Total ginsenosides induce autophagic cell death in cervical cancer cells accompanied by downregulation of bone marrow stromal antigen-2

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Abstract. Ginsenosides are important active components in *Panax ginseng*. In the present study, total ginsenosides (TGNs) were demonstrated to enhance autophagy by promoting acidic vacuole organelle formation, recruitment of enhanced green fluorescent protein-microtubule-associated protein light chain 3 and expression of autophagy-related factors in cervical cancer cell lines. TGN markedly increased the expression of p62 at the transcriptional level, but decreased p62 protein expression in the presence of actinomycin D. The autophagic regulatory effect was reversible. TGN (≤120 μg/ml) did not affect the proliferation of cervical cancer cells under normal culture conditions, but markedly inhibited the growth of serum-deprived cells. Treatment with an inhibitor of autophagy (3-methyladenine) impaired TGN-induced cell death. This suggested that TGN caused autophagic cell death. In addition, western blot analysis demonstrated that the protein level of bone marrow stromal antigen-2 (BST-2) was downregulated by TGN. Upregulation of BST-2 reduced cell death. The results of the combined actions of various monomeric ginsenosides in TGN provide the molecular basis to develop TGN as a promising candidate for cancer therapy.

Introduction

Autophagy is a vital metabolic process in eukaryotic cells, which plays a significant role in regulation of cell survival and death (1). Excessive autophagy disrupts cellular functions, which directly results in autophagic programmed cell death or apoptotic cell death. In certain cases, autophagy counteracts apoptotic cell death via a cell survival pathway (2). Cervical cancer is the second most prevalent type of malignancy in females (3). The primary and most common anticancer therapy for cervical cancer is chemotherapy. However, tumour cells develop intrinsic resistance against chemotherapy. Plant-derived compounds characterised by low toxicity and a wide range of anticancer activities present as promising novel anticancer agents (4).

Panax ginseng C.A. Meyer is a type of traditional medicinal plant that has been widely used in Asian regions for thousands of years (5). Ginsenosides, some of the most important active components in *Panax ginseng*, exert numerous pharmacological actions, including marked suppression of the proliferation and migration of tumour cells (5). Different ginsenoside monomers have different effects on autophagy in cancer cells and act on different pathways (6,7). A recent study suggested that total ginsenoside (TGN) extract induces autophagic cell death in non-small cell lung cancer cells (8). However, the TGN concentrations used in the study were considered to be high (0.125-1 mg/ml) and the changes in p62 expression were not discussed.

Bone marrow stromal antigen 2 (BST-2), also referred to as CD317, tetherin and HM1.24 is a multifunctional protein. It is an interferon-inducible type II transmembrane glycoprotein that functions as an NF-κB activator (9), host restriction factor that tethers virions on the cell membrane (10) and survival protein that increases cancer cell adhesion and resistance to apoptosis (11). Under various disease conditions, particularly malignancies, BST-2 has been reported to be upregulated (12). The increased expression of BST-2 mediates tumour growth, invasion and metastasis (12,13). Therefore, BST-2 is upregulated in autophagy knockdown cells and associated with CD63, which may inhibit hepatitis C virus assembly or release (14). Another study indicated that a non-canonical autophagy pathway reminiscent of microtubule-associated protein light chain 3 (LC3)-associated phagocytosis contributes to viral protein U counteraction of BST-2 restriction (15). These data indicate that BST-2 is an autophagy-associated factor.

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The effect of TGN on autophagy of cervical cancer cells remains unclear. In the present study, the effect of TGN on autophagy in HeLa cells was investigated and TGN treatment was found to induce irreversible autophagy in a concentration- and time-dependent manner. TGN increased the expression of p62 at transcriptional and protein expression levels. Further experiments revealed that TGN promoted autophagic cell death under serum-deprived conditions accompanied by downregulation of BST-2, which is important for the survival of cancer cells.

Materials and methods

Antibodies and reagents. The following antibodies were used in the present study: Mouse anti-tubulin monoclonal antibody (mAb; cat. no. 627901; BioLegend, Inc.), rabbit anti-LC3 mAb, rabbit anti-beclin 1 mAb (cat. nos. 12741 and 3498, respectively; Cell Signaling Technology, Inc.) and rabbit anti-BST-2 pAb (cat. no. BS5634; Bioworld Technology, Inc.). The secondary antibodies were HRP-conjugated goat anti-mouse and anti-rabbit immunoglobulin G (cat. nos. 115-035-003 and 111-585-003, respectively; Jackson ImmunoResearch Laboratories, Inc.). Ginsenoside Rb_1, Rb_2, Rc, Rd, Rg_1, Rg_2 and Rf (purity, >98%) were purchased from Chengdu Must Bio-Technology Co., Ltd. Acridine orange, lysis buffer and a Braford assay kit were purchased from Beyotime Institute of Biotechnology. Cell counting kit-8 (CCK-8) was purchased from Boster Biological Technology. Rapamycin, bafilomycin A1 and 3-methyladenine (3-MA) were purchased from InvivoGen. Actinomycin D was purchased from MedChemEpxress. Other ginsenosides were purchased from Chengdu Must Bio-Technology Co., Ltd. TRIzol® was purchased from Thermo Fisher Scientific, Inc. SYBR Premix Ex Taq™ and a reverse transcription kit were purchased from Takara Biotechnology Co., Ltd. The protein extraction buffer was purchased from Beyotime Institute of Biotechnology. Earle's Balanced Salt Solution (EBSS) was purchased from Beijing Solarbio Science & Technology Co., Ltd.

Preparation of TGN. Ginseng crude powder (1 kg) was soaked in water overnight and extracted four times in boiling water for 3 h each time. The water-soluble substances were collected, applied to a D101 macroporous resin column and eluted with EtOH:H_2O (0:100, 75:25; v/v). The eluent was condensed and evaporated to obtain TGN.

Analysis of TGN by high-performance liquid chromatography (HPLC). To analyse ginsenoside monomers, an Agilent 1260 series high performance liquid chromatograph, Agilent system chemistry workstation and Agilent 1260 UV-visible wavelength detector were used. A Sepax Bio-C18 HPLC column (5 μm; 4.6x250 mm) was used for ginsenoside separation. The temperature of the column was maintained at 40˚C. The mobile phase consisted of solvent A (acetonitrile) and solvent B (water). The gradient elution program was as follows: 0-45 min, A 19%; 45-50 min, A 19-27%; 50-60 min, A 27-31%; 60-70 min, A 31-28%; 70-85 min, A 28-35%; 85-100 min, A 35%. The flow rate was set at 1 ml/min. The quantitative method used was an external standard method (16).

Cell culture and transfection. The following cell lines were obtained from the American Type Culture Collection: HeLa (cat. no. CCL-2), MS751 (cat. no. HTB-34) and C-33A (cat. no. HTB-31) and were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum at 37˚C with 5% CO_2. Culture under serum deprivation condition was culture in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. Enhanced green fluorescent protein (EGFP)-LC3, BST-2 IHA and VR1012 have been described previously (17,18). Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transient plasmid transfections. EGFP-LC3-II plasmid (500 ng) was transfected into HeLa, MS751 and C-33A cells. After 37˚C for 24 h, the cells were treated with dimethylsulfoxide (DMSO) or TGN for an additional 7 h and then analysed for fluorescence. ImageJ Software (v1.8.0; National Institutes of Health) was used for densitometric analysis. VR1012 or BST-2 IHA plasmid (50 ng) was transfected into HeLa, MS751 and C-33A cells. After incubation at 37˚C for 24 h, cells were treated with DMSO or TGN for an additional 24 h and then analysed via CCK-8 assay. Following overnight culture in 6-well plates, HeLa cells were treated with Actinomycin D (80 mM) at 37˚C with 5% CO_2 for 24 h. Following overnight culture in 6-well plates, cells were treated with 1 ml EBSS at 37˚C for 3 h.

Western blotting. After overnight culture in 6-well plates, HeLa cells were treated with DMSO or TGN (40/60/80 μg/ml) for 24 h. Cells were harvested by centrifugation (800 x g; 25˚C; 5 min), resuspended in RIPA total protein extraction lysis buffer (cat. no. BD0031; Bioworld Technology, Inc.) and BCA was used to detect the protein content. The loading buffer was added and boiled for 15 min. A total of 8 μg protein/lane was separated by SDS-PAGE on a 12% gel and the separated proteins were transferred to nitrocellulose membranes (Whatman plc; Cytiva). The membranes were blocked with 5% dry non-fat milk (BD Biosciences) for 30 min at room temperature. After washing in PBST 3 times, the membranes were incubated with the primary antibodies detailed in *Antibodies and reagents* (dilution, 1:1,000) overnight at 4˚C, washed with in PBST three times and then incubated with a secondary antibody as detailed in *Antibodies and reagents* (dilution, 1:1,000) for 1 h at room temperature. Protein bands were visualised using the Ultra High Sensitivity ECL Substrate kit (Beyotime Institute of Biotechnology). Immunoreactivity was visualised by chemiluminescence and densitometric analysis was performed with ImageJ Software (v1.8.0). The western blots in Fig. 3 are from cells following treatment with 80 μg/ml TGN for 16 h or EBSS at 37˚C for 3 h. The compounds were washed out and proteins were extracted at the indicated time points.

Reverse transcription-quantitative PCR (RT-qPCR). Following overnight culture in 6-well plates, HeLa cells were treated with DMSO or TGN for 24 h. Following centrifugation (800 x g; 25˚C; 5 min), the cells were collected and washed in PBS three times. Total RNA was extracted with TRIzol and reverse transcribed into cDNA using the reverse transcription kit. The PCR primers used were as follows: p62 forward, 5'-GCCAGAGGAACAGATGGAGT-3' and reverse, 5'-TCC

Table I. Main components and contents of total ginsenosides.

Type	Content (%)
Rb_1	16.60
Rb_2	11.53
Rc	9.90
Rd	6.80
Rg_1	3.30
Rg_2	0.98
Rf	1.40
Total	50.51

Table II. Retention time of total ginsenosides.

Type	Retention time (min)
Rg_1	31.863
Re	33.853
Rf	60.595
Rg2	65.816
Rb1	70.277
Rb2	82.749
Rb3	84.051
Rd	88.941

GATTCTGGCATCTGTAG-3'; BST-2 forward, 5'-CTGCAACCACACTGTGATG-3' and reverse, 5'-ACGCGTCCTGAAGCTTATG-3'; GAPDH forward, 5'-GGTGAAGGTCGGAGTCAACGGA-3' and reverse, 5'-GAGGGATCTCGCTCCTGGAAGA-3'. RT-qPCR was performed using a SYBR Premix Ex Taq kit and the CFX Connect™ Real-Time system (Bio-Rad Laboratories, Inc.). The thermal cycling conditions were as follows: 95˚C for 3 min; 40 cycles of 95˚C for 10 sec and 55˚C for 30 sec; 95˚C for 10 sec; 65˚C for 5 sec and 95˚C for 5 sec. Data were calculated relative to a calibrator according to the $2^{-\Delta\Delta Cq}$ method (19).

Acidic vesicular organelle (AVO) staining assay. After rinsing in PBS and fixing with 4% paraformaldehyde 25˚C for 10 min, the cells were stained for AVOs in the dark 37˚C for 30 min. Stained cells were observed and imaged by fluorescence microscopy (excitation, 488 nm).

Cell Counting Kit (CCK)-8 assay. Cells were cultured overnight in 96-well plates. The next day, the culture supernatant was replaced with medium containing DMSO or TGN and the cells were incubated 37˚C for 40 min. For analysis, CCK-8 substrate was added to the 96-well plates, followed by incubation at 37˚C for 1 h. Absorbance was measured at 450 nm using an Infinite 200 PRO microplate reader (Tecan Group, Ltd.). The cells in Fig. 4C were treated with 80 μg/ml TGN and 3-MA for 24 h and cell viability detected via Cell Counting Kit-8 assays.

Statistical analysis. All data represent at least three independent experiments, which were evaluated statistically by one-way ANOVA and Dunnett's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference. The statistical analysis software was GraphPad Prism 7.0 (GraphPad Software Inc.).

Results

TGN induces cervical cancer cell autophagy in a time- and concentration-dependent manner. A TGN extract of ginseng root was analysed by HPLC. The chromatograms, including standard and sample chromatograms are presented in Fig. S1. The main components and contents of TGN are presented in Table I. The retention times specified are presented in Table II. To determine the effect of TGN on autophagy, the expression levels of autophagy factors in TGN-treated HeLa, MS751 and C-33A cells were investigated. TGN was observed to increase the processing of LC3-I to LC3-II in a time- and dose-dependent manner, as well as increase the expression of autophagy-related factor Beclin-1 in the three types of cervical cancer cells (Fig. 1A and B).

Next, an EGFP-LC3-II plasmid (500 ng) was transfected into HeLa, MS751 and C-33A cells. After 24 h, the cells were treated with DMSO or TGN for an additional 7 h and then analysed for fluorescence. As shown in Fig. 2A, TGN promoted an increase in EGFP-LC3-II puncta. To further detect autophagy activation, the formation of AVOs in the three types of TGN-treated cervical cancer cells was analysed by AVO staining. Fig. 2B shows that a large number of AVOs appeared following treatment in a dose-dependent manner. These results suggested that TGN induced cervical cancer cell autophagy in a time- and concentration-dependent manner.

TGN increases transcription of p62 independent of autophagy. SQSTM1/p62 is a substrate for autophagy, which should be degraded following autophagy activation. However, p62 changes can be specific to the cell type and context. Occasionally, the expression level of p62 changes independent of autophagy (20-22). Furthermore, p62 may be transcriptionally upregulated under certain conditions (23). In the present study, western blotting demonstrated increases in p62 protein levels following TGN treatment (Fig. S2A). RT-qPCR analysis was performed to evaluate the transcriptional level of p62 after TGN treatment, which confirmed that p62 accumulation was due to transcriptional activation (Fig. S2B). To assess the change in p62 protein level, transcription inhibitor actinomycin D was used to eliminate the interference caused by increased transcription. TGN markedly decreased p62 protein levels in the presence of actinomycin D (Fig. S2C and D). These results further confirmed that TGN induced autophagy and increased transcription of p62 independent of autophagy.

The effect of TGN on autophagy promotion is irreversible. Subsequently, whether autophagy activation was reversible in the three cervical cancer cell lines was determined. EBSS is

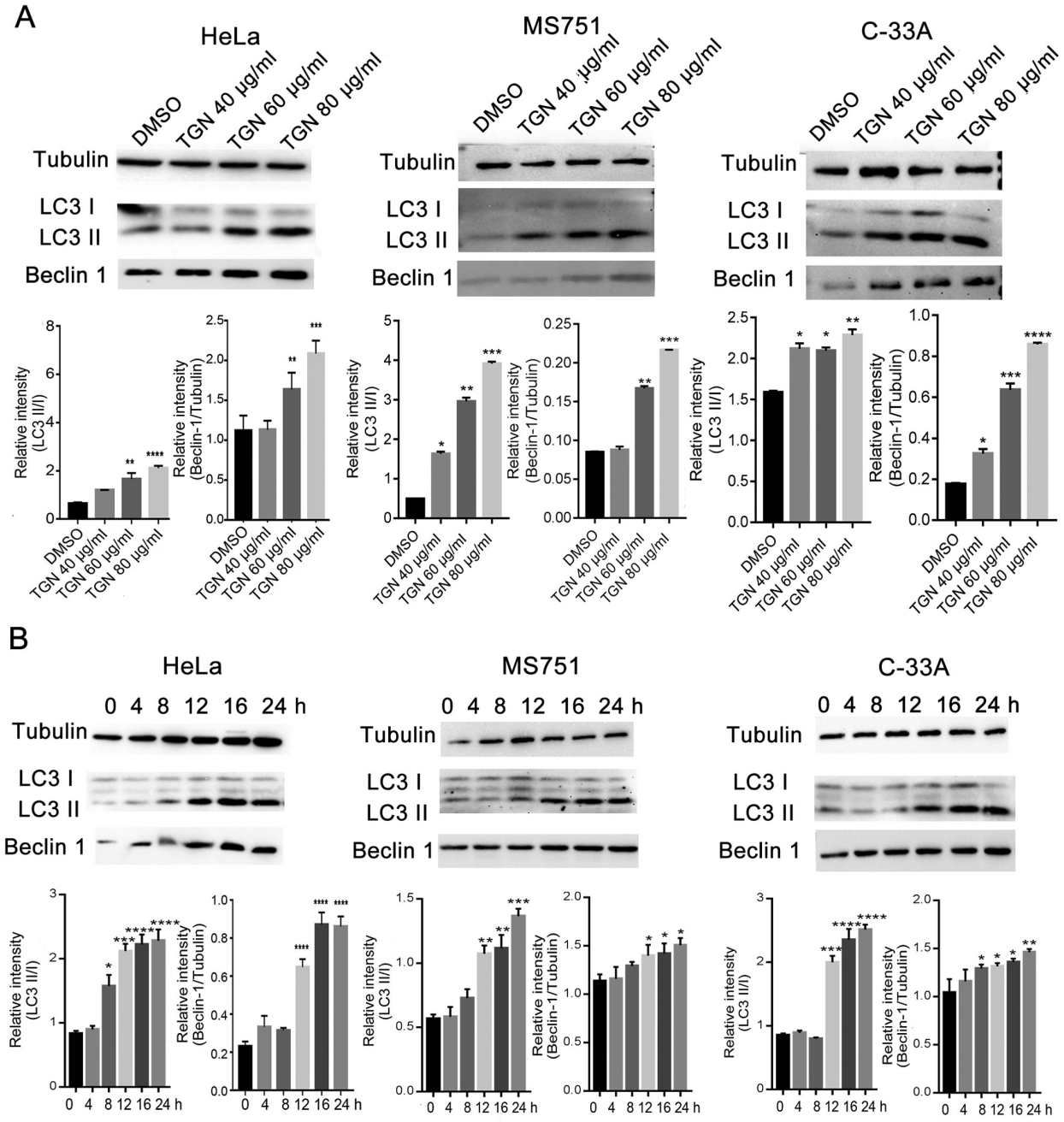

Figure 1. TGN induces cell autophagy in cervical cancer cell lines, HeLa, MS751 and C-33A in a (A) dose- and (B) time-dependent manner. Western blot analysis was performed with antibodies specific for Beclin-1, LC3 I/II and control protein, tubulin, followed by statistical analysis of the results; the values are presented as means ± SD. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. DMSO. DMSO, dimethylsulfoxide; TGN, total ginsenoside; LC3, microtubule-associated protein light chain 3.

used for the short-term maintenance of cells in a CO_2 environment and induces reversible autophagy (6). As shown in Fig. 3A-C, the activation of autophagy was not relieved even at 6 h after removal of TGN. Furthermore, the ratio of LC3-II/I continued to increase. However, EBSS-induced autophagy was rapidly relieved after replacing the medium. These data indicated that the effect of TGN on autophagy was irreversible over a short time period. The effects of ginsenoside monomers on autophagy showed that major components of TGN promoted autophagy, except ginsenoside Rf (Fig. 3D). These results differed from previous studies (8), which may be due to the use of different cell lines.

Reduction of BST-2 enhances cervical cancer cell death. Next, the cytotoxicity of TGN in the three types of cervical cancer cells was assessed via CCK-8 assay. TGN (~120 μg/ml) had no effect on the proliferation of HeLa, MS751 or C-33A cells (Fig. 4A). Serum deprivation is often used to emulate the tumour microenvironment. Therefore, the cells were exposed to TGN under serum deprivation and then cell viability was assessed. As a result, TGN notably suppressed cell growth in a dose- and time-dependent manner (Fig. 4B). To determine whether cell death was caused by the combination of nutrient deficiency and TGN, autophagy inhibitor 3-MA was introduced into the

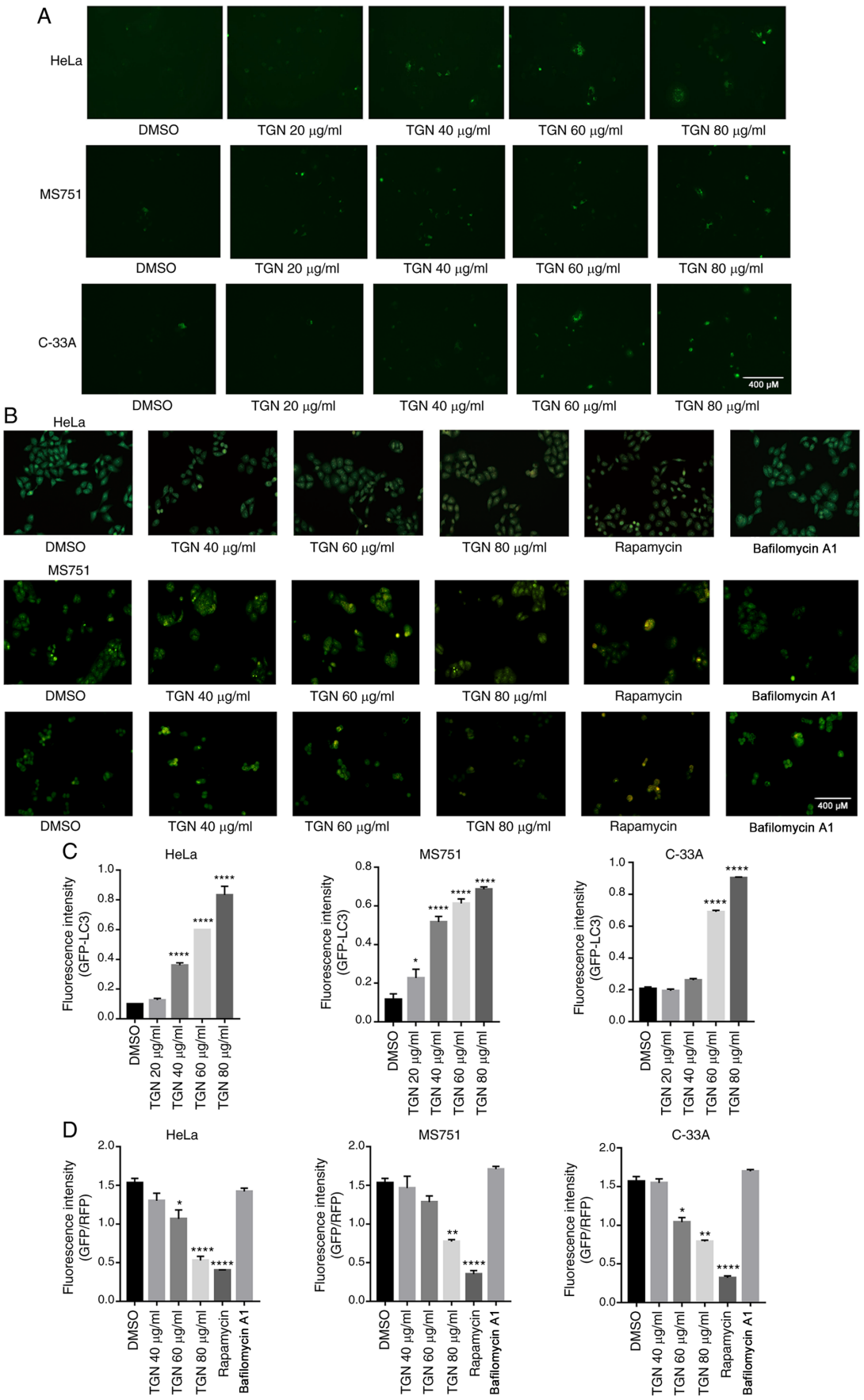

Figure 2. TGN increased EGFP-LC3 puncta and the quantity of AVOs. (A) TGN treatment markedly increased EGFP-LC3 puncta as observed by fluorescence microscopy. (B) AVOs were examined by incubating HeLa, MS751 and C-33A cells with acridine orange and observed by fluorescence microscopy. (C) Quantification of EGFP-LC3 puncta observed by fluorescence microscopy. (D) Quantification of AVOs stained with acridine orange and observed by fluorescence microscopy. Values are presented as means ± SD. *P<0.05, **P<0.01 and ****P<0.0001 vs. DMSO. TGN, total ginsenoside; EGFP-LC3, enhanced green fluorescent protein microtubule-associated protein light chain 3; AVO, acidic vesicular organelle; DMSO, dimethylsulfoxide.

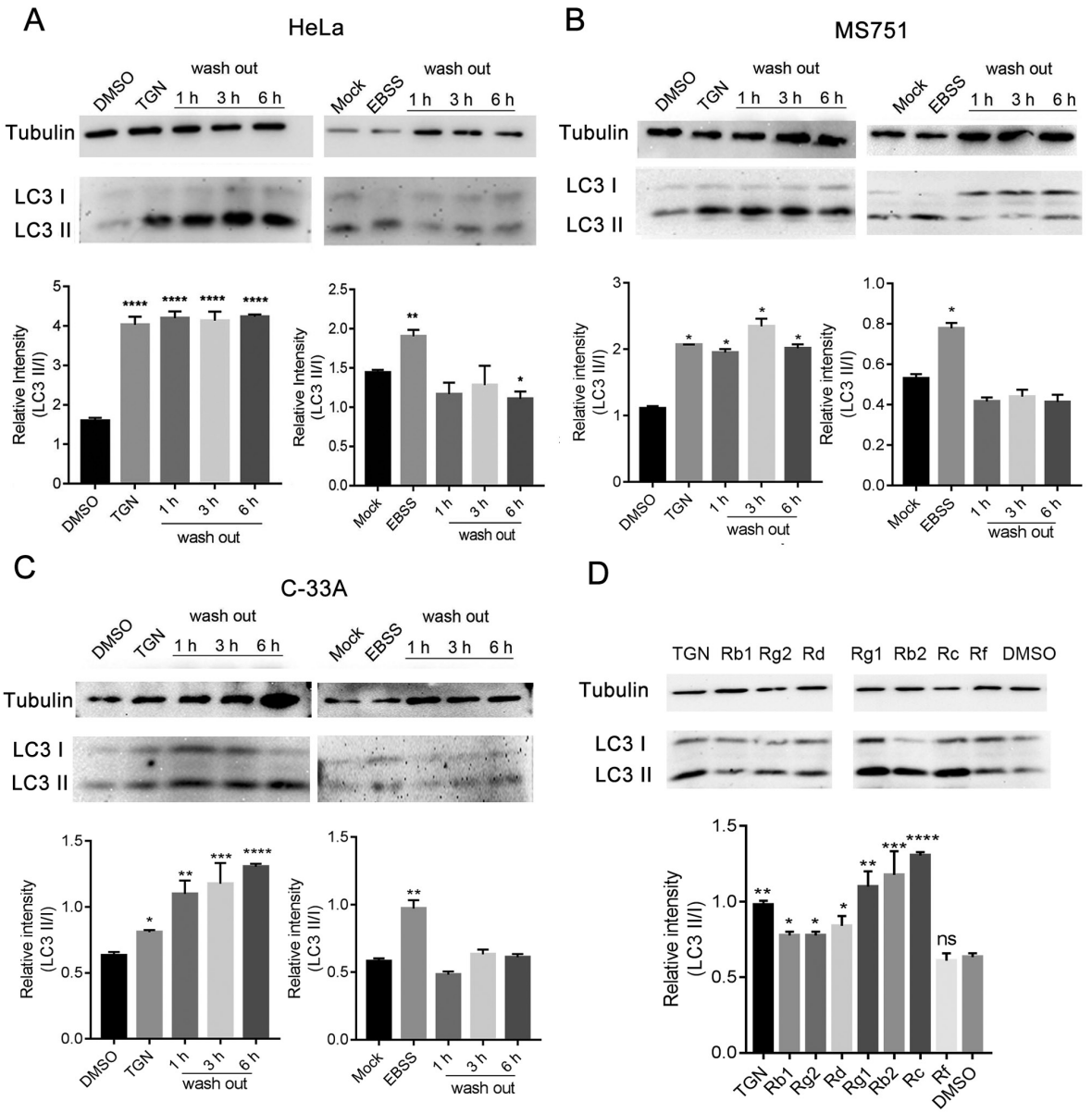

Figure 3. Autophagy is maintained for a long duration even after removing TGN. Western blots of (A) HeLa, (B) MS751 and (C) C-33A cells following treatment with 80 μg/ml TGN for 16 h or EBSS for 3 h. The compounds were washed out and proteins were extracted at the indicated time points. Statistical analysis of the western blots is also presented. (D) HeLa cells were treated with different ginsenosides for 16 h (60 μg/ml TGN; 70 μM ginsenosides monomer), cell lysates were detected with an LC3 antibody and tubulin served as the loading control. Values are presented as means ± SD. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. DMSO or Mock; ns, not significant. TGN, total ginsenoside; LC3, microtubule-associated protein light chain 3; DMSO, dimethylsulfoxide; EBSS, Earle's Balanced Salt Solution.

experiment. TGN-induced cell death was partly weakened by 3-MA, indicating that TGN induced autophagic cell death (Fig. 4C).

The expression of BST-2 is associated with various types of cancer. Therefore, the protein level of BST-2 was analysed in TGN-treated cells. The transcription and protein expression levels of BST-2 were observed to be downregulated in TGN-treated cervical cancer cells in normal culture (Fig. 5A). To further confirm the association of BST-2 downregulation with cell death, a BST-2 expression plasmid was introduced into the experiment and a CCK-8 assay was used to evaluate cell viability. The result demonstrated that upregulation of BST-2 reduced cell death in a serum-deprived culture (Fig. 5B). These results indicated that TGN induced downregulation of BST-2 and promoted cervical cancer cell death in serum-deprived cultures.

Discussion

In recent years, there has been a rise in the morbidity and mortality of cancer, presenting a global health issue (24). Botanical medicines have been used to treat various types of disease in Asia for thousands of years, and ginseng is one of the most well-known and widely used oriental medicinal plants (24). Dried, steamed or heated ginseng is distributed in 35 countries in various forms (24). Previous studies have reported the beneficial effects of ginseng on diseases, such as cancer, immune disorders, diabetes, as well

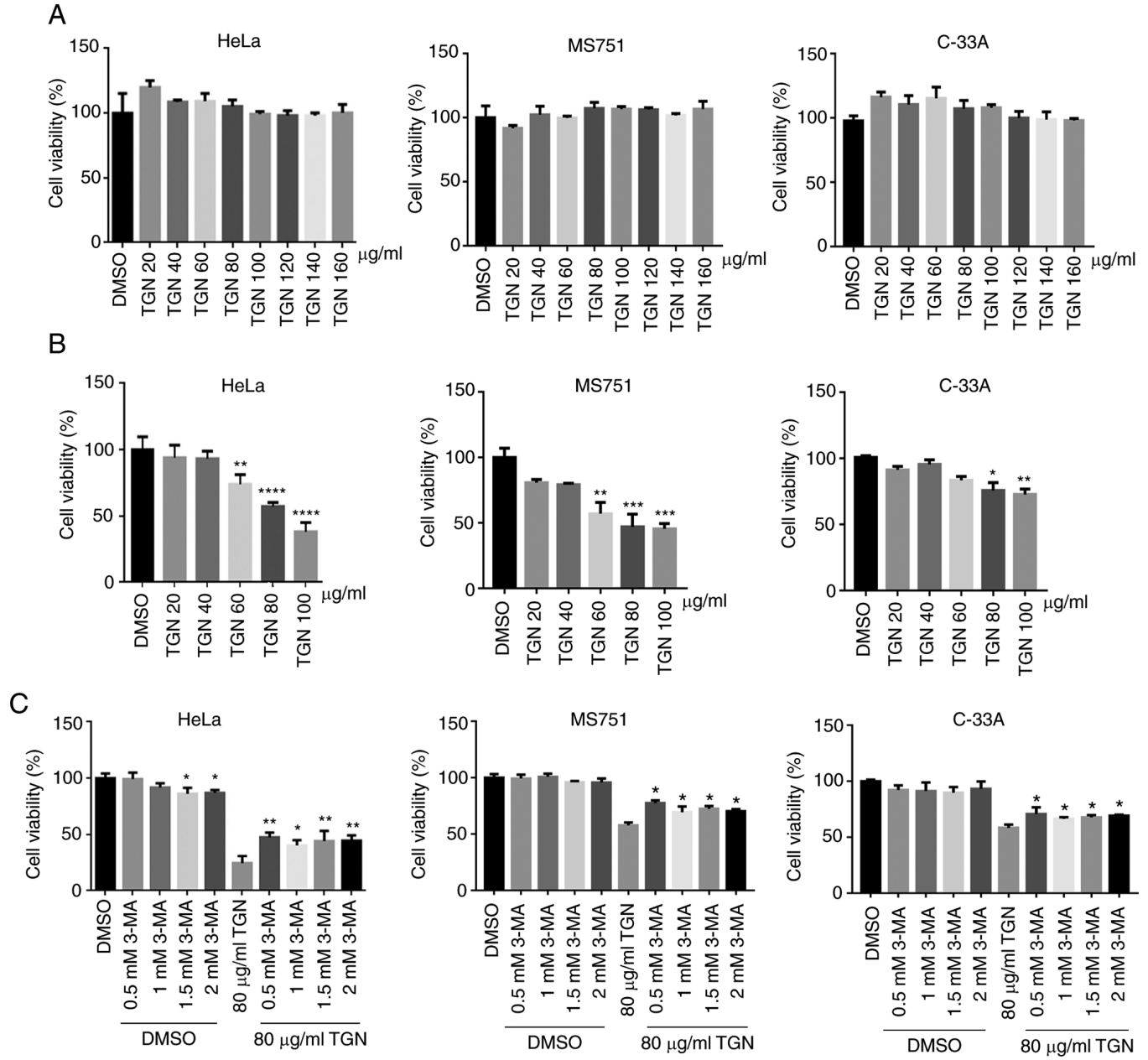

Figure 4. TGN induces autophagic cell death of cervical cancer cells under serum deprivation. (A) Effects of treatment with TGN at various concentrations on HeLa, MS751 and C-33A cells under normal conditions. (B) Effects of treatment with TGN at various concentrations on HeLa, MS751 and C-33A cells under serum-deprived conditions. (C) HeLa, MS751 and C-33A cells were treated with 80 μg/ml TGN with 3-MA for 24 h and then cell viability was detected via Cell Counting Kit-8 assays. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. DMSO. TGN, total ginsenoside; 3-MA, 3-methyladenine; DMSO, dimethylsulfoxide.

as on liver, nervous system, cardiovascular and infectious diseases (25-31).

BST-2 is an innate immune gene that is upregulated in various types of cancer, such as breast cancer, mammary tumours and bladder cancer (32-34). Dimers of BST-2 promote cell-cell and cell-matrix adhesions, cell motility, survival and growth (35). It also protects cervical cancer cells from serum deprivation-induced death (35). A BST-2-based peptide, known as B49 and its analogue, B49Mod1 inhibit adhesion and growth of breast cancer cells (35,36). Therefore, targeting BST-2 presents a potential therapeutic strategy against cancer (37-39). BST-2 is an autophagy-associated protein. It is hypothesized to be a substrate of autophagy. In the present study, the expression level of BST-2 was identified to be decreased in TGN-treated cells and accompanied by increasing cell death. The underlying mechanism may be that BST-2 was degraded through induced autophagy and increased the death of serum-deprived cells.

Therefore, TGN enhanced irreversible autophagy in cervical cancer cell lines and caused significant autophagic cell death in serum-deprived cells. TGN markedly increased the expression of p62 at the transcriptional level, but decreased p62 protein levels in the presence of actinomycin D. Furthermore, protein expression levels of BST-2 were downregulated by TGN and upregulation of BST-2 reduced the

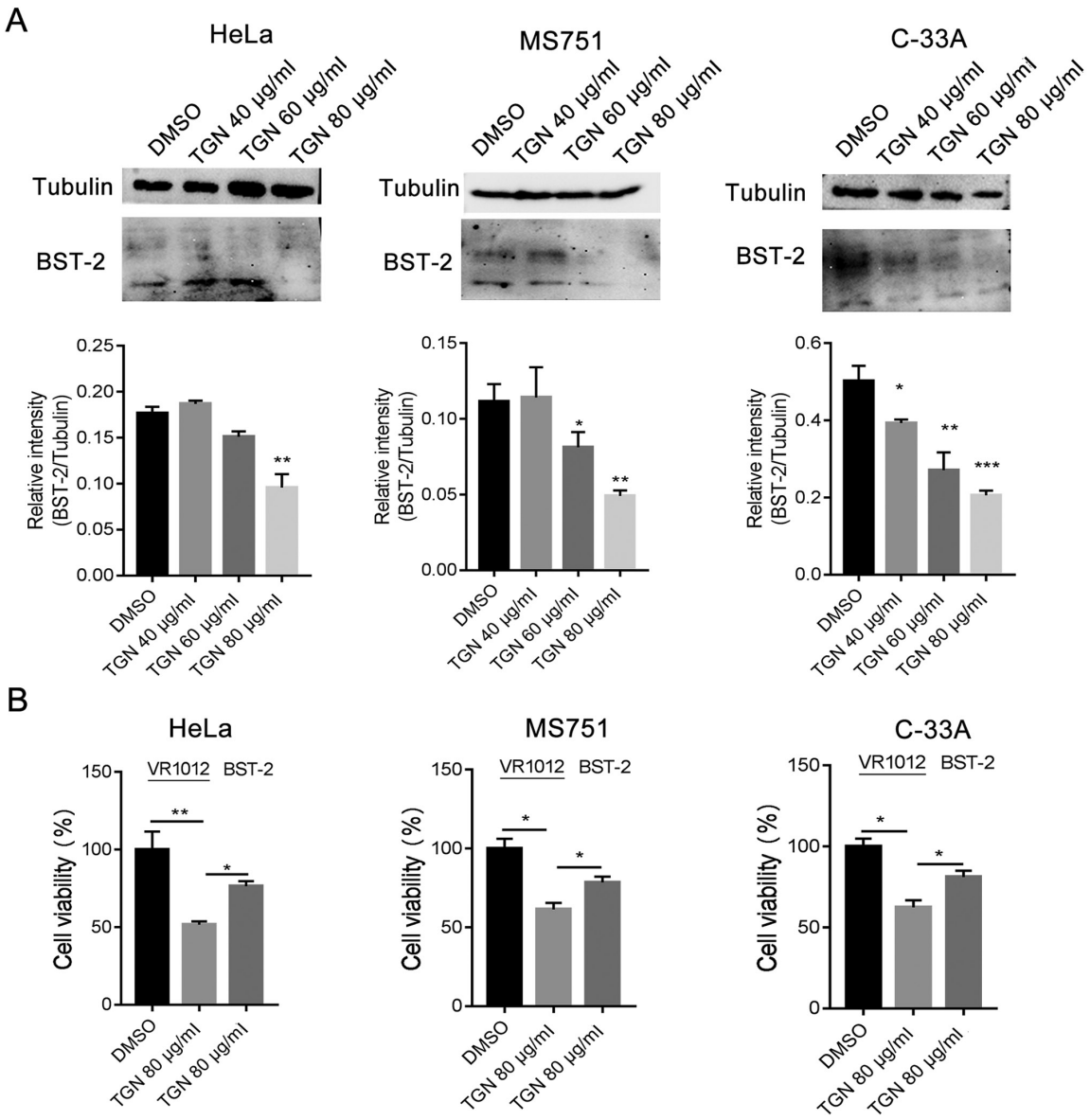

Figure 5. TGN induced downregulation of BST-2 and promoted cervical cancer cell death in serum-deprived cultures. (A) After cells were treated with 80 μg/ml TGN for 16 h, the protein expression levels of BST-2 was determined by western blotting. (B) Cell viability in the serum-deprived culture was detected via Cell Counting Kit-8 assays following BST-2 overexpression. *P<0.05, **P<0.01, ***P<0.001 vs. DMSO. TGN, total ginsenoside; BST-2, bone marrow stromal antigen-2; DMSO, dimethylsulfoxide.

cell death that was caused by TGN. These results provide the molecular basis to develop TGN as a promising candidate for cancer therapy.

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Availability of data and materials

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

Authors' contributions

JW, ML and SB designed the experiments. SB, YZ, FL, SL, ZH and SW performed the experiments. ML, SW, XB, DZ analysed the experiments. JW wrote the paper. SB and JW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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