Co-inhibition of BMI1 and Mel18 enhances chemosensitivity of esophageal squamous cell carcinoma *in vitro* and *in vivo*

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Received April 11, 2018; Accepted February 20, 2019

DOI: 10.3892/ol.2019.10160

Abstract. Esophageal squamous cell carcinoma (ESCC) accounts for almost 90% of esophageal cancer cases and is the sixth most common cause of cancer-associated mortality worldwide. Cisplatin is the standard therapeutic reagent for ESCC; however, chemoresistance frequently occurs after a few weeks, which leads to ESCC recurrence. Aberrant expression of B lymphoma Mo-MLV insertion region 1 homolog (BMI1) has been reported to activate multiple growth-regulatory pathways, induce antiapoptotic abilities in numerous types of cancer cells and promote chemoresistance. However, to the best of our knowledge, the role of BMI1 in cisplatin-resistant ESCC, and the interaction between BMI1 and its homologue melanoma nuclear protein 18 (Mel18) remain unknown. The present study identified that knockdown of BMI1 promoted cytotoxic effects of cisplatin, and co-inhibition of Mel18 and BMI1 enhanced cisplatin-induced apoptosis and cytotoxicity. Inhibition of BMI1 and Mel18 also suppressed the expression of c-Myc. Furthermore, this combined inhibition sensitized esophageal xenograft tumors to cisplatin to a greater extent compared with BMI1 inhibition alone. In summary, the current study demonstrated that inhibition of BMI1 and Mel18 could increase the sensitivity of esophageal cancer cells to cisplatin via inhibition of c-Myc. Therefore, combined targeting of BMI1 and Mel18 may serve as a promising therapeutic strategy for sensitizing ESCC to chemotherapy.

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Key words: esophageal squamous cell carcinoma, chemoresistance, apoptosis, c-Myc, B lymphoma Mo-MLV insertion region 1 homolog, melanoma nuclear protein 18

Introduction

Esophageal cancer was the ninth most common cancer type and the sixth most common cause of cancer-associated mortality globally in 2012 (1). Esophageal squamous cell carcinoma (ESCC) accounts for ~90% of all cases of esophageal cancer worldwide (2). The incidence rate of ESCC is particularly high in the so-called 'esophageal cancer belt', which stretches from northern China, where the annual incidence rate is 1/100,000, through central Asia to northern Iran (1,2). Despite significant advancements in treatment options, including surgery and chemotherapy, the overall survival rate has not significantly improved (3). Due to a lack of symptoms during early stages of ESCC and a lack of non-invasive detection strategies, the majority of cases are diagnosed during advanced stages of the disease. Platinum-based therapeutic regimens are currently employed for the clinical management of esophageal cancer and are associated with high rates of clinical responses (4). However, a large number of human malignancies are intrinsic or become insensitive to the cytostatic effects of platinum, which causes a prominent challenge for the use of platinum. Therefore, there is a requirement to develop new therapeutic strategies that improve chemosensitivity.

B lymphoma Mo-MLV insertion region 1 homolog (BMI1) is a member of the polycomb repressive complex 1 (PRC1). BMI1 functions as an epigenetic regulator and represses gene transcription via its participation in histone modification and DNA methylation (5). BMI1 expression is increased in numerous types of human cancer; therefore, it can be used as a predictive biomarker of progression and poor prognosis (6). A number of studies have demonstrated that aberrant overexpression of BMI1 is associated with advanced stages, aggressive clinicopathological behaviors, therapeutic resistance and poor prognosis in melanoma, glioma and other types of tumor (6,7). BMI1 also serves a crucial role in cisplatin chemoresistance and inhibition of BMI1 can reverse cisplatin insensitivity (6,8). A recent study demonstrated that targeting BMI1-positive cancer stem cells effectively inhibited head and neck squamous cell carcinoma (HNSCC) growth and eliminated chemoresistance (9). The small molecule complex PTC-209 targeting BMI1 can inhibit tumor growth and enhance chemosensitivity in colorectal cancer and HNSCC (10,11). ESCC shares numerous biological

characteristics with HNSCC (12); however, to the best of our knowledge, understanding of BMI1 regarding chemotherapy sensitivity of esophageal cancer remains absent. Therefore, the current study investigated the association between BMI1 and cisplatin chemosensitivity in ESCC.

Melanoma nuclear protein 18 (Mel18), a homologue of BMI1, contains a zinc finger structure and is involved in histone methylation, ubiquitination, SUMOylation and chromatin remodeling (13,14). Mel18 is implicated in the regulation of cell proliferation, differentiation, tumorigenesis, senescence, apoptosis, cancer stem cell activity, angiogenesis and invasion in a number of cancer types (15-17). Although the Mel18 gene product is structurally highly similar to the BMI1 protein, the role of Mel18 in cancer remains controversial. Our previous study demonstrated that BMI1 expression is significantly upregulated in ESCC tissues compared with adjacent noncancerous tissues, and a strong negative association was identified between Mel18 and BMI1 expression in ESCC (18). Previous studies have also revealed that Mel18 acts as a tumor suppressor and is downregulated in certain types of human cancer, including breast, gastric, prostate and colorectal cancer (19-22). By contrast, Mel18 may act as an oncogene as it is highly expressed in several types of tumor, including Hodgkin's lymphoma, medulloblastoma, salivary gland adenoid cystic carcinoma and salivary gland myoepithelial tumor (23-26). Jung *et al* (27) demonstrated that silencing Mel18 inhibits endothelial cell migration and tube formation. In addition, Park *et al* (28) identified that Mel18 inhibition promotes tube formation in human umbilical endothelial cells. A number of studies have suggested that Mel18 downregulates BMI1 in several types of human tumor (29-31). However, certain studies have indicated that BMI1 and Mel18 exhibit synergistic roles in the regulation of homeobox (HOX) genes, skeletal patterning, H3K27 trimethylation and colitis-associated cancer development (15,32,33). These observations indicate that the biological functions of BMI1 and Mel18 may be different or redundant in different cancer microenvironments. BMI1 is upregulated in ESCC tissues and cells, and the expression of Mel18 is negatively associated with BMI1 in gastric cancer and ESCC (18,31,34). To the best of our knowledge, the interaction and involvement of Mel18 and BMI1 in the chemoresistance of ESCC has not been evaluated. We hypothesized that Mel18 and BMI1 cooperate to regulate the intrinsic chemosensitivity of ESCC.

Our pilot study suggested that inhibition of BMI1 significantly effects cisplatin-induced proliferation and clonal growth of ESCC cells. This effect may be strengthened by co-inhibition of BMI1 and Mel18 in ESCC cells. The present study investigated the combinational effects of Mel18 and BMI1 on apoptosis and key molecules of apoptosis. To do so, it was hypothesized that combined inhibition of BMI1 and Mel18 could enhance the effects of BMI1-induced cell proliferation inhibition by regulating apoptosis and associated proteins.

Materials and methods

Cell culture and treatment with cisplatin. Human ESCC cell lines (EC109 and TE1) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). ESCC cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin mix (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Cells were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. Cisplatin (MedChemExpress, Monmouth Junction, NJ, USA) was dissolved in dimethyl sulfoxide (DMSO; Beyotime Institute of Biotechnology, Shanghai, China) at 50 mM and stored at -80˚C until use.

Using a 96-well plate, $1x10^4$ stably transfected cells in 100 μl of complete growth medium were seeded 1 day prior to treatment. Cells were treated with cisplatin (1, 2, 4, 8, 16 and 32 μM) dissolved in complete growth medium containing <1% DMSO and control cells were treated with complete growth medium containing the same concentration of DMSO.

Plasmids construct and transfection. BMI1 short hairpin RNA (shRNA) was designed and cloned into the pcDNA3.1-EGFP vector with the neomycin resistant gene. The BMI1 shRNA target sequence was as follows: 5'-GGTCATCAGCAACTT CTTCT-3'. Mel18 shRNA was designed and cloned into the psi-LVRU6GP vector with the puromycin resistant gene. The Mel18 shRNA target sequence was as follows: 5'-GGCTCT GAGTGATGATGAGAT-3'. Human full-length Mel18 (reference sequence no. NM_007144.2) was isolated from the human complementary DNA library and connected to the pEZ-M13 vector with the neomycin resistant gene. All plasmids, including Negative control (NC) shRNA, were purchased from GenePharma (Shanghai, China). Transfections of all vectors were performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The mass/concentration of all plasmid transfected was 2 mg/ml. Stably transfected TE1 and EC109 cells were selected and maintained in DMEM containing 700 μg/ml G418 (Beyotime Institute of Biotechnology) or 1 μg/ml puromycin (Beyotime Institute of Biotechnology). After 2 weeks, stable transfected cells were used for subsequent experiments. Transfection efficiency was evaluated by western blot analysis.

Measurement of cytotoxicity. The cytotoxic effects of cisplatin in ESCC cells were measured using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Stably transfected cells were plated in 96-well plates at a density of $1x10^4$ cells/well. Following incubation at 37˚C for 24 h, cells were treated with various concentrations of cisplatin (1, 2, 4, 8, 16 and 32 μM) at 37˚C for 24 h. Following 24 h, the complete growth medium was replaced with serum-free medium, 10 μl CCK-8 was added to each well and the cell mixtures were incubated at 37˚C for 2 h. Using a fine needle the bubbles were punctured and the absorbance was then measured at 450 nm using a microplate reader. The half maximal inhibitory concentration (IC_{50}) was defined as the concentration resulting in a 50% reduction in growth compared with the growth of the control cells. Cell viability was calculated according to the following equation: Cell viability = [A450(drug)-A450(blank)]/[A450(control)-A450(blank)]. Six replicate wells were set up in each group and three independent experiments were performed. The IC_{50} dose-response curves were plotted with GraphPad Prism Version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Colony formation assay. Following stable transfection of TE1 and EC109 cells, exponentially growing cells were harvested and placed into 60 mm plates ($1x10^3$ cells/well) and cultured with 5 μM cisplatin at 37˚C. The medium was changed every 3 days. After 2-3 weeks, cells were washed in PBS three times, fixed in 4% formalin (Beyotime Institute of Biotechnology) at room temperature for 15 min and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) at room temperature for 10 min. The number of colonies was counted and analyzed using ImageJ software version 1.0 (National Institutes of Health, Bethesda, MD, USA).

Apoptosis detection. Apoptosis was determined with the Annexin V-APC/7-AAD apoptosis kit (MultiSciences, Hangzhou, Zhejiang, China). ESCC cells were seeded in 6-well plates and incubated with 5 μM cisplatin and serum-free medium at 37˚C for 24 h. The cells were digested with 0.25% trypsin without EDTA (Thermo Fisher Scientific, Inc.) and resuspended in binding buffer to a density of $1x10^6$ cells/ml. Annexin V-APC (5 μl) and 7-AAD (10 μl) were mixed prior to incubation in the dark at room temperature for 10 min. Apoptotic cells were detected using a flow cytometer (FACSAriaII; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). FlowJo software (version 10; Becton, Dickinson and Company) was used for data analysis.

Western blot analysis. Both ESCC cells were seeded in 6-well plates at a density of $2x10^5$ cells/well and treated with cisplatin (5 μM) at 37˚C for 24 h. Cells were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) supplemented with phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) for 30 min at 4˚C and centrifuged at 10,000 x g for 15 min in 4˚C. The supernatant was then collected. Tumor tissues were cut into 100 mg pieces, incubated with 500 μl lysis buffer, homogenized completely with a tissue homogenizer for 5 min and lysed for 30 min on ice. Samples were then centrifuged at 10,000 x g for 15 min in 4˚C, and supernatant was collected. Protein concentration was determined using bicinchoninic acid assay (Beyotime Institute of Biotechnology). Proteins (30 μg) were separated by 10% SDS-PAGE and transferred onto 0.22 μm polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 2 h at 37˚C and incubated with the appropriate primary antibodies at 4˚C overnight. The following antibodies were used: Anti-Mel18 (cat. no. ab5267, Abcam, Cambridge, UK), anti-BMI1 (cat. no. 6964; Cell Signaling Technology, Danvers, MA, USA), anti-B-cell lymphoma-2 (Bcl-2) (cat. no. 4223; Cell Signaling Technology), anti-Bcl-2-associated X protein (BAX) (cat. no. 5023; Cell Signaling Technology), anti-caspase3 (cat. no. 9662; Cell Signaling Technology), anti-nuclear factor-κB (NF-κB) (cat. no. 8242; Cell Signaling Technology), anti-c-Myc (cat. no. 5605; Cell Signaling Technology), anti-Akt (cat. no. 4685; Cell Signaling Technology), anti-phosphorylated-Akt (cat. no. 4060; Cell Signaling Technology) and anti-GAPDH (cat. no. 5174; Cell Signaling Technology). Subsequently, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. ab97200; Abcam) at room temperature for 1 h. Bands were detected using enhanced chemiluminescence substrate (EMD Millipore) and Bio-Rad ChemiDoc MP High-end imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Relative expression level of all proteins was normalized to endogenous control GAPDH using ImageJ version 1.0 (National Institutes of Health, Bethesda, MD, USA).

Tumor xenograft model. All animal procedures were approved by the Ethics Committee of Qianfoshan Hospital (Jinan, China). Mice were housed (five mice per cage) under specific pathogen-free conditions, at a constant room temperature of 22-24˚C, with a 12-h light/dark cycle and had unlimited access to food and water. The significance of BMI1 and Mel18 inhibition in the sensitization of cisplatin in ESCC *in vivo* was studied by subcutaneous injection of cancer cells into nude mice. A total of 20 male BALB/c nude mice (3-week old) were purchased from Vital River Laboratories Co., Ltd. (Beijing, China). All 20 nude mice were randomly divided into four groups. Exponentially growing transfected EC109 cells, including Mel18 shRNA and BMI1 shRNA-transfected cells, Mel18 and BMI1 shRNA-transfected cells, BMI1 shRNA-transfected cells, and NC shRNA-transfected cells, were harvested and resuspended in sterile PBS. Equal cell numbers ($5x10^6$) of each group were injected subcutaneously in the right flank of BALB/c nude mice. Tumor volume was calculated using the following formula: Tumor volume (mm^3) = length x $width^2$ x0.5. When the tumor volume reached approximately 200 mm^3, cisplatin was intraperitoneally injected every 3 days with a dose of 5 mg/kg, according to the manufacturer's protocol and a previously published study (35). After 3 weeks of treatment, mice were sacrificed in a chamber with increasing concentrations of carbon dioxide. The tumor volume was measured by a caliper and calculated using the aforementioned formula.

Statistical analysis. All statistical analysis was performed using SPSS software (version 20; IBM Corp., Armonk, NY, USA). Unless otherwise indicated, data were presented as the means ± standard deviation. An unpaired Student's t-test of two independent samples was used for statistical comparison between two groups. Analysis of variance was performed to compare the mean among multiple groups and Student-Newman-Keuls method was used for pairwise comparison between different treatment groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

BMI1 knockdown increases the sensitivity of cells to cisplatin. To study the biological role of BMI1 on cell survival in ESCC with the treatment of cisplatin, two BMI1 shRNA stably transfected cell lines were established. As demonstrated in Fig. 1A, BMI1 expression was markedly lower in the transfected EC109 and TE1 cells compared with the negative control cells. To examine the effects of cisplatin on the survival of BMI1 knockdown cells, a CCK-8 assay was performed following treatment of the cells with cisplatin for 24 h. Following treatment with various concentrations of cisplatin, it was identified that the BMI1 shRNA-transfected cells demonstrated lower cell viabilities compared with the NC shRNA-transfected

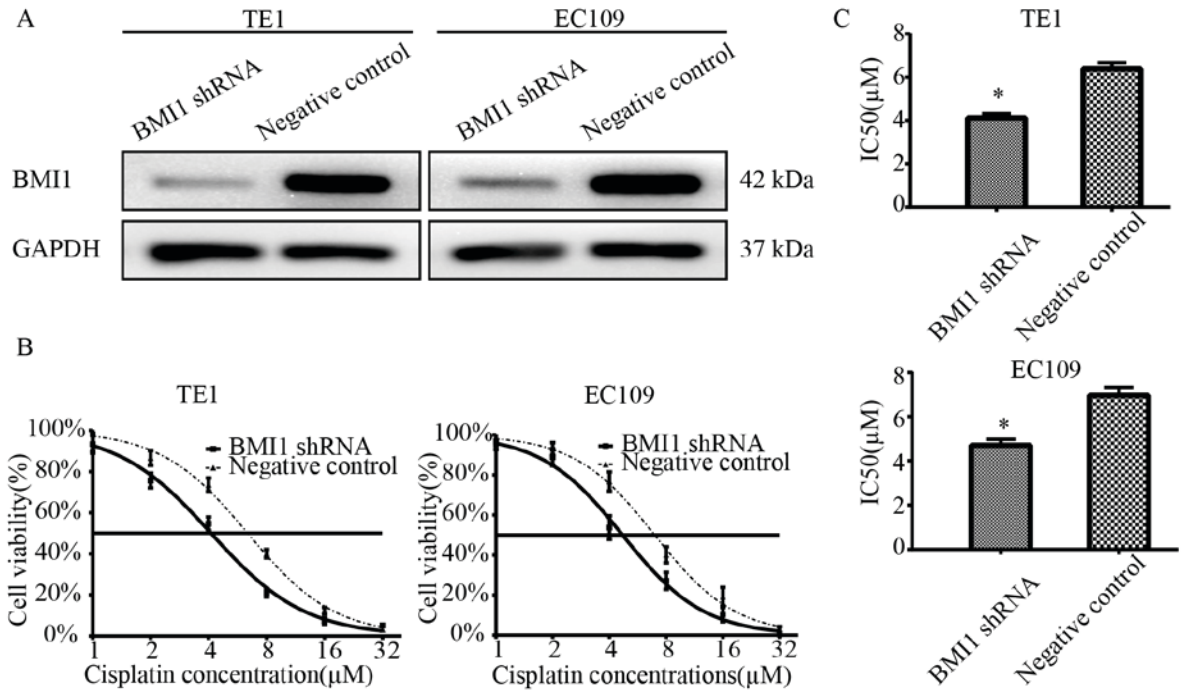

Figure 1. Effects of BMI1 downregulation on cisplatin chemosensitivity of TE1 and EC109 cells. (A) The inhibitory effects of BMI1 shRNA in EC109 and TE1 cells. The protein expression of BMI1 was detected by western blot analysis. (B) TE1 and EC109 cells were treated with increasing concentrations of cisplatin for 24 h, and cell viability was determined using a Cell Counting Kit-8 assay. The horizontal line indicates the IC_{50} value. (C) The IC_{50} values of cisplatin in TE1 and EC109 cells. Data were presented as the means ± standard deviation. *P<0.05 vs. negative control. IC_{50}, half maximal inhibitory concentration; shRNA, short hairpin RNA; BMI1, B lymphoma Mo-MLV insertion region 1 homolog.

cells (Fig. 1B). The IC_{50} values of cisplatin in the BMI shRNA-transfected and the NC-transfected EC109 cells were 4.695±0.287 and 6.953±0.369 μM, respectively (P<0.05). The IC_{50} values of cisplatin in the BMI shRNA-transfected and the NC-transfected TE1 cells were 4.117±0.192 and 6.376±0.294 μM, respectively (P<0.05; Fig. 1C). These results indicate that BMI1 knockdown increases sensitivity to cisplatin.

Co-inhibition of Mel18 and BMI1 further sensitizes ESCC cells to cisplatin compared with inhibition of BMI1 alone. To determine the effects of Mel18 and BMI1 on the chemosensitivity of ESCC cells, the present study established four stably transfected ESCC cell lines for each TE1 and EC109 cells, including Mel18 shRNA and BMI1 shRNA-transfected cells, Mel18 and BMI1 shRNA-transfected cells, BMI1 shRNA-transfected cells, and NC shRNA-transfected cells. As presented in Fig. 2A-C, the transfection efficiency was verified by western blot analysis.

Subsequently, a survival assay was performed with the transfected cells treated with different concentrations of cisplatin. As expected, the cell viability was significantly lower for the BMI1 shRNA-transfected cells compared with the NC cells following treatment with cisplatin in EC109 cells (P<0.05; Fig. 2D and E; right panel). A combination of Mel18 and BMI inhibition significantly enhanced the sensitivity to cisplatin compared with BMI inhibition alone in EC109 cells (P<0.05, Fig. 2D and E; right panel). Overexpression of Mel18 combined with BMI1 inhibition did not enhance the sensitivity to cisplatin compared with BMI inhibition alone in EC109 cells (Fig. 2D and E; right panel). Similar results were observed in TE1 cells (Fig. 2D and E; left panel). In comparison with the NC group, the three remaining groups demonstrated significantly lower IC_{50} values (all P<0.05; Fig. 2E). The EC109 cells with the most significant reduction in IC_{50} were those in which both Mel18 and BMI1 had been inhibited (P<0.01; Fig. 2E). Similar results were observed in TE1 cells (Fig. 2E).

To further determine the effects of Mel18 and BMI1 inhibition on cisplatin sensitization, the current study investigated the long-term effects of BMI1 and Mel18 inhibition by colony formation assay. It was revealed that depletion of BMI1 increased sensitivity to cisplatin (Fig. 3A and B). Furthermore, depletion of Mel18 and BMI1 in the two ESCC cell lines further increased sensitivity to cisplatin compared with individual depletion of BMI1 (Fig. 3A and B). In summary, the current results indicate that combined inhibition of Mel18 and BMI1 sensitizes ESCC cells to cisplatin. By contrast, overexpression of Mel18 combined with BMI1 inhibition did not markedly reduce cell survival compared with individual knockdown of BMI1.

Inhibition of BMI1 and Mel18 enhances cisplatin-induced apoptosis. Cisplatin-induced cytotoxic effects predominantly occur via apoptosis (36). To evaluate the effect of BMI1 and Mel18 knockdown on cisplatin-induced apoptosis, the apoptotic rates of stably transfected ESCC cells were examined following treatment with 5 μM of cisplatin for 24 h. Consistent with the cytotoxic effects observed with the cell viability assay, combined inhibition of BMI1 and Mel18 increased the rate of

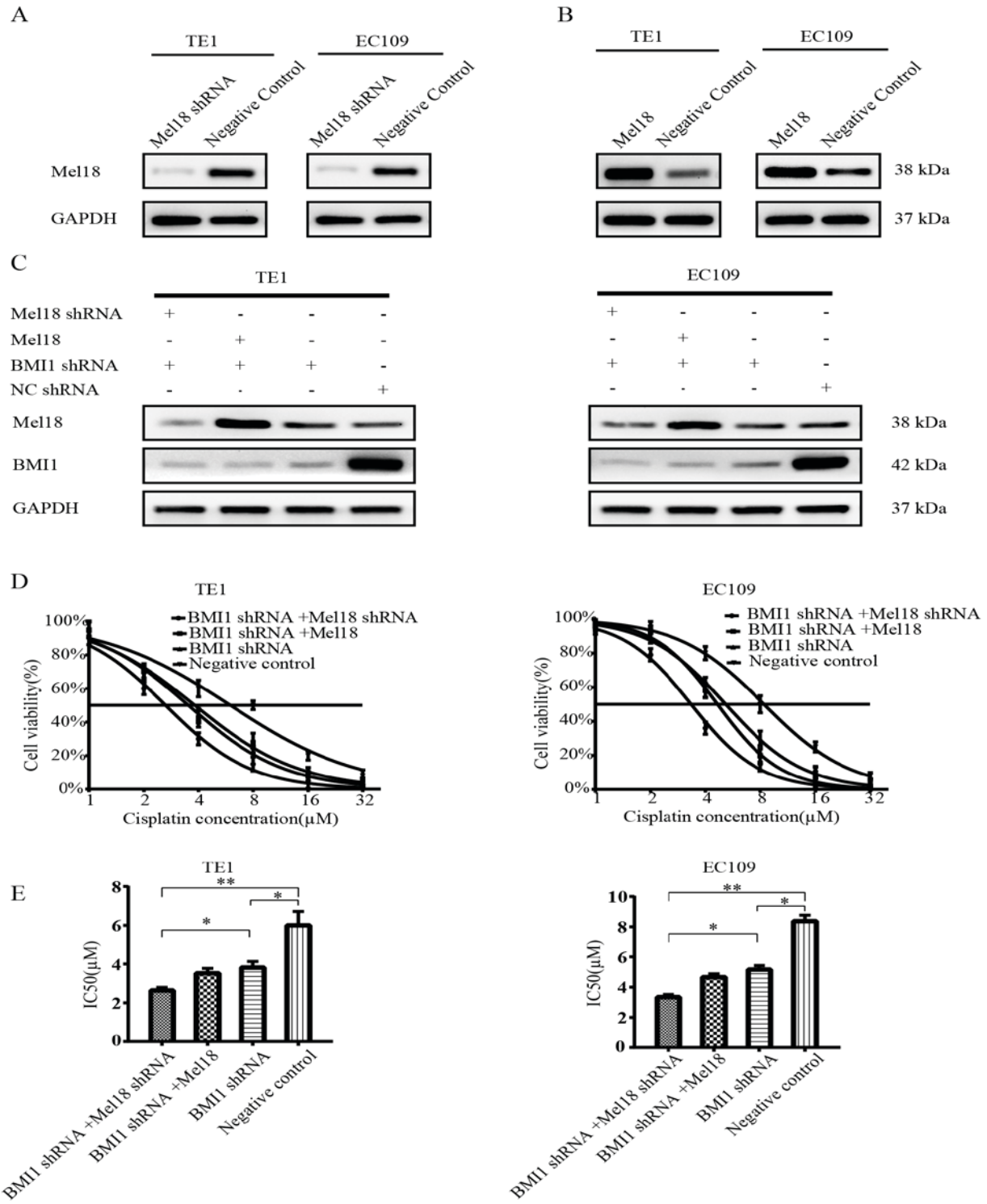

Figure 2. Knockdown of Mel18 and BMI1 further increases sensitivity of esophageal squamous cell carcinoma cells to cisplatin compared with knockdown of BMI1 alone. (A) Western blot analysis was used to determine the protein level of Mel18 in TE1 (left) and EC109 (right) cell lines following Mel18 shRNA plasmid transfection. (B) Western blot analysis was used to evaluate the protein level of Mel18 in TE1 (left) and EC109 (right) cell lines following transfection with Mel18 expression plasmid. (C) Transfection validation was achieved by western blot analysis. (D) Stably transfected TE1 (left panel) and EC109 (right panels) cells growing in 96-well plates were treated with the indicated doses of cisplatin. A Cell Counting Kit-8 assay was used to determine cell survival. The horizontal line indicates the IC_{50} value. (E) The IC_{50} values of cisplatin in transfected TE1 (left) and EC109 (right) cells are presented by histograms. Data were presented as the means ± standard deviation. $^{*}P<0.05$, $^{**}P<0.01$. IC_{50}, half maximal inhibitory concentration; shRNA, short hairpin RNA; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; Mel18, melanoma nuclear protein 18.

apoptosis compared with the BMI1 inhibition group (P<0.05) or the NC group (P<0.01), as presented in Fig. 4A and B. Similar results were revealed in the EC109 and TE1 cells. In summary, Mel18 and BMI1 inhibition can prompt an increase in the rate of apoptosis with BMI1 inhibition alone in ESCC cells.

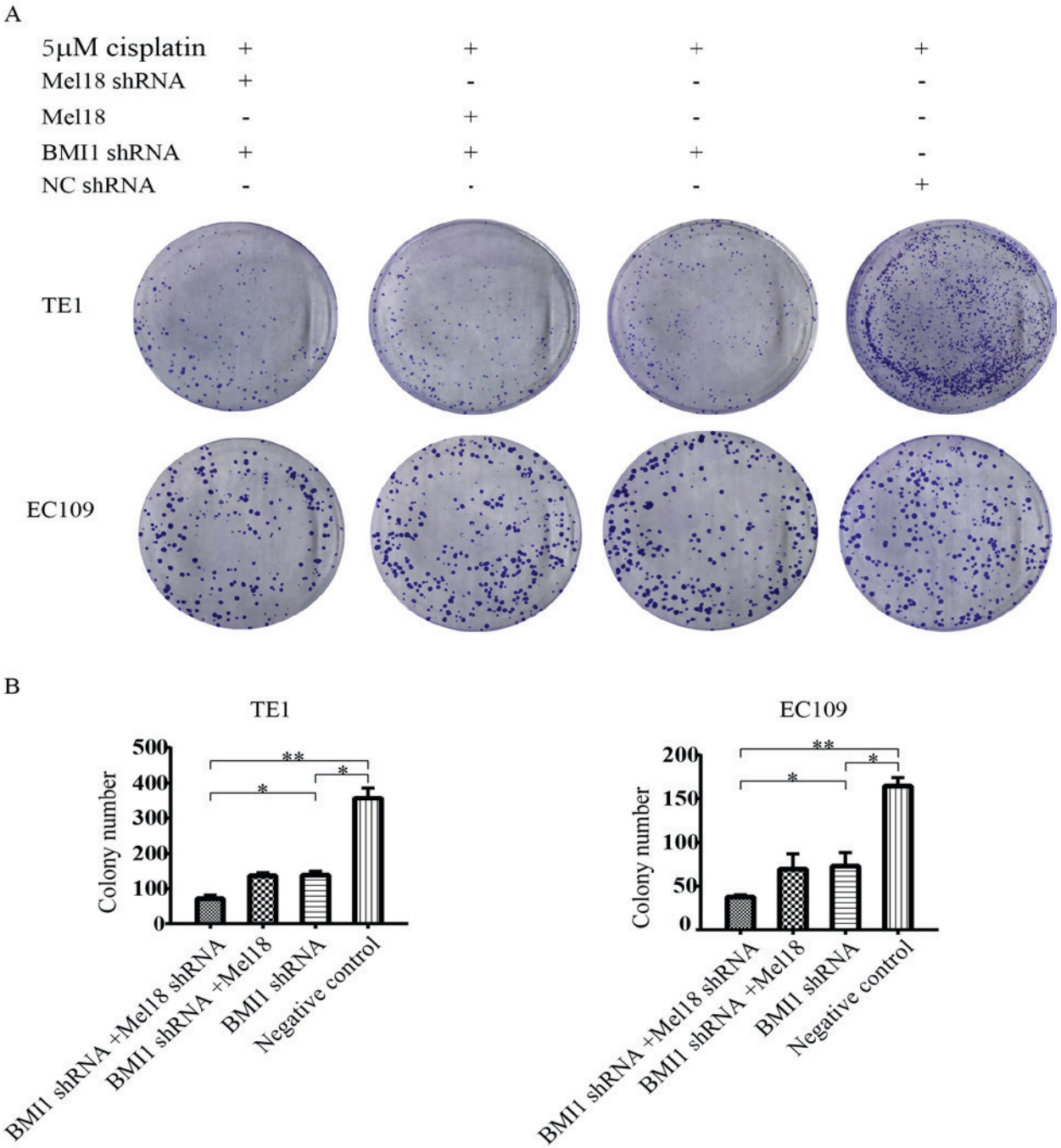

Figure 3. Knockdown of Mel18 and BMI1 further increases long-term sensitivity of ESCC cells to cisplatin. (A) Representative images of colony formation following treatment of ESCC cells with 5 μM cisplatin. (B) The numbers of colonies are presented by histograms. Data were presented as the means ± standard deviation. *P<0.05, **P<0.01. ESCC, esophageal squamous cell carcinoma; shRNA, short hairpin RNA; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; Mel18, melanoma nuclear protein 18.

c-Myc may be essential for BMI-1 and Mel18-induced sensitization to cisplatin. Cisplatin-induced apoptosis predominantly occurs via phosphoinositide 3-kinase/Akt, NF-κB and c-Myc dysregulation (36). Mel18 and BMI1 can regulate proliferation, apoptosis, angiogenesis, tumorigenesis and development via these pathways (28,37,38). To further investigate the mechanism underlying the enhancement of cisplatin-induced apoptosis by the silencing of BMI1 and Mel18, the present study examined the expression levels of proteins associated with these pathways, including total NF-κB, total-Akt, phosphorylated-Akt, c-Myc, caspase-3, BAX and Bcl-2, in the stably transfected cells. The protein expression levels were evaluated by western blot analysis. As presented in Fig. 5, the expression levels of caspase-3, BAX markedly increased and Bcl-2 markedly decreased in cells transfected with Mel18 shRNA and BMI1 shRNA compared with cells transfected with NC or BMI shRNA (all P<0.05), which indicated that BMII1 and Mel18-induced apoptosis may be closely associated with the mitochondrial apoptotic pathway. Inhibition of BMI1 was identified to reduce the expression levels of proteins

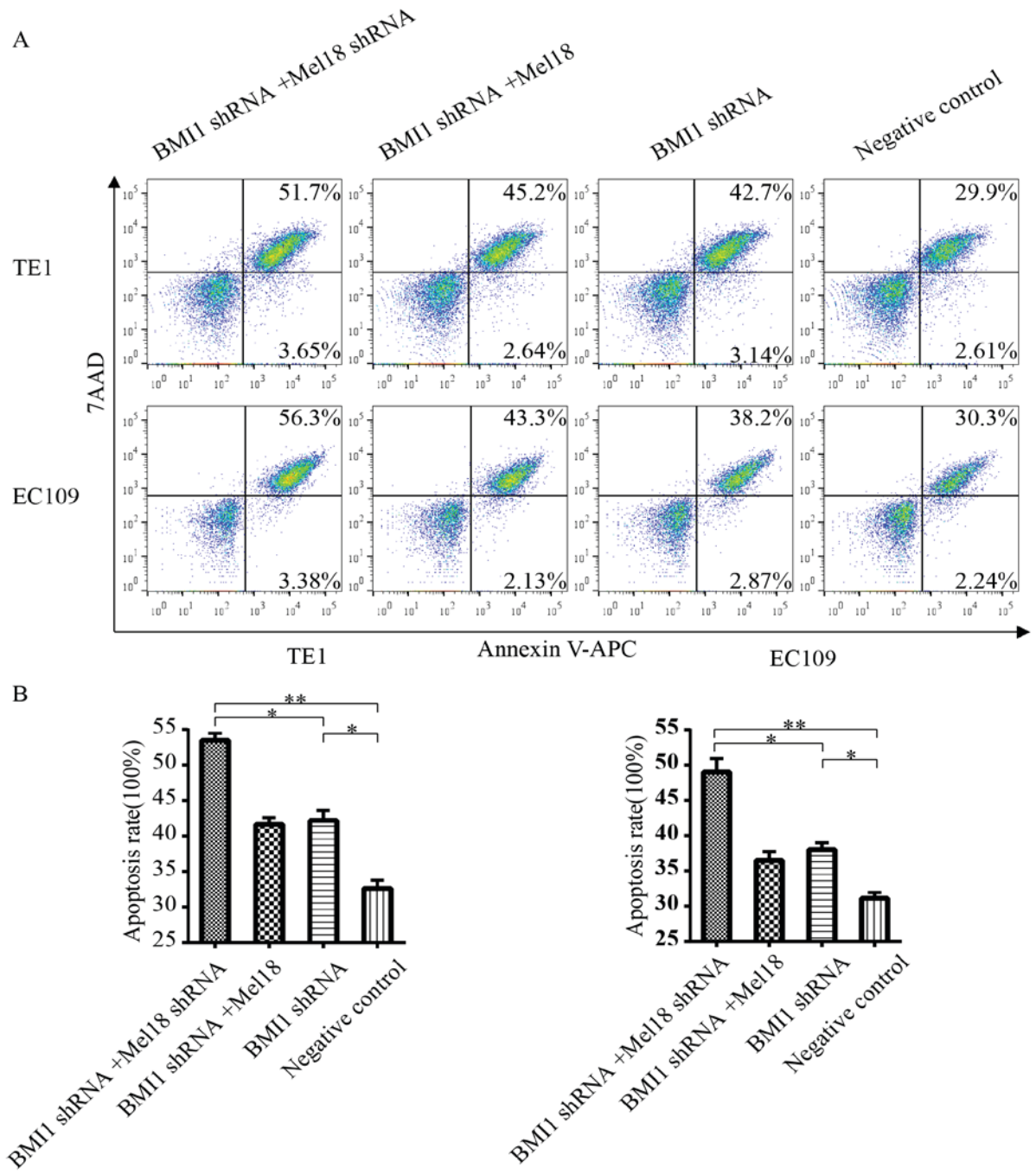

Figure 4. Inhibition of BMI11 and Mel18 increases apoptosis of ESCC cells. (A) Representative histograms present the apoptotic status of EC109 and TE1 cells using the Annexin V-APC/7-AAD method. The upper right and the lower right quadrants were quantified to indicate the number of apoptotic cells. (B) The quantified apoptosis rates of ESCC cells. Data were presented as the means ± standard deviation. *P<0.05, **P<0.01. ESCC, esophageal squamous cell carcinoma; shRNA, short hairpin RNA; APC, allophycocyanin; 7-AAD, 7-amino-actinomycin D; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; Mel18, melanoma nuclear protein 18.

associated with these signaling pathways to different extents, which is consistent with previous studies (6,7,9). Compared with BMI1 inhibition or NC group, the effect of inhibiting BMI1 and Mel18 on c-Myc was more notable (P<0.05), while the effect on NF-κB and Akt was limited.

BMI1 and Mel18 inhibition sensitize esophageal xenograft tumors to cisplatin. The effect of cisplatin treatment in combination with BMI1 and Mel18 inhibition on the growth of esophageal tumors was further determined *in vivo*. An ESCC xenograft model was established by subcutaneous injection of ESCC cells into the right flank of nude mice. The treatments were initiated once tumor volumes reached ~200 mm^3 (Fig. 6A). With the same does of cisplatin, the size of EC109 xenograft tumors in the BMI1 shRNA group were significantly smaller compared with the NC group (P<0.05; Fig. 6B). In addition, the tumor volume was markedly lower in the BMI1 and Mel18 shRNA group compared with the BMI1 inhibition

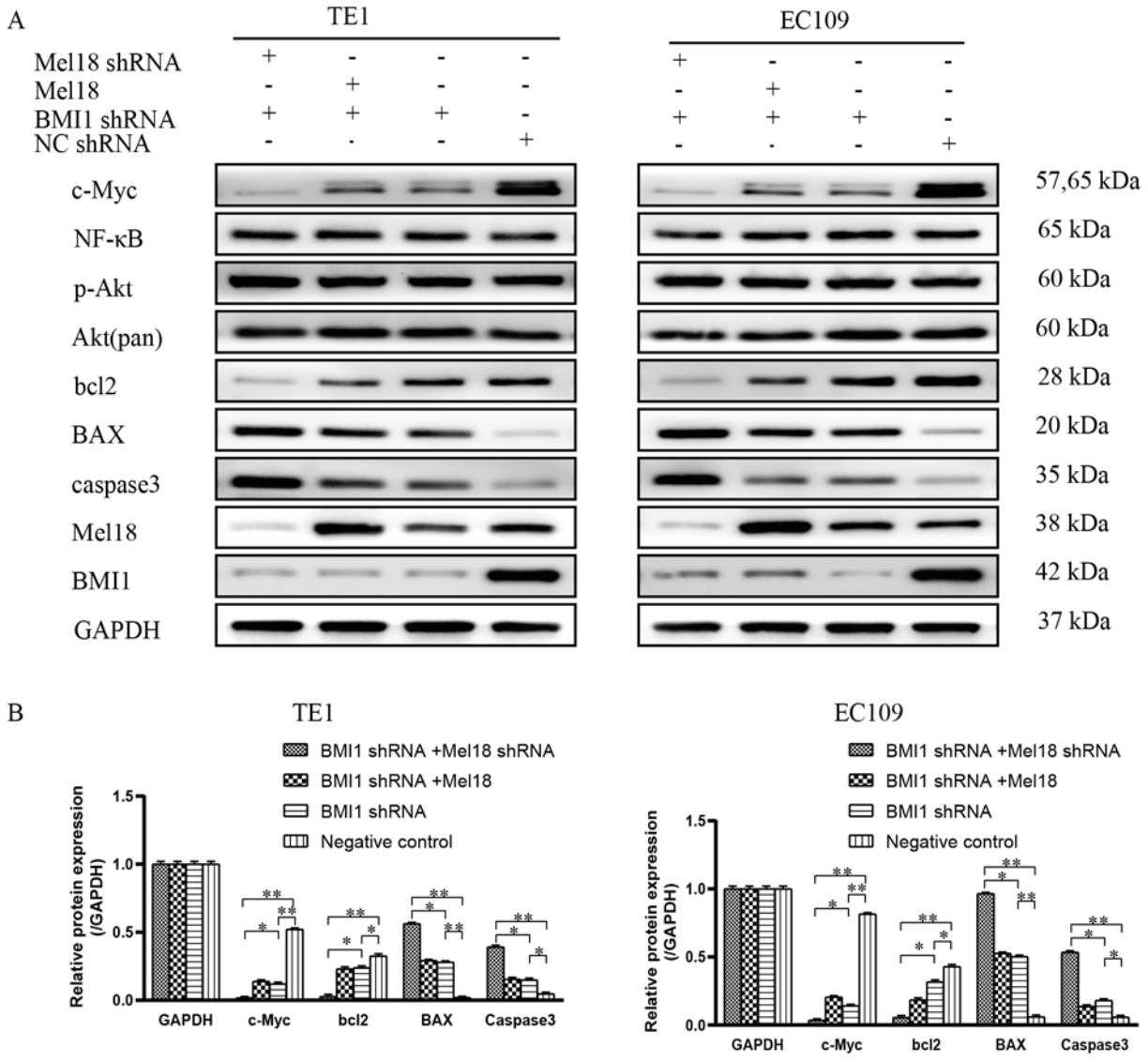

Figure 5. BMI1 and Mel18 inhibition regulates apoptosis-associated protein expression. Esophageal cancer cells transfected with BMI1 shRNA or Mel18 shRNA were treated with cisplatin. The expression level of c-Myc was markedly different among the different groups of cells. (A) Representative western blots images of three separated experiments. (B) Western blots quantification. Data were presented as the mean ± standard deviation. *P<0.05, **P<0.01. shRNA, short hairpin RNA; NF-κB, nuclear factor-κB; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; Mel18, melanoma nuclear protein 18; p, phosphorylated; Bcl-2, B-cell lymphoma-2; BAX, Bcl-2-associated X protein.

alone group (P<0.05; Fig. 6B). The expression levels of Mel18 and BMI1 in xenograft tumors were verified by western blot analysis (Fig. 6C). These results suggest that Mel18 inhibition can cooperate with BMI1 inhibition to enhance chemosensitivity of EC109 cells *in vivo*.

Discussion

Platinum-based chemoradiotherapy is the standard treatment for advanced esophageal cancer and postoperative recurrent esophageal cancer (4,39). A limitation of this therapy is the frequent occurrence of resistance to platinum. BMI1 is an important epigenetic regulator and stem cell marker (7,10). Previous studies have demonstrated that inhibition of BMI1 can regulate the expression of MDR1, affect platinum transport and hydration, and promote the chemotherapy sensitivity of ovarian, breast and pancreatic cancer (40-42). As a homologue of BMI1, the function of Mel18, and the interaction of BMI1 and Mel18 have been widely studied. However, the role of Mel18 remains controversial. The present study demonstrated that Mel18 inhibition, combined with BMI1 knockdown, can promote the chemosensitivity of esophageal cancer cells via cisplatin-induced apoptosis.

The current study established BMI1 knockdown EC109 and TE1 cells by transfection with BMI1 shRNA, followed by treatment with an increasing gradient of cisplatin. Compared with the negative control group, the cell survival rate and the IC_{50} value of the BMI1 inhibition group was significantly lower, which indicates that BMI1-inhibited esophageal cancer cells are sensitive to cisplatin. Therefore, there is a requirement to further investigate the association between BMI1 and cisplatin chemosensitivity.

Mel18 is a homologue of BMI1 and a component of PRC1. The structures of BMI1 and Mel18 are similar, and their

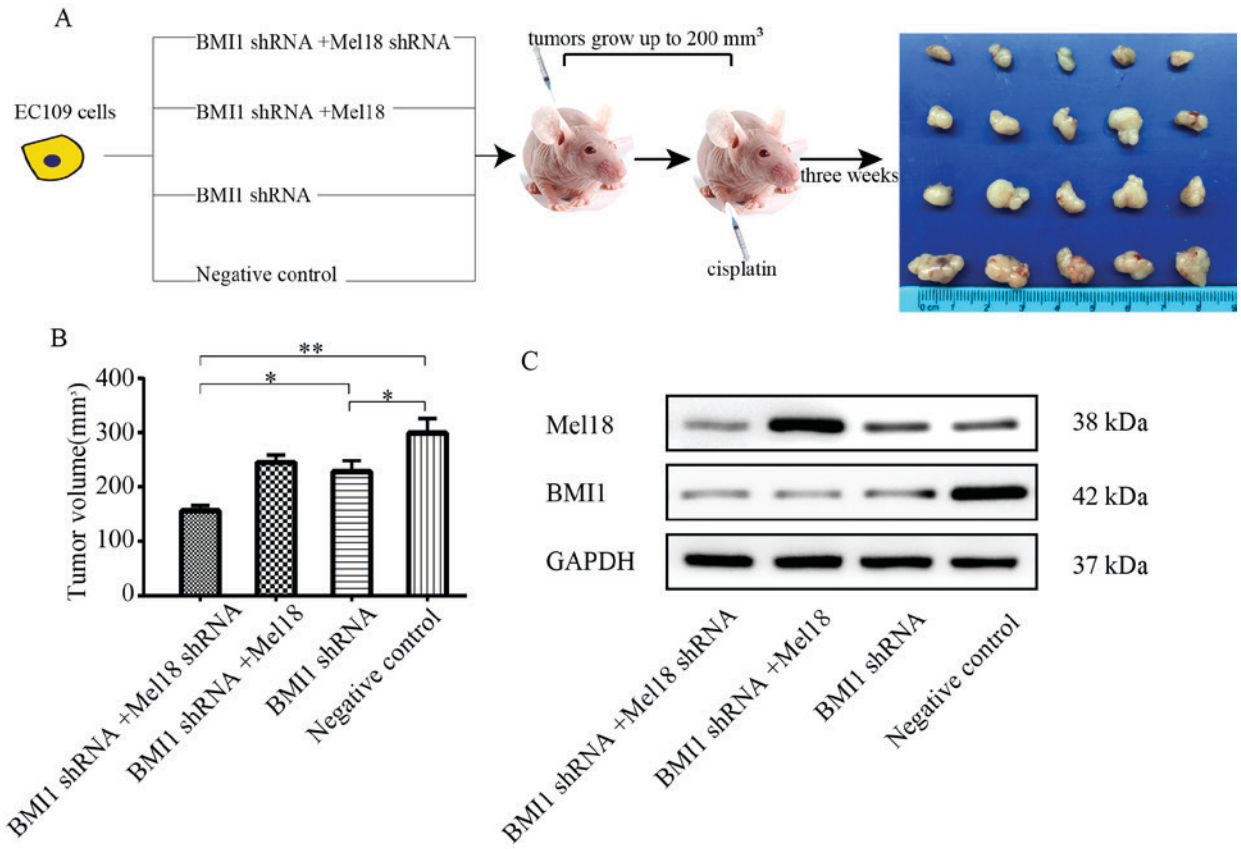

Figure 6. BMI1 and Mel18 inhibition sensitizes esophageal xenograft tumors to the effects of cisplatin. (A) The mice were randomly divided into four groups for different treatment strategies. Photographs of the tumors were obtained after ~3 weeks of treatment. (B) Tumor volumes were measured following treatment. (C) The expression levels of proteins of interest were verified by western blot analysis. Data were presented as the means ± standard deviation. *P<0.05; **P<0.01. shRNA, short hairpin RNA; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; Mel18, melanoma nuclear protein 18.

roles are similar in embryonic development; however, the association between Mel18 and BMI1 remains unknown (32). Previous studies have suggested that Mel18 can inhibit BMI1, which subsequently inhibits tumorigenesis, angiogenesis and tumor progression (20,31,43). By contrast, Liu *et al* (15) confirmed that BMI1 and Mel18 contribute to the development of colorectal cancer by promoting proliferation and reducing apoptosis via suppression of Reg3b expression. The interaction between BMI1 and Mel18, and the effects on tumor sensitivity to chemotherapy remain unknown and require further investigation.

In the present study, Mel18 was knocked down or overexpressed in esophageal cancer cell lines by stable transfection with BMI1 shRNA. Compared with the BMI1-inhibited group and the negative control group, inhibition of Mel18 and BMI1 markedly enhanced the short-term and long-term sensitivity of esophageal cancer cells to cisplatin, and these effects were consistent with the impacts on apoptosis and changes of protein levels associated with the mitochondrial apoptotic pathway. During an investigation of the core proteins associated with the mitochondrial apoptosis pathway, the expression level of c-Myc was identified to exhibit the most notable change following Mel18 and BMI1 inhibition. A number of studies have demonstrated that PRC1s, including BMI1 and Mel18, can regulate c-Myc to affect neoplastic cell proliferation and apoptosis (5,44). BMI1 has been identified to cooperate with c-Myc within the cell nucleus and BMI1 overexpression can inhibit c-Myc-induced apoptosis via negative regulation of the Ink4a-Arf pathway (5). Mel18 can regulate the cell cycle via a c-Myc/Cdc25 cascade (44). Combined with these previous studies, the results of the present study indicate that the regulation of chemotherapy sensitivity of esophageal cancer by BMI1 and Mel18 may be achieved via c-Myc regulation of the mitochondrial apoptosis pathway.

In summary, the present study confirmed the role of BMI1 in the regulation of tumor chemosensitivity and revealed a combined effect of Mel18 and BMI1 on tumor chemosensitivity, and confirmed this effect *in vivo*. To the best of our knowledge, the current study was the first to demonstrate that inhibition of BMI1 can increase the chemosensitivity of ESCC to platinum-based chemotherapy. In addition, it was demonstrated that Mel18 inhibition can enhance the effects of BMI1 inhibition. These effects were identified to be achieved via apoptosis-associated pathways. However, the current study also had numerous limitations and complete understanding of the associated mechanisms remains unknown. Liu *et al* (15) confirmed that BMI1 and Mel18 synergistically promote the development of colon cancer; however, the present study did not verify that the combined effects of the two molecules were achieved through synergy. In future studies it may be beneficial to study whether there is a direct interaction between the two molecules; this could be achieved by investigating whether

the combined effects of the two molecules are achieved via synergistic or additional effects. Although further clinical studies are required, Mel18 and BMI1 may serve as prominent therapeutic targets for ESCC chemotherapy.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Shandong Science and Technology Department Science and Technology Development Foundation (grant no. 2013GSF11836) and the Natural Science Foundation of Shandong Province (grant no. ZR2016HQ50).

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

JW and HJ designed the study and performed all *in vitro* experiments. QZ, JD and XY performed the animal experiments. ZJ designed the experiments and performed the statistical analysis.

Ethics approval and consent to participate

All animal procedures were approved by Qianfoshan Hospital Ethics Committee (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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