich, WB 1:10,000) or rabbit anti-GAPDH (#2118, CST, 1:5,000) used as a loading control was detected alongside the experimental samples. Subsequently, the membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. The protein signals were detected using ECL detection kit (Bio-Rad, Hercules, CA, USA) after washing the membranes three times with TBST. Blots were analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

Immunocytochemistry

Immunofluorescence staining and quantitative analyses were performed as described previously [30]. Cells were rinsed once with PBS and fixed in 4% paraformaldehyde for 20 min. Afterward, cells were permeabilized with 0.1% Triton X-100 for 5 min and then blocked in PBS containing 5% bovine serum albumin (BSA) at room temperature for 1 h. Subsequently, cells were incubated with primary antibodies at 4°C overnight, washed 3 times with PBS and incubated with secondary antibodies at room temperature for 1 h. The primary antibodies included rabbit anti-cleaved caspase-3 (#9579, CST, 1:200), rabbit anti-Lamin B1 (ab16048, Abcam, 1:200), mouse anti-PH3 (ab14955, Abcam, 1:2,500), rabbit anti-Ki67 (AB9260, Millipore, 1:200) and mouse anti-YAP (WH0010413M1, Sigma, 1:1,000). The secondary antibodies included anti-rabbit Alexa Fluor488 (A21206, Invitrogen, 1:1,000), anti-rabbit Alexa Fluor546 (A10040, Invitrogen, 1:1,000), anti-mouse Alexa Fluor488 (A21202, Invitrogen, 1:1,000) and anti-mouse Alexa Fluor546 (A10036, Invitrogen, 1:1,000). Cells were mounted on glass slides after washing three times with PBS. Images were acquired using a fluorescence microscope (Nikon, Tokyo, Japan) or Olympus SLIDEVIEW™ VS200 microscope (Olympus, Japan) and analyzed using Image J software.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzolTM reagent (#15596026, Ambion, Shanghai, China) according to the instructions provided by the manufacturer to evaluate mRNA expression levels. A total of 2 μg RNA was transcribed into cDNA using a SuperScript™ One-Step Reverse Transcription Kit (#10928-034, Invitrogen). The expression levels of mRNA were quantified using the iTaq™ Universal SYBR® Green Supermix (#172-5122, Bio-Rad) on a real-time PCR detection System (Applied Biosystems, Alameda, CA, USA). The relative mRNA levels were quantified using the iTaq™ Universal SYBR® Green Supermix (#172-5122, Bio-Rad) on a real-time PCR detection System (Applied Biosystems, Alameda, CA, USA) and were presented as ΔCt = Ct gene - Ct reference, and the fold change of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

β-actin was used as the endogenous control. The primers used in this study were synthesized by Shanghai Sangon Biotech and presented as follows: *p16*, 5'-TGCAGATAGACTAGCCAGGGGA-3' and 5'-CTTCCAGCAGTGCCCGCA-3' [31]; *p21*, 5'-GTGAGACACCAGAGTGCAAGA-3' and 5'-ACAGCGATATCGAGACACTCA-3' [32]; *p53*, 5'-CACAGTCGGATATGAGCATC-3' and 5'-GTCGTCCAGATACTCAGCAT-3' [32]; *IL-1β*, 5'-TACCTATGTCTTGCCCGTGG-3' and 5'-TAGCAGGTCGTCATCATCCC-3' [32]; *IL-6*, 5'-CTGCTCTGGTCTTCTGGAGT-3' and 5'-TGGAAGTTGGGGTAGGAAGG-3' [32]; *IL-10*, 5'-AGACCCACATGCTCCGAGAG-3' and 5'-GGGCATCACTTCTACCAGGT-3' [32]; *TNF-α*, 5'-TCCCAACAAGGAGGAGAAGT-3' and 5'-TGGTATGAAGTGGCAAATCG-3' [32]; *NF-κB*, 5'-CATCCACCTTCATGCTCAGC-3' and 5'-CCACCACATCTTCCTGCTTG-3' [32]; *MMP-2*, 5'-CAACGGTCGGGAATACAGCA-3' and 5'-AGGCCATGGGTTGGATCTTCA-3' [33]; *MMP-9*, 5'-AACGTCTTTCACTACCAAGACAAG-3' and 5'-TTGTGGAAACTCACACGCCA-3' [33]; *β-actin*, 5'-AAGTCCCTCACCCTCCCAAAAG-3' and 5'-AAGCAATGCTGTCACCTTCCC-3' [34].

In vivo glioma model and administration of β-elemene

Six-week-old BALB/C-nude male mice weighing approximately 18 g were purchased from GemPharmatech Co., Ltd. (Nanjing, China). A total of 3×10^6 C6 cells were suspended in serum-free DMEM medium and inoculated subcutaneously into nude mice. Mice were randomly divided into two groups: the vehicle control group and the β-elemene group. Mice were intraperitoneally injected with vehicle control or 100 mg/kg/d β-elemene, respectively. Tumor size was measured after every 3 days to evaluate tumor growth. Tumor volume was calculated as follows: Tumor volume = $(\text{tumor length})^2 \times \text{tumor width} \times 0.5$. All nude mice were euthanized 23 days after injection with the drug.

Statistical analysis

Data values were expressed as mean ± standard error of the mean (SEM), which was derived from at least three independent experiments. Student's *t*-test or analysis of variance (ANOVA) was used to compare means between groups and statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., CA, USA). A P value of < 0.05 was considered statistically significant.

Results

Low concentration of β-elemene promotes premature senescence of C6 cells

Cell growth was initially evaluated using C6 cells, a glioma cell line to determine the effects of β-elemene on glioma cells. The growth of C6 cells was inhibited in a concentration-dependent and time-dependent manner (**Figure 1A**). In addition, when the C6 cells were treated with β-elemene at a concentration of 50 μg/mL for 1 day, the cells began to die, and cell death was more pronounced when cells were treated with β-elemene at a concentration of 100 μg/mL for 2 days (**Figure 1A**). Afterward, cell viability of glioma cell lines, U87, DBTRG and C6 cells treated with β-elemene for 1 day was examined using CCK-8 assay. The cell viability of U87, DBTRG and C6 cells was significantly decreased in a dose-dependent manner (**Figure 1B-D**). The effects of β-elemene on C6 cell senescence were investigated because cell growth inhibition is one of the key features of senescent cells. Results of SA-β-Gal staining to detect SA-β-Gal activity, which is a common standard method for detecting senescence [35], revealed that the percentage of SA-β-Gal positive C6 cells was significantly increased by β-elemene treatment in a dose- and time-dependent manner, with the highest effect recorded at a concentration of 10 μg/mL for 2 days (**Figure 1E**, **1F**). Furthermore, the percentages of SA-β-Gal positive C6 cells were decreased at concentrations of 50 μg/mL and 100 μg/mL for 2 days, and were lower than the percentage recorded at a concentration of 10 μg/mL for 2 days, which might be due to an increase in cell death (**Figure 1E**, **1F**). Therefore, a concentration of 10 μg/mL β-elemene was considered as an appropriate concentration for inducing C6 cell senescence. C6 cells were treated with β-elemene at different time intervals to establish the optimal action time of β-elemene. Cells began to die when C6 cells were treated with 10 μg/mL β-elemene for more than 2 days (**Figure 1G**). Therefore, a concentration of 10 μg/mL β-elemene for 2 days was selected as the optimal condition for investigating cell senescence in subsequent experiments. The percentage of SA-β-Gal positive C6 cells was approximately 70% at the optimal concentration (**Figure 1H**, **1I**). Overall, the results suggested that a low concentration of

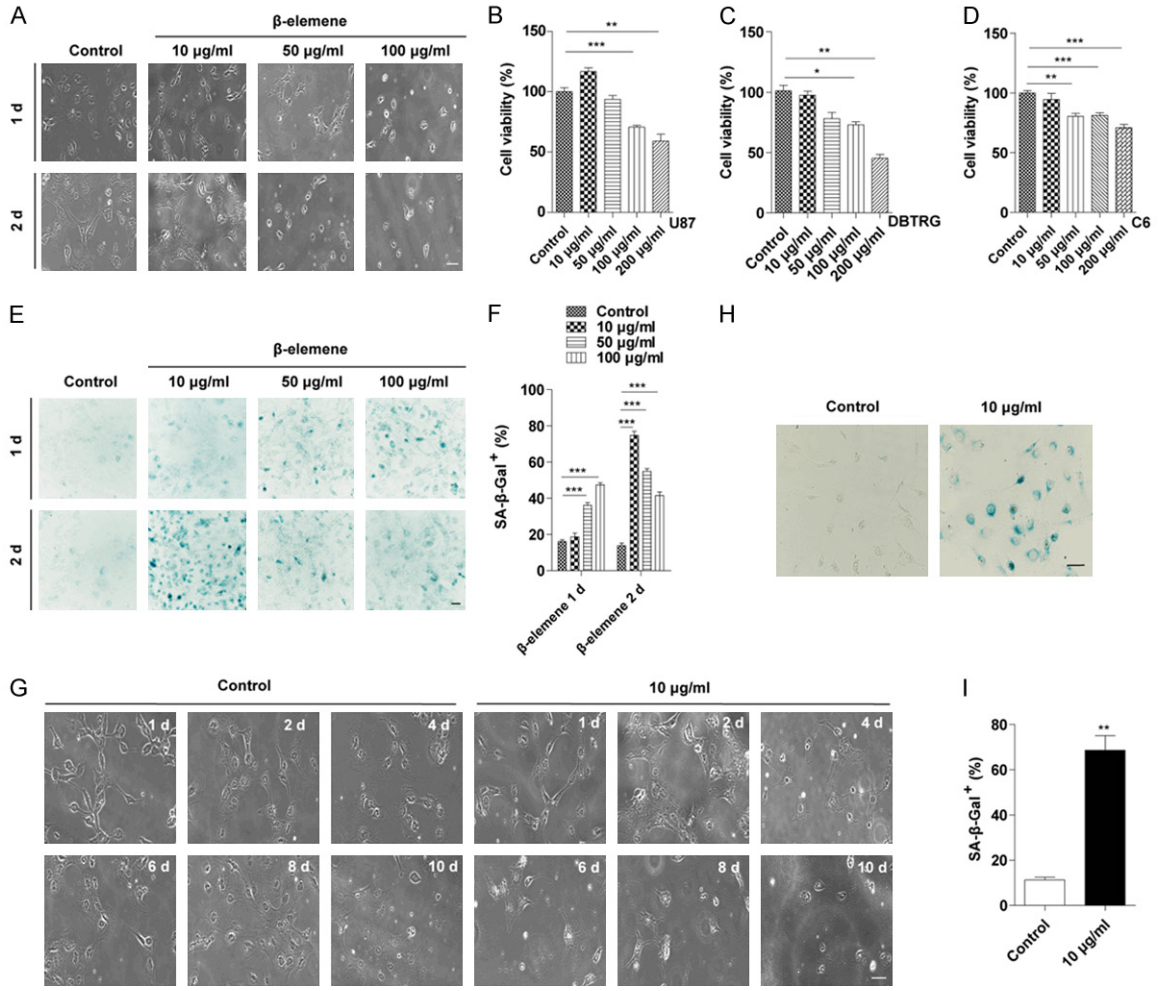

Figure 1. β-elemene promoted the premature senescence of C6 cells. (A) Representative images of C6 cells in the bright field treated with control or different concentrations of β-elemene for 1 day or 2 days. (B-D) The effects of β-elemene (treated at 10 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL for 1 day) on U87 (B), DBTRG (C) and C6 (D) cell viability detected by CCK8 (n=5). (E) Representative images of SA-β-Gal staining of C6 cells treated with control or different concentrations of β-elemene for 1 day or 2 days. (F) Quantification of the percentage of SA-β-Gal$^+$ C6 cells over total cells as shown in (E) (n=15). (G) Representative images of C6 cells in the bright field treated with control or 10 μg/mL β-elemene for 1, 2, 4, 6, 8, or 10 days. (H) Representative images of SA-β-Gal staining of C6 cells treated with control or 10 μg/mL β-elemene for 2 days. (I) Quantification of the percentage of SA-β-Gal$^+$ C6 cells over total cells as shown in (H) (n=15). d=day. Scale bars, 20 μm. Data were mean ± s.e.m, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

β-elemene induced premature senescence in glioma cells.

Low concentration of β-elemene induces changes in senescent makers in glioma cells

Biochemical cellular analyses were performed to further investigate β-elemene-induced senescence in C6 cells. Cells exhibit increased expressions of *p16*, *p21*, and *p53* [36-38], and decreased expression of Lamin B1 during aging [39, 40]. The mRNA levels of *p16*, *p21*, and *p53* were significantly increased in C6 cells treated with β-elemene when compared to the control cells (**Figure 2A-C**). Western blot results revealed that the protein level of *p53* treated with β-elemene was increased significantly, whereas Lamin B1 expression was decreased significantly (**Figure 2D-F**). Furthermore, immunostaining results revealed a significant decrease in Lamin B1 expression in C6 cells treated with β-elemene (**Figure 2G**). Inhibition of cell proliferation is one of the key characteristics of cell senescence. PH3 and Ki67 (a cell marker of proliferation) staining showed that PH3 and Ki67 positive percentages were sig-

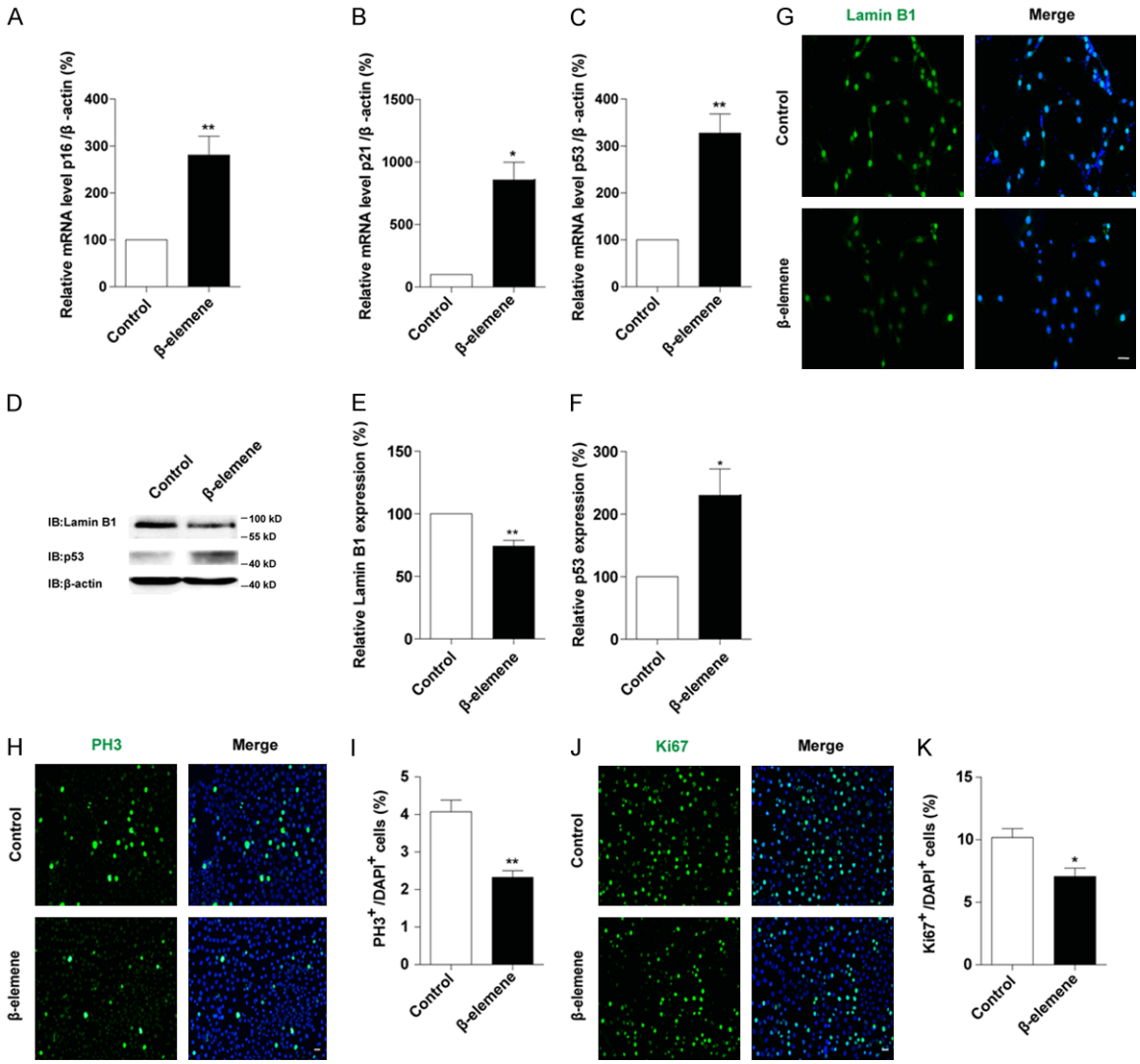

Figure 2. Low concentration of β-elemene induced the changes of senescence makers in glioma cells. (A-C) qPCR analysis of p16 (n=8), p21 (n=3) and p53 (n=5) mRNA levels in C6 cells treated with control or 10 μg/mL β-elemene for 2 days. (D) Western blot detected the expression of p53 and Lamin B1 in C6 cells treated with control or 10 μg/mL β-elemene for 2 days. (E, F) Quantification of Lamin B1 (E, n=7) and p53 (F, n=5) expression as shown in (D). (G) Immunostaining analysis of Lamin B1 (green) in C6 cells treated with control or 10 μg/mL β-elemene for 2 days. (H, J) Immunostaining analysis of PH3 (green) (H) and Ki67 (green) (J) in C6 cells treated with control or 10 μg/mL β-elemene for 2 days. (I, K) Quantification of the percentage of $PH3^+$ (I) or $Ki67^+$ (K) C6 cells over total cells as shown in (H, J) (n=15). d=day. Scale bars, 20 μm. Data were mean ± s.e.m, $^*P < 0.05$, $^{**}P < 0.01$.

nificantly decreased by treated with 10 μg/mL β-elemene for 2 days (**Figure 2H-K**), which indicated that cell proliferation was inhibited in C6 cells treated with β-elemene. These results suggested that a low concentration of β-elemene induced the changes of senescent makers in glioma cells.

Low concentration of β-elemene induces expression of SASP in glioma cells

The expression of SASP is a common outcome of cell senescence [41-43], and factors associated with SASP, such as cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6), pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α), matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) [44], and transcription factor, nuclear factor-κB (NF-κB) [45], usually are increased during cell senescence. The mRNA levels of *IL-1*, *IL-6*, *TNF-α*, *NF-κB*, *MMP-2* and *MMP-9* were significantly increased in C6 cells after treatment with β-elemene (**Figure 3A-F**). Furthermore, the protein level of NF-κB was significantly increased

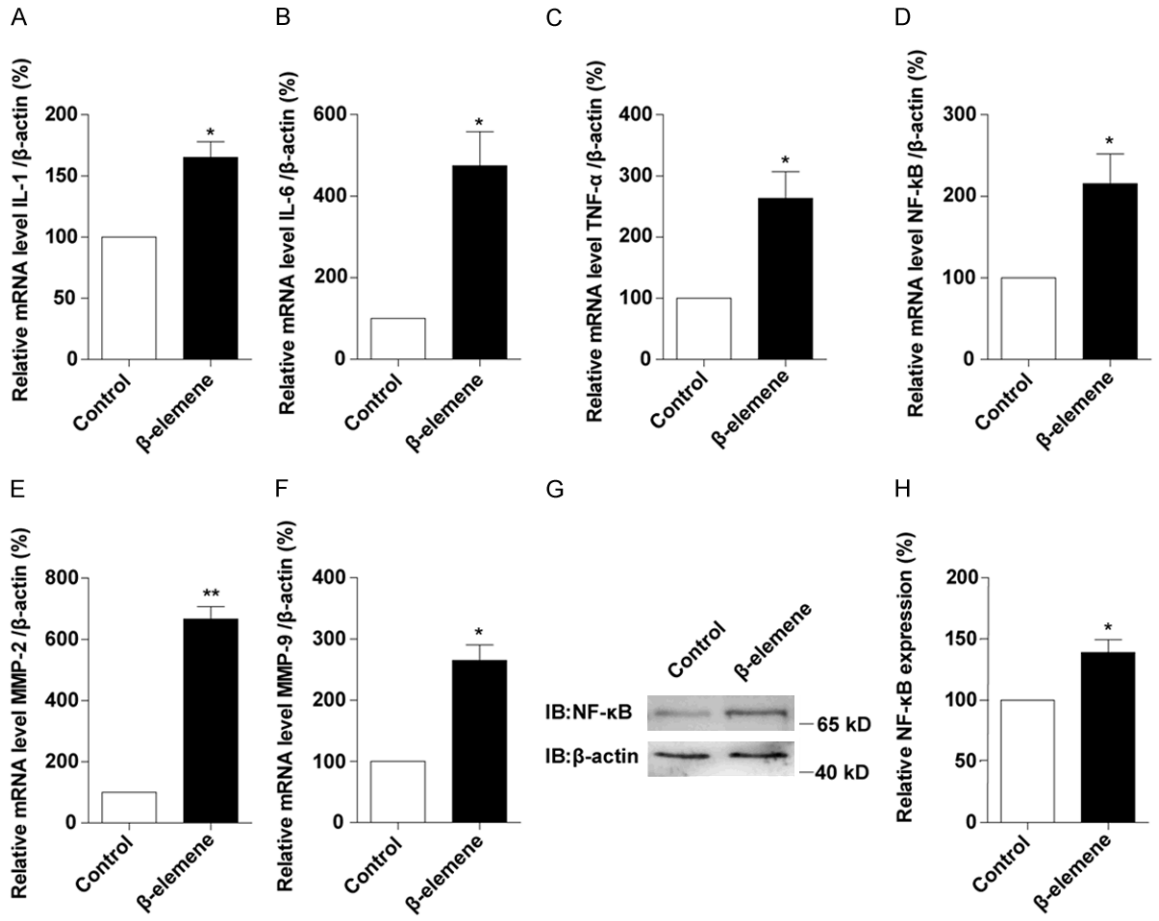

Figure 3. Low concentration of β-elemene induced the senescence-associated secretory phenotype in glioma cells. (A-F) qPCR analysis of IL-1 (n=6), IL-6 (n=3), TNF-α (n=6), NF-κB (n=6), MMP-2 (n=3) and MMP-9 (n=3) mRNA levels in C6 cells treated with control or 10 μg/mL β-elemene for 2 days. (G) Western blot detected the expression of NF-κB in C6 cells treated with control or 10 μg/mL β-elemene for 2 days. (H) Quantification of NF-κB expression as shown in (G) (n=3). Data were mean ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

in C6 cells treated with β-elemene (**Figure 3G**, **3H**). Taken together, these results further suggested that a low concentration of β-elemene induced senescence in glioma cells.

Low concentration of β-elemene induces cell senescence but does not cause apoptosis in glioma cells

Anti-apoptosis is another characteristic of senescent cells. Cell viability, western blot and immunostaining analyses were performed to determine whether C6 cell senescence induced by β-elemene (10 μg/mL for 2 days) caused cell apoptosis. No statistically significant difference was observed in cell viability of C6 cells treated with β-elemene at a concentration of 10 μg/mL for 1 and 2 days (**Figure 4A**, **4B**). Western blot analyses results revealed that the protein level of Lamin B1 was decreased; however, cleaved-caspase-3 (c-caspase-3)/caspase-3 level remained unchanged, indicating that cell apoptosis did not occur in these senescent C6 cells (**Figure 4C-E**). In addition, immunostaining of c-caspase-3 revealed that a treatment of 10 μg/mL β-elemene for 2 days did not increase the percentage of c-caspase-3 positive cells. Nevertheless, etoposide, a DNA-damage drug [46], which can also be used to induce cell senescence and has been demonstrated to cause cell apoptosis at high concentrations [47], caused cell apoptosis in C6 cells (**Figure 4F**, **4G**). These results suggested that a low concentration of β-elemene induced senescence in glioma cells, but does not induce apoptosis in glioma cells.

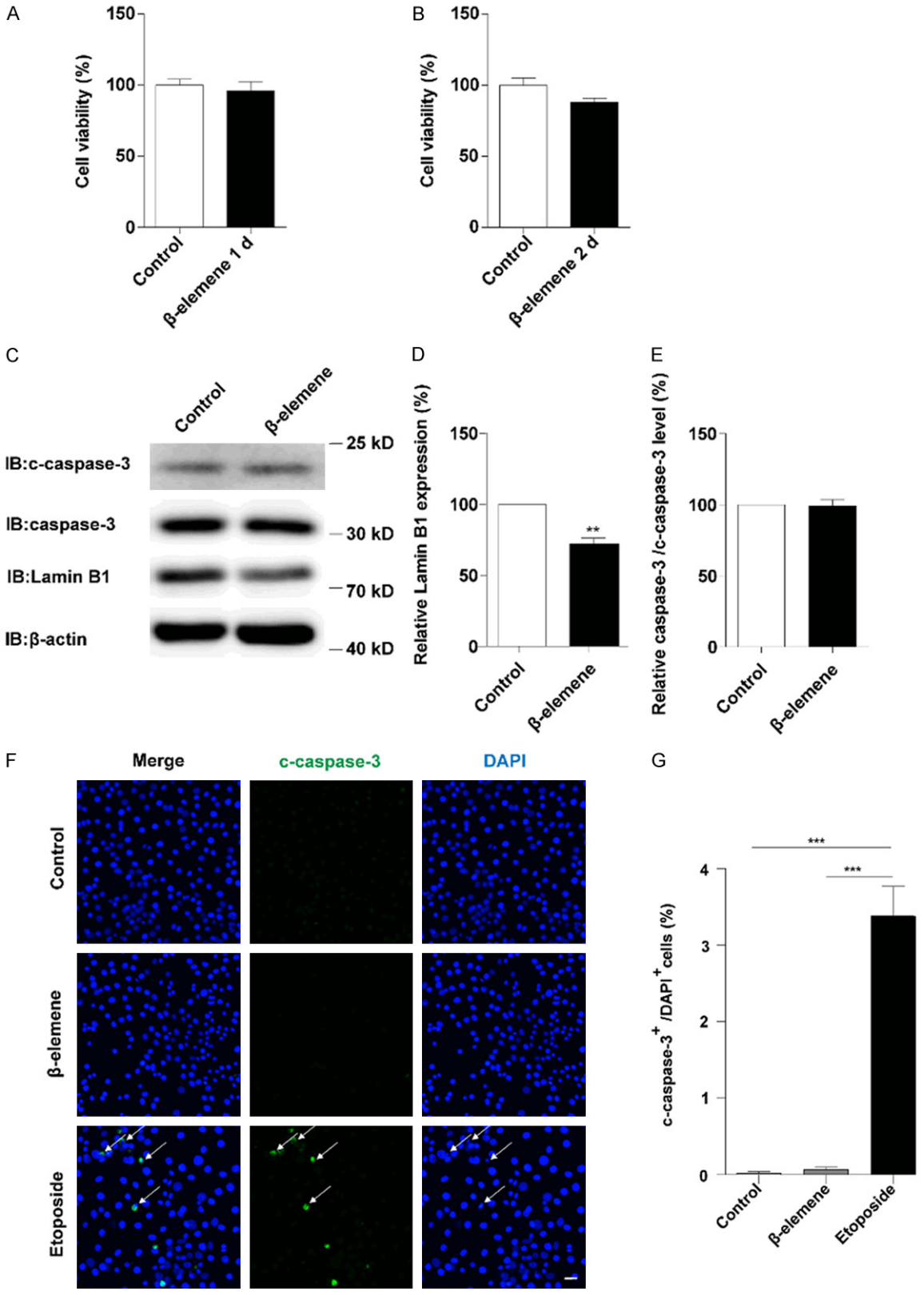

Figure 4. Low concentration of β-elemene induced senescence of glioma cells, but didn't cause apparent cell apoptosis. (A, B) The effects of 10 μg/mL β-elemene for 1 day or 2 days on C6 cell viability detected by CCK8 (n=3). (C) Western blot detected the expression of and c-caspase-3, caspase-3 and Lamin B1 in cells treated with control or

10 μg/mL β-elemene at for 2 d. (D, E) Quantification of Lamin B1 and c-caspase-3/caspase-3 level as shown in (C) (n=4). (F) Immunostaining analysis of c-caspase-3 (green) in C6 cells treated with control and 10 μg/mL β-elemene for 2 days or with 12 μM etoposide for 1 day, and recovered for 4 days. (G) Quantification of the percentage of c-caspase-3$^+$ C6 cells over total cells as shown in (F) (n=15). d=day. Scale bars, 20 μm. Data were mean ± s.e.m, $^{**}P < 0.01$, $^{***}P < 0.001$.

High concentration of β-elemene causes apoptosis in glioma cells coupled with cell senescence

The percentage of SA-β-Gal positive C6 cells was less than 40% when cells were treated with 100 μg/mL β-elemene for 1 day (Figure S1A, S1B), which could be due to cell death. Western blot analyses revealed that treatment with 100 μg/mL β-elemene decreased the expression of Lamin B1, increased expression of p53, and considerably increased the level of c-caspase-3/caspase-3, which suggested that a high concentration of β-elemene induced cell apoptosis coupled with cell senescence (Figure S1C-F). Furthermore, the percentage of SA-β-Gal positive C6 cells was similar to the control when C6 cells were treated with β-elemene at a concentration of 100 μg/mL for 2 days (Figure S1G, S1H). Western blot and immunostaining results revealed that the expression of Lamin B1 remained unchanged in C6 cells treated with 100 μg/mL β-elemene for 2 days (Figure S1I-L); however, immunostaining results revealed that the percentage of c-caspase-3 cells was increased significantly (Figure S1M, S1N). These results suggested that a high concentration of β-elemene induced cell apoptosis coupled with cell senescence in glioma cells.

Low concentration of β-elemene promotes senescence in other glioma cell lines

DBTRG cells were investigated to determine whether β-elemene-induced C6 senescence was also applicable to other glioma cell lines. As expected, the percentage of SA-β-gal positive DBTRG cells was significantly increased to approximately 50% when DBTRG cells were treated with 10 μg/mL β-elemene for 2 days (**Figure 5A**, **5B**). Western blot results further revealed that the protein level of Lamin B1 in DBTRG cells treated with β-elemene was significantly decreased (Figure S2A, S2B). In addition, the mRNA levels of *p16*, *p21*, *p53*, *IL-1*, *IL-6* and *NF-κB* were significantly increased in C6 cells after treatment with β-elemene (**Figure 5C-H**). These results suggested that a low concentration of β-elemene also induced the senescence in other glioma cells, DBTRG cells.

β-elemene promotes senescence in glioma cells through inactivation of YAP-CDK6 signaling in vitro

Previous studies have revealed that inhibition of YAP-CDK6 signaling promotes senescence in human fibroblast cells [22]. Therefore, we subsequently investigated whether the YAP-CDK6 signaling pathway mediated β-elemene-induced senescence in glioma. As expected, the protein levels of p-YAP, YAP, Lamin B1 and CDK6 were decreased, whereas p53 expression was increased in β-elemene-induced senescent C6 cells (**Figure 6A-F**). The protein levels of Lamin B1 and CDK6 were also decreased in β-elemene-induced senescent DBTRG cells (Figure S2A-C). Moreover, the mRNA level of CDK6 was decreased in β-elemene-induced senescent C6 cells (**Figure 6G**), suggesting that YAP-CDK6 pathway could be involved in glioma senescence. To further verify the hypothesis, we subsequently investigated whether YAP overexpression in senescent C6 cells could rescue β-elemene-induced senescence. Results of the analyses revealed that overexpression of YAP indeed rescued β-elemene-induced inhibition of cell proliferation (**Figure 7A-C**) and senescence in C6 cells (**Figure 7D**, **7E**). Furthermore, overexpression of YAP significantly rescued the decrease of CDK6 and Lamin B1 expressions induced by β-elemene treatment (**Figure 7F-H**). These results suggested that overexpression of YAP rescued β-elemene-induced senescence in glioma cells. Similarly, overexpression of CDK6 rescued β-elemene-induced senescence in C6 cells (**Figure 8A-C**), and significantly rescued β-elemene-induced decrease of Lamin B1 expression (**Figure 8D-F**). Taken together, these results suggested that β-elemene induced senescence in glioma cells through inactivation of YAP-CDK6 signaling pathway.

β-elemene induced the senescence in glioma cells in vivo through inactivation of YAP-CDK6 signaling pathway.

In view of *in vitro* experiments showing the effect of β-elemene on glioma cells, we further

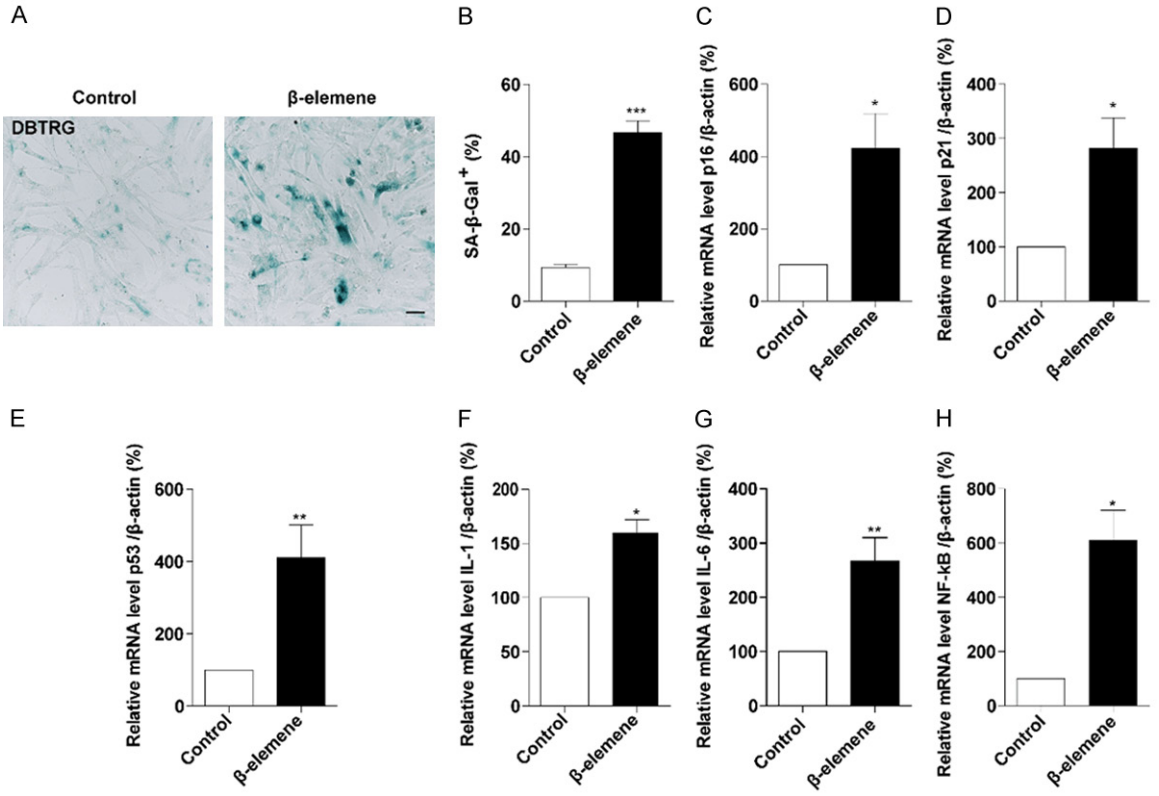

Figure 5. Low concentration of β-elemene promoted the senescence of DBTRG-05MG glioma cells. (A) Representative images of SA-β-Gal staining of DBTRG-05MG cells treated with control or 10 μg/mL β-elemene for 2 d. (B) Quantification of the percentage of SA-β-Gal$^+$ C6 cells over total cells as shown in (A) (n=15). (C-H) qPCR analysis of p16 (C, n=4), p21 (D, n=4) and p53 (E, n=5), IL-1 (F, n=3), IL-6 (G, n=5) and NF-κB (H, n=4) mRNA levels in C6 cells treated with control or 10 μg/mL β-elemene for 2 days. Scale bar, 20 μm. Data were mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

confirmed the effect of β-elemene in xenograft glioma models. C6 glioma cells (3×10^6) were subcutaneously injected into nude mice, and then the vehicle control or β-elemene (100 mg/kg) were injected intraperitoneally to treat tumor-bearing mice for up to 23 days. Compared with the vehicle control group, glioma in the β-elemene-treated group showed significant growth inhibition (**Figure 9A**). Moreover, the tumor size in the β-elemene-treated group was significantly decreased, compared to the control group (**Figure 9B**). Consistent with the results of *in vitro* experiments, the percentage of SA-β-Gal-positive glioma cells was increased significantly by β-elemene treatment, compared with the vehicle control group (**Figure 9C**, **9D**). Furthermore, western blot showed that the p-YAP, YAP, Lamin B1 and CDK6 protein expression levels were significantly decreased by β-elemene treatment, compared with the vehicle control treatment group (**Figure 9E-I**). In addition, immunostaining also showed that the immunofluorescence intensity of Lamin B1 expression was significantly decreased by β-elemene treatment, compared with the control treatment (**Figure 9J**). Taken together, these results suggested that β-elemene treatment also induced the senescence in glioma cells *in vivo* through inactivation of YAP-CKD6 signaling pathway, which might be responsible for inhibition of glioma growth.

Discussion

The present study revealed that β-elemene induced glioma senescence through inactivation of YAP-CDK6 signaling both *in vitro* and *in vivo* (**Figure 10**). This study has revealed a new role and mechanisms of β-elemene in glioma cell senescence, which will enhance our understanding of the mechanisms underlying glioma senescence and provide novel ideas for glioma treatment.

Notably, β-elemene is highly effective in clinical application to treat a wide variety of tumors [48-51]; however, several clinical and experi-

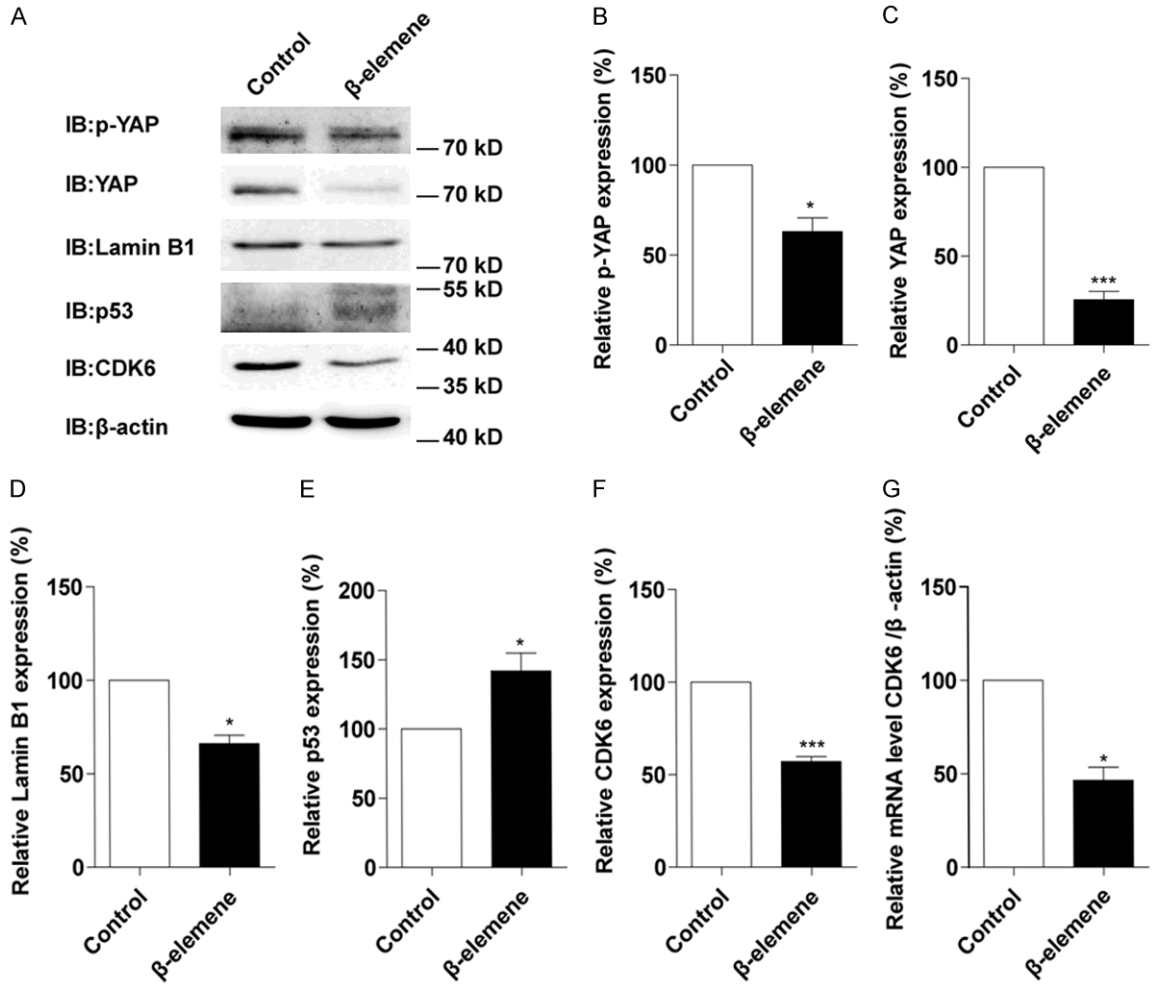

Figure 6. Low concentration of β-elemene inactivated YAP-CDK6 signaling in C6 glioma cells. (A) Western blot detected the expression of p-YAP, YAP, Lamin B1, p53 and CDK6 in C6 cells treated with control or 10 μg/mL β-elemene for 2 days. (B-F) Quantification of relative p-YAP (B), YAP (C), Lamin B1 (D), p53 (E) and CDK6 (F) levels as shown in (A) (n=3). (G) qPCR analysis of relative CDK6 mRNA level in C6 cells treated with control or 10 μg/mL β-elemene for 2 days (n=3). Data were mean ± s.e.m, $^{*}P < 0.05$, $^{***}P < 0.001$.

mental studies both *in vivo* and *in vitro* have largely focused on the anti-proliferation functions [52], pro-apoptotic effects [53], and how to enhance radiotherapeutic [54] and chemotherapeutic sensitivity of β-elemene [55]. Killing of glioma cells directly [2, 3] has not yielded promising effects because gliomas exhibit very strong heterogeneity. Considering that cell proliferation is essential for tumorigenesis, blocking cell cycle progression and triggering extensive senescence in glioma cells could be beneficial in the prevention of cancer development [7, 56, 57].

In the present study, we found that a low concentration of β-elemene inhibited growth of glioma cells but did not affect its cell viability *in vitro*. Multiple senescence markers have been widely used to identify senescent cells (Vizioli & Adams, 2016) [7, 58], including accumulating β-galactosidase and cyclins such as p16, p21, and p53 [36, 59]. SASP that is associated with aging response is expressed after extensive changes in genes such as inflammatory cytokines and chemokines [60]. We found that the expression of SASP genes (IL-1, IL-6, TNF-α, NF-κB, MMP-2, MMP-9 and NF-κB) remarkably increased upon treatment with β-elemene, which implies that low concentration of β-elemene induces senescence in glioma cells. The present study also revealed that an apoptotic phenotype in glioma cells was gradually

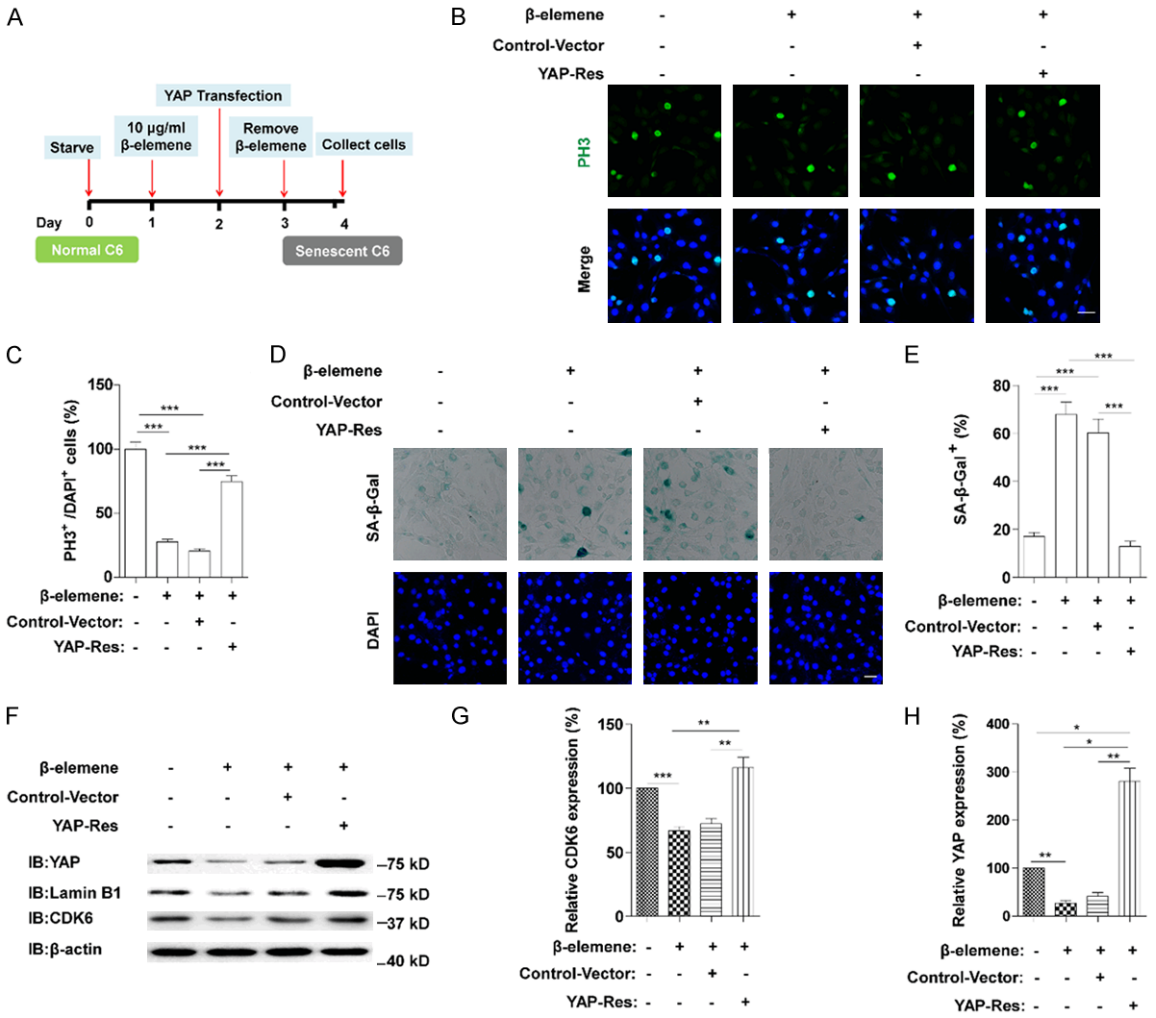

Figure 7. Overexpression of YAP partially rescued the senescence phenotypes of C6 cells induced by β-elemene. (A) The schematic of YAP transfection in senescent C6 cells. (B) Representative images of PH3 (green) staining of control C6 cells, senescent C6 cells (treated with 10 μg/mL β-elemene for 2 days) without transfection, or senescent C6 cells (treated with 10 μg/mL β-elemene for 2 days) transfected with Flag or YAP-Flag plasmid (YAP-Res). (C) Quantification of the percentage of $PH3^+$ cells over total cells as shown in (B) (n=15). (D) Representative images of SA-β-Gal staining of C6 cells under various conditions. (E) Quantification of the percentage of SA-β-Gal^+ cells over total cells as shown in (D) (n=15). (F) Western blot detected the expression of YAP, Lamin B1, and CDK6 in C6 cells under various conditions. (G, H) Quantification of CDK6 (G) and YAP (H) level as shown in (F) (n=10). Scale bars, 20 μm. Data were mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

expressed in a time-dependent manner under a high concentration of β-elemene treatment *in vitro*, which was consistent with previous reports that β-elemene could trigger tumor cell apoptosis [1]. Moreover, a widespread anti-cancer drug, etoposide, which has also been used to induce cell senescence, activates apoptosis at high concentrations [47, 61].

Current studies have revealed that cell senescence is predominantly caused by oncogene mutation, dysfunctional telomeres, and DNA damage [62]. The loss of oncogenes and the activation of tumor suppressor could evoke stagnation of cell cycles in malignant cells [36], thereby interrupting tumor formation and progression [63, 64], and are therefore considered ideal outcomes during cancer treatment [65, 66]. Strikingly, as a powerful proto-oncogene [67-69], down-regulation of YAP expression in senescent glioma cells is caused by β-elemene. According to a previous study, in senescent IMR90 human fibroblasts, the YAP-CDK6 signaling pathway was inhibited and

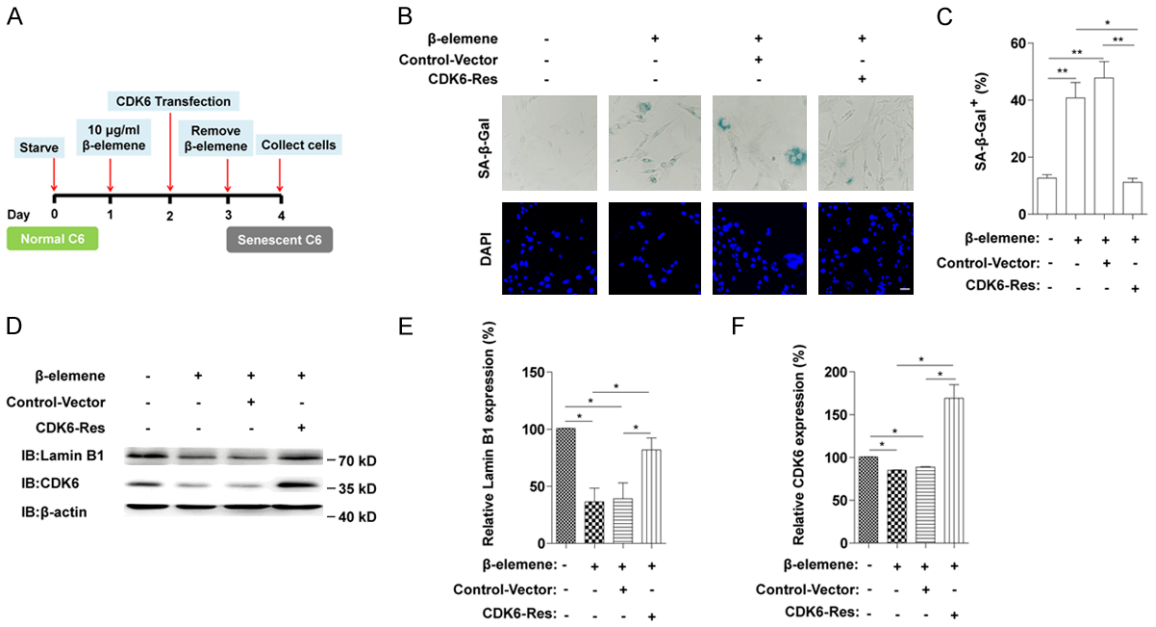

Figure 8. Overexpression of CDK6 partially rescued the senescence phenotypes of C6 cells induced by β-elemene. (A) The schematic of CDK6 transfection in senescent C6 cells. (B) Representative images of SA-β-Gal staining of control C6 cells, senescent C6 cells (treated with 10 μg/mL β-elemene for 2 days) without transfection, or senescent C6 cells (treated with 10 μg/mL β-elemene for 2 days) transfected with pcDNA3.1 or pcDNA3.1-CDK6 plasmid (CDK6-Res). (C) Quantification of the percentage of SA-β-Gal$^+$ cells over total cells as shown in (B) (n=15). (D) Western blot detected the expression of Lamin B1 and CDK6 in C6 cells under various conditions. (E, F) Quantification of Lamin B1 (E) and CDK6 (F) level as shown in (D) (n=10). Scale bar, 20 μm. Data were mean ± s.e.m. $^*P < 0.05$, $^{**}P < 0.01$.

CDK6 expression was downregulated, which causes cell cycle arrest [22]. In *in vitro* and *in vivo* experiments, we found that β-elemene could mediate senescence in glioma cells through inactivation of the YAP-CDK6 signaling pathway. Based on an evolutionary perspective, senescence in cancer cells is a feasible measure of preventing early tumor growth [12], and enhancement of chemotherapy and radiotherapy efficiency in killing tumor cells [70]. However, long-term aging could cause chronic inflammation, which in turn increases neovascularization to promote tumor development [71], paracrine immune growth factors to establish an immunosuppressive microenvironment and secrete chemokines that facilitate escape of cancer cells from tumor surveillance [72], thereby increasing the formation of secondary tumors or occurrence of cancer relapses [73]. We found that β-elemene treatment could induce the senescence of glioma cells and significantly inhibit growth of xenograft glioma, and thus senescence of glioma cells induced by β-elemene is an effective strategy to curb the development of glioma, which presents a promising glioma therapy for future studies. Nevertheless, it is noteworthy that the persistence of therapy-induced senescent cells is detrimental [74, 75]. A few studies have proposed the concept of two-step anti-cancer strategy, that is, inducing glioma cell senescence after chemoradiation followed by serotherapy [7], which could result in tumor stalling and initial tumor regression. In the current clinical therapies, β-elemene is mostly used as an ancillary drug with other medications or with radiotherapy, such as in combination with Class I anti-tumor drug platinum-based chemotherapy or radiotherapy for non-small cell lung cancer, and cetuximab for deficient colorectal cancer, which effectively increases sensitivity of the drug and prolongs the life of patients. This could be the most feasible solution to enhance therapeutic efficiency and patient prognosis, but application of such strategies in the treatment of glioma requires further research and testing.

In summary, the present study has demonstrated that β-elemene-induced glioma cell senescence is mediated by inactivation of the YAP-CDK6 signaling pathway. Senescence in glioma

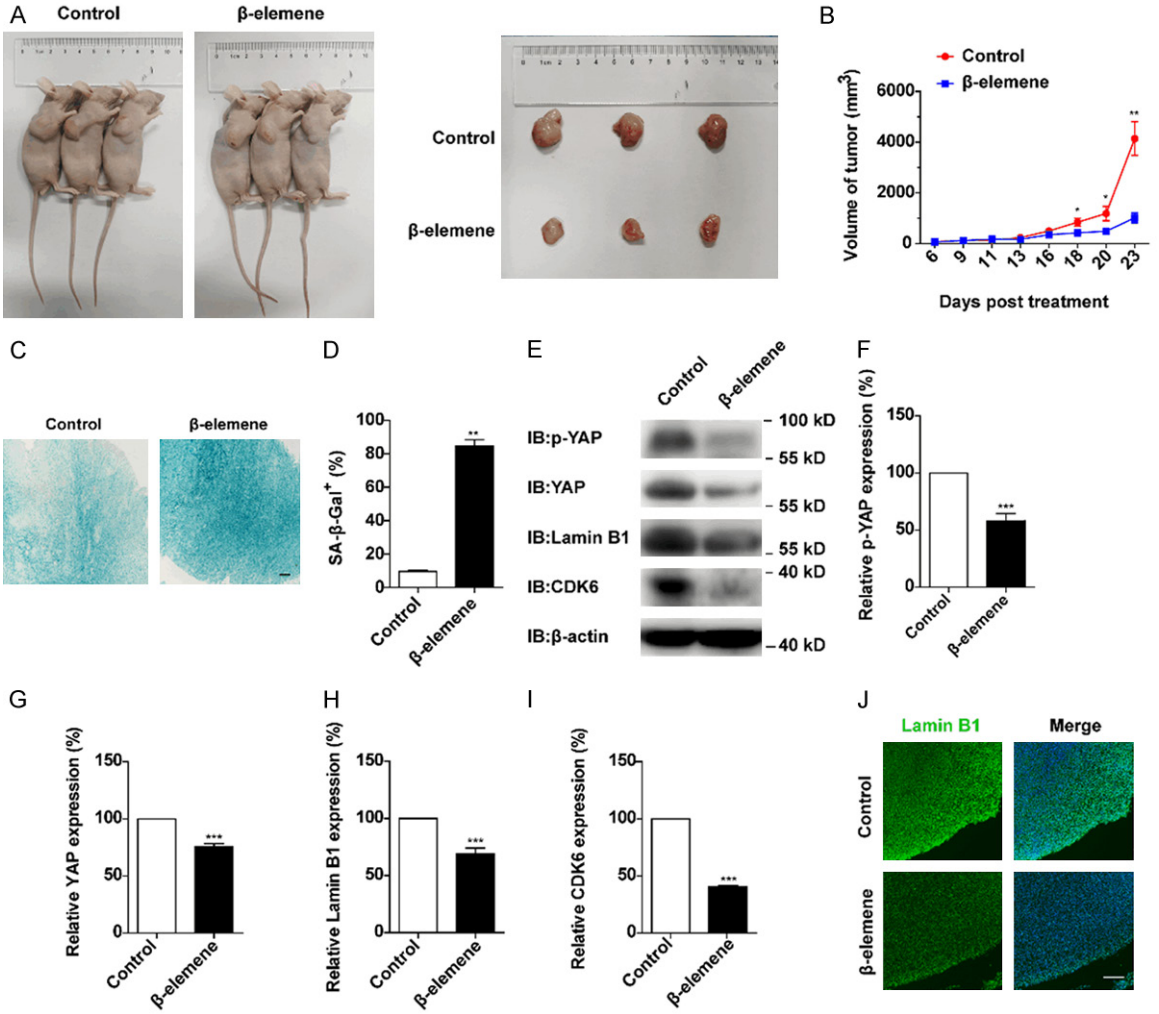

Figure 9. β-elemene induced the senescence in glioma cells in vivo through inactivation of YAP-CDK6 signaling pathway. (A) Representative images of the vehicle- and β-elemene-treated (100 mg/kg) xenografts derived from nude mice were shown. (B) Tumor volume curve of the vehicle- and β-elemene-treated (100 mg/kg) xenografts derived from nude mice as shown in (A) (n=5 each group). (C) Representative images of SA-β-Gal staining of glioma tumors treated with vehicle control or 100 mg/kg β-elemene on 23 days post treatment. (D) Quantification of SA-β-Gal staining (percentage of cells) as shown in (C) (n=3). (E) Western blot detected the expression of p-YAP, YAP, Lamin B1 and CDK6 in glioma tumors treated with vehicle control or 100 mg/kg β-elemene on 23 days post treatment. (F-I) Quantification of relative p-YAP (F), YAP (G), Lamin B1 (H) and CDK6 (I) levels as shown in (E) (n=3). (J) Immunohistochemistry for Lamin B1 (green) staining in tumor treated with vehicle control or 100 mg/kg β-elemene on 23 days post treatment. Scale bar, 100 μm. Data were mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$.

cells induced by β-elemene that is obtained from traditional Chinese medicine extracts can be a potential treatment intervention for gliomas. The present study also provides new insights into future research of β-elemene in the treatment of gliomas.

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Disclosure of conflict of interest

None.

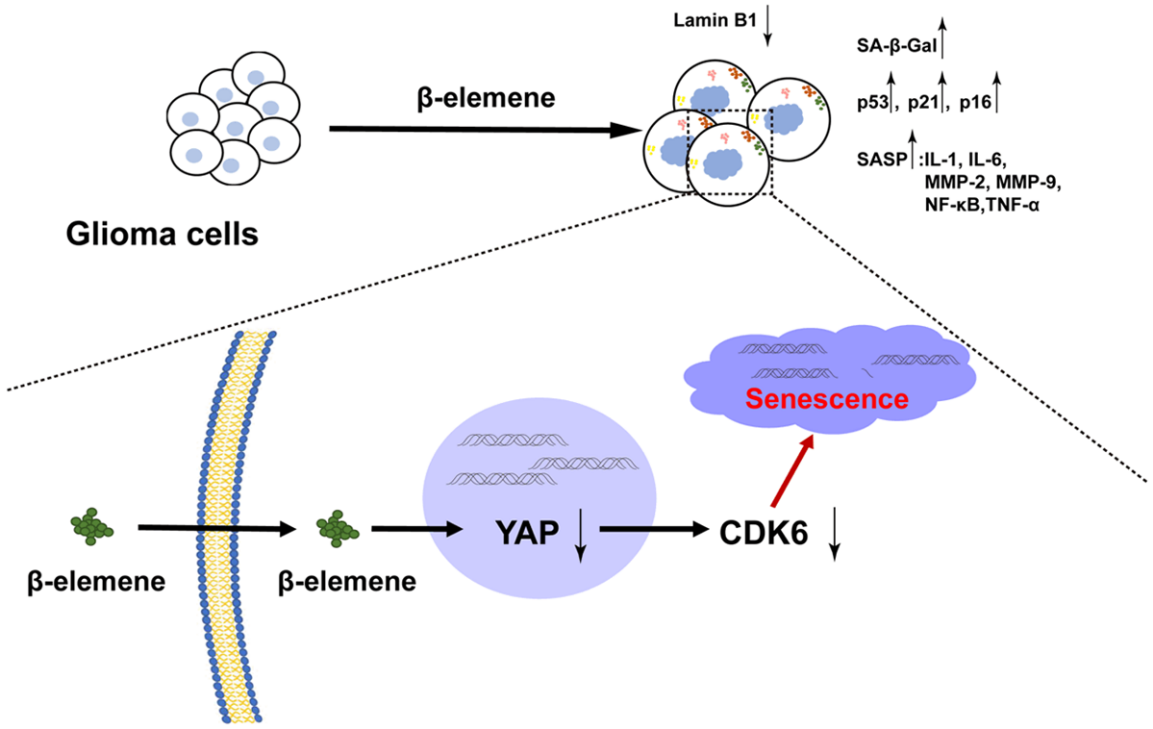

Figure 10. Working model of β-elemene-induced senescence of glioma cells. β-elemene treatment inactivates YAP-CDK6 pathway in glioma cells, which may result into typical characteristics of senescent cells, including reduced cell proliferation, hypertrophy morphology, increased level of SA-β-Gal activity, increased expressions of p16, p21, and p53, upregulation of several senescence-associated genes such as IL-1, IL-6, MMP-2, MMP-9, NF-κB, and TNF-α, and downregulation of Lamin B1.

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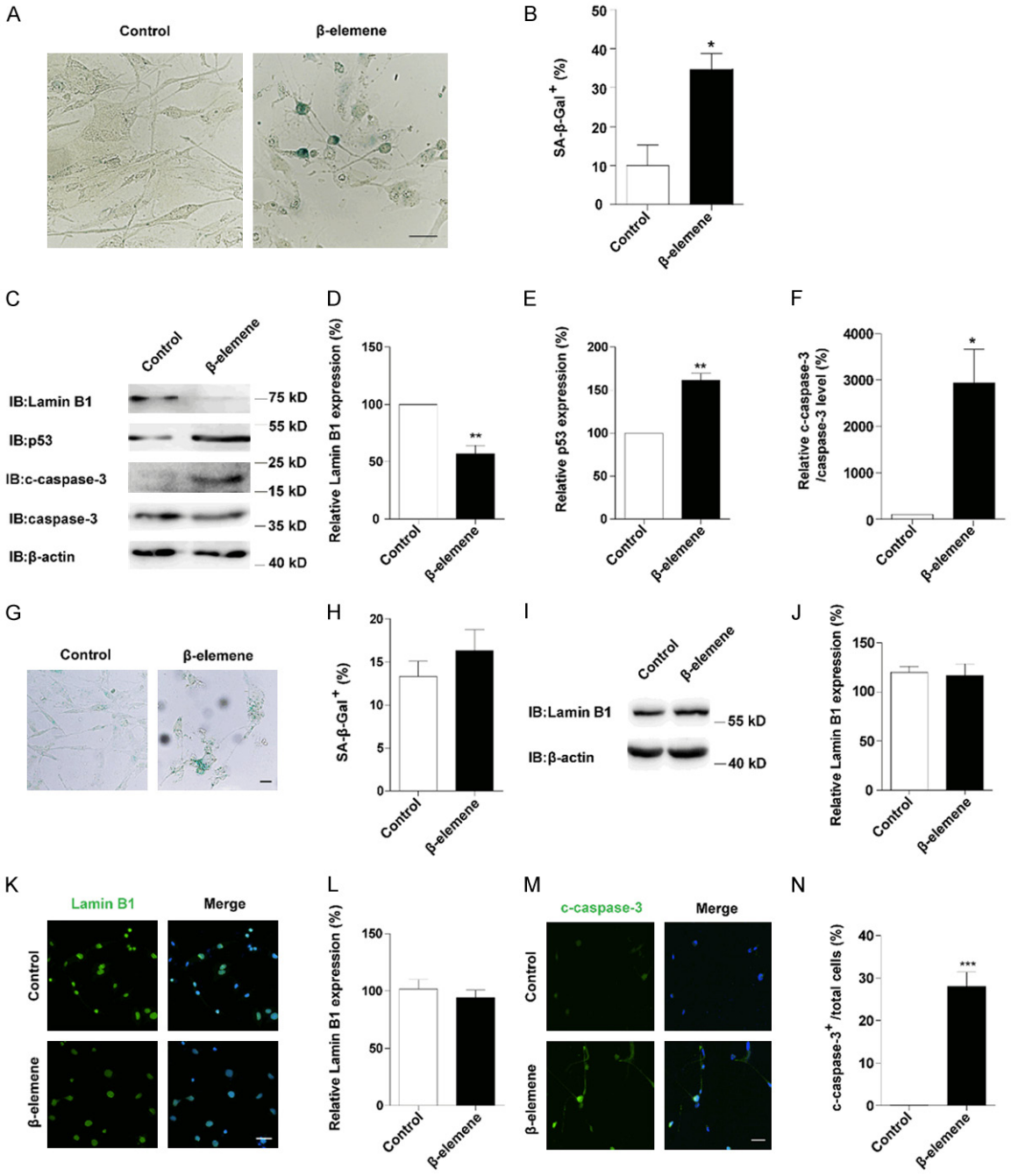

Figure S1. High concentration of β-elemene increased apoptosis of glioma cells. (A) Representative images of SA-β-Gal staining of C6 cells treated with control or 100 μg/mL β-elemene at for 1 day. (B) Quantification of the percentage of SA-β-Gal$^+$ C6 cells over total cells as shown in (A) (n=15). (C) Western blot detected the expression of Lamin B1, p53, caspase-3, and c-caspase-3 in C6 cells treated with control or 100 μg/mL β-elemene for 1 day. (D-F) Quantification of Lamin B1 (D), p53 (E), and c-caspase-3/caspase-3 (F) (n=8) levels as shown in (C). (G) Representative images of SA-β-Gal staining of C6 cells treated with control or 100 μg/mL β-elemene for 2 days. (H) Quantification of the percentage of SA-β-Gal$^+$ C6 cells over total cells as shown in (G) (n=15). (I) Western blot detected the expression of Lamin B1 in C6 cells treated with control or 100 μg/mL β-elemene for 2 days. (J) Quantification of Lamin B1 level as shown in (I) (n=8). (K) Representative images of Lamin B1 staining of C6 cells treated with control or 100 μg/mL β-elemene for 2 days. (L) Quantification of the percentage of Lamin B1$^+$ C6 cells over total cells as shown in (K) (n=15). (M) Representative images of c-caspase-3 staining of C6 cells treated with control or 100 μg/mL β-elemene for 2 days. (N) Quantification of the percentage of c-caspase-3$^+$ C6 cells over total cells as shown in (M) (n=15). Scale bars, 20 μm. Data were mean ± s.e.m, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

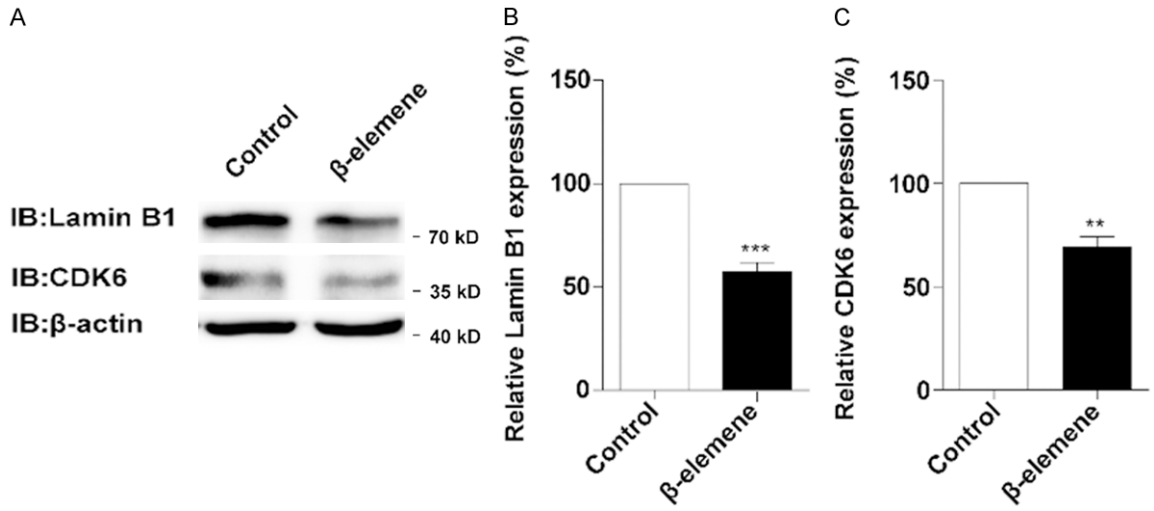

Figure S2. β-elemene induced changes of senescence makers in other glioma cell line. (A) Western blot detected the expression of Lamin B1 and CDK6 in DBTRG cells treated with control or 10 μg/mL β-elemene for 2 days. (B, C) Quantification of the relative Lamin B1 (B) and CDK6 (C) levels as shown in (A) (n=3). Data were mean ± s.e.m. $^{**}P < 0.01$, $^{***}P < 0.001$.