Received: 10 July 2017 | Revised: 9 November 2017 | Accepted: 5 December 2017

DOI: 10.1111/ajco.12841

ORIGINAL ARTICLE

WILEY

# Resveratrol induced apoptosis in human gastric carcinoma SGC-7901 cells via activation of mitochondrial pathway

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**Funding information**
Project of Jiang-su Province Health and Family Planning Commission, Grant/Award Numbers: H2017035, H201510

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## Abstract

**Background:** Resveratrol is a natural polyphenolic compound and its anticancer effect has been receiving considerable attention. Previous studies showed that resveratrol could inhibited the growth of human gastric carcinoma cells and apoptosis induction was an important mechanism. However, whether mitochondrial pathway was involved in resveratrol-induced apoptosis in human gastric cancer was not very clear.

**Methods:** The cells were examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, Annexin V/PI staining assay, mitochondrial membrane depolarization, cell morphological assessment, cytochrome *c* release assay, and Western blotting assay.

**Results:** In this study, we found that resveratrol induced apoptosis in human gastric carcinoma SGC-7901 cells. Cleaved PARP was observed and caspase-3 was activated by resveratrol. Next, the mitochondrial membrane potential of cells dissipated after the cells were treated by resveratrol. Moreover, we found that pro-caspase 9 was downregulated and cytochrome *c* released from mitochondrial to the cytosol. We also found that the expression ratio of Bax/Bcl-2 was increased in the treated cells. We finally showed that resveratrol inhibited the proliferation of SGC-7901 xerograph *in vivo*.

**Conclusions:** Collectively, our findings demonstrate that resveratrol triggers apoptosis via mitochondrial pathway in SGC-7901 cells, which provide more basis for resveratrol acting as antitumor agents in cancer therapy.

KEYWORDS
apoptosis, mitochondrial pathway, resveratrol

## 1 | INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second cause leading of cancer-related mortality in the world. According to the global estimation, a total of 951 600 new GC cases were diagnosed and a minimum of 723 100 patients died from this disease in 2012, accounting for 10% of total deaths from cancer.[1] Despite the improvement of conventional therapies for advanced GC, including surgery, chemotherapy, and radiotherapy, the length or quality of life of patients with advanced GC is still poor.[2,3] Therefore, the exploration of new preventive drugs or therapeutic targets of GC is urgently needed.

A promising source of therapeutic agents is that traditional medicine is derived from natural compounds. A wide variety of natural compounds derived from medicinal plants have been extensively studied for the treatment of human disease including different types of cancer. Resveratrol (trans-3,5,4-trihydroxystilbene), a natural polyphenolic compound, is found in mulberries, grapes, red wine, peanuts, knotweed and other plants.[4,5] Previous reports reveal that resveratrol has exhibited anticancer effect in several cancers,[6–12] including GC, and apoptosis induction is an important mechanism involved in its anticancer effect.

Apoptosis is mediated through two major pathways, the death receptor pathway and the mitochondrial pathway. The mitochondrial pathway is a complex course with mitochondria as central gateway controllers and the Bcl-2 family of proteins[13,14] as executioners. The Bcl-2 proteins can be divided into anti-apoptotic (Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1, Nr-13 and others) and pro-apoptotic members[15,16] The pro-apoptotic members are represented by two subgroups: the multidomain or Bax subfamily (Bax, Bak, and Bok) that contains multiple BH domains and the BH3-only subfamily (Bad, Bid, Bim,

  

Noxa, Hrk and others).[17,18] It is clear that the molecules upstream, like the BH3-only proteins, capture various apoptotic stimuli or signals and subsequently activate the multidomain pro-apoptotic proteins. Activation of the intrinsic pathway involves the release of pro-apoptotic factors, like cytochrome *c*, Smac/Diablo, Endonuclease G and AIF, from the mitochondrial intermembrane space, which amplifies the apoptotic cascades to the final destruction of the cell.[19,20] The apoptosome/caspase-9 pathway is a signaling route downstream of the mitochondrion. The release of cytochrome *c* triggers the formation of apoptosome complex and the activation of caspase-9.[21]

Previously, Liu reported that resveratrol could induce apoptosis in human GC SGC-7901 cells.[22] However, there have no studies showing that whether mitochondrial pathway is involved in resveratrol-induced apoptosis in human GC SGC-7901. The aim of present study is to investigate the potential effect of resveratrol on mitochondrial pathway in SGC-7901 cells.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

Resveratrol was obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA), and it was dissolved in Dimethyl sulfoxide (DMSO) at 100 mM and stored at −20°C. The concentrations used in this study were 25 and 50 $\mu$M, and the basal medium was diluted freshly with a final DMSO concentration of 0.1%. Controls were always treated with the same amount of DMSO as used in the corresponding experiments.

Antibodies to caspase-3(sc-56052), caspase-9 (sc-56073), Bax (sc-526) and Bcl-2(sc-7382) were obtained from Santa Cruz (Santa Cruz, CA, USA); antibody to cytochrome *c* was from CALBIOCHEM (Merck, Darmstadt, Germany); antibodies to PARP were from Cell Signaling (Danvers, MA, USA) and antibody to $\beta$-actin (BM0627) was from Boster (Wuhan, China). IRDye$^{TM}$800 conjugated secondary antibodies were obtained from Rockland Inc. (Philadelphia, PA, USA), and other chemicals were all obtained from Sigma unless otherwise stated.

### 2.2 | Cell line

Human gastric carcinoma SGC-7901 cells were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in 90% RPMI1640 medium (Gibco, NY, USA) supplemented with 10% FBS (Sijiqing, Hangzhou, China), 100 U/mL penicillin and 100 $\mu$g/mL streptomycin. Exponentially growing cultures were maintained in a humidified atmosphere of 5% $CO_2$ at 37°C.

### 2.3 | MTT

Cell toxicity was measured by a colorimetric assay using MTT as described previously.[23] Experiments were performed in triplicate in a parallel manner for each concentration of resveratrol used for 24 h. Control cells were treated with culture media containing 0.1% DMSO. Absorbance (*A*) was measured at 570 nm. Cell death (%) was calculated using the following equation: cell death $(\%) = (1 - A_{\text{treatment}}/A_{\text{control}}) \times 100\%$.

### 2.4 | Animal model

Male athymic BALB/c nude mice (35–40 days old) with body weight ranging from 18 to 22 g were supplied by the Academy of Military Medical Sciences of the Chinese People's Liberation Army (Certificate No. SCXK-(Army) 2017–004). The animals were maintained at 22 ± 2°C with 55–65% humidity in stainless steel cages under controlled light (12 h light/day) and were fed with standard laboratory food and water. Animal care was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health, USA.

Twenty nude mice were inoculated subcutaneously with $1 \times 10^7$ SGC-7901 cells into the right axilla. After 12 days of growth, tumor sizes were determined using micrometer calipers. Mice-inoculated SGC-7901 cells with similar tumor volumes were randomly divided into the following two groups (five mice/group): saline control and resveratrol (50 mg/kg, i.v., every 2 days). Tumor sizes were measured every 3 days using micrometer calipers, and tumor volume was calculated using the following formula: $\text{TV}(\text{mm}^3) = d^2 \times D/2$, where *d* and *D* were the shortest and the longest diameters, respectively. Mice were sacrificed on day 21, and tumor tissues were used for immunohistochemistry assay.

### 2.5 | Annexin V/PI staining

Apoptotic cells were identified by Annexin V-FITC Apoptosis Detection Kits (BioVision, Moutain View, CA, USA) according to the manufacturer's instructions. In brief, $1 \times 10^6$ cells were harvested and washed with PBS. Cells were resuspended in 500 $\mu$L binding buffer and then 5 $\mu$L Annexin V-FITC and 1 $\mu$L PI were added. Flow cytometric analysis was performed immediately after double supravital staining. Data acquisition and analysis were performed using a Becton Dickinson FACSCalibur flow cytometer and CellQuest software.

### 2.6 | Mitochondrial membrane depolarization

Mito PT Apoptosis Detection Kit (B-Bridge International Inc., San Jose, CA, USA) was used to measure the mitochondrial membrane potential according to the manufacturer's protocol. Briefly, cells were incubated with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzamidazolo carbocyanin iodide (JC-1) for 30 mins at 37°C, then washed twice with cold buffer and resuspended in washing buffer. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 590 nm using laser scanning confocal microscope and FACSCalibur flow cytometer.

### 2.7 | Cell morphological assessment

To observe the morphological evidence of apoptosis, cell nuclei was visualized following DNA staining with the fluorescent dye DAPI. Briefly, cells were seeded in six-well tissue culture plates at a

concentration of $1 \times 10^5$ cells/well and they were treated with 25 or 50 $\mu$M resveratrol for 24 h. At the end of incubation, the morphology of cells was monitored under an inverted light microscope. Cells were fixed with 4% paraformaldehyde for 20 mins and washed with PBS, and then incubated with DAPI (1 $\mu$g/mL) for 10 min. After being washed with PBS, cells were observed by using fluorescent microscopy (Olympus, Tokyo, Japan) with a peak excitation wave length at 340 nm.

### 2.8 | Cytochrome *c* release assay

The fractionation of the mitochondrial protein and cytosolic protein was extracted according to cytochrome *c* release Apoptosis Assay Kit (CALBIOCHEM) instruction. Briefly, $5 \times 10^7$ cells were collected by centrifugation at 600 × *g* for 5 min at 4°C and washed with ice-cold PBS. Cells were resuspended with 1 mL of 1 × cytosol extraction buffer mix containing dithiothreitol and protease inhibitors and incubated on ice for 10 min. Then cells were homogenized in an ice-cold grinder and the homogenate was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 700 × *g* for 10 min at 4°C. The supernatant was transferred to a fresh 1.5 mL microcentrifuge tube and centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant was collected as cytosolic fraction and the pellet resuspended in 0.1 mL mitochondrial extraction buffer mix containing DTT and protease inhibitors and then vortexed for 10 s and saved as mitochondrial fraction. Western blotting was used to detect cytochrome *c* of cytosolic fraction and mitochondrial fraction with cytochrome *c* antibody (CALBIOCHEM).

### 2.9 | Western blotting assay

Briefly, after washing twice with PBS, the cultured cells were collected and lysed in lysis buffer (100 mM Tris-Cl, pH 6.8, 4% [m/v] SDS, 20% [v/v] glycerol, 200 mM b-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and 1 g/mL aprotinin). Lysates were centrifuged at 13 000 × *g* for 15 min at 4°C. The concentration of total proteins were measured by BCA assay method with Varioskan spectrofluorometer and spectrophotometer (Thermo) at 562 nm. Protein samples were separated with 15% SDS-PAGE gel and transferred onto the PVDF membranes (Millipore). Immune complexes were formed by incubation of proteins with primary antibodies overnight at 4°C followed by IRDyeTM 800 conjugated second antibody for 1 h at 37°C. Immunoreactive protein bands were detected with an Odyssey Scanning System (LI-COR Inc., Lincoln, Nebraska, USA).

### 2.10 | TUNEL assay

Apoptosis induction in the tissue specimen was analyzed by TUNEL assay. It was performed as per instructions given *in situ* Cell Death Kit. The slides were counter stained with hematoxylin, dehydrated and sealed with a cover slip and sealant. The slides were photographed with a confocal laser scanning microscope (Fluoview FV1000; Olympus). Visceral tissue resected from control and test mice were fixed in formalin. Paraffin embedding steps were performed and then tissue sections (4 $\mu$m thick) were prepared and stained with hematoxylin eosin stain (H&E) for tissue pathology.

### 2.11 | Statistical analysis

All results were presented as the mean ± SEM of triplicate experiments performed in parallel unless otherwise indicated. Statistical analyses were performed using an unpaired two-tailed Student's *t*-test or one-way Analysis of Variance (ANOVA). All comparisons were made relative to untreated controls; significant differences were indicated as $^*P < 0.05$ and $^{**}P < 0.01$.

## 3 | RESULTS

### 3.1 | Resveratrol inhibits the viability of SGC-7901 cells

MTT assay showed that resveratrol significantly inhibited the viability of SGC-7901 cells (Figure 1A). After the cells incubated continuously in the absence or presence of different concentrations of resveratrol for 24 h, the inhibitory effect of resveratrol on SGC-7901 cells was in a concentration-dependent manner and statistically significant compared with the control group ($P < 0.01$). The value of $IC_{50}$ is 48.28 ±5.6 $\mu$M for 24 h resveratrol treatment. As shown in Figure 1B, untreated SGC-7901 cells grew well with clear skeletons while the cells treated with resveratrol were equivocal and some of them were turned around. With the enhancement of drug concentration, the floated cells increased.

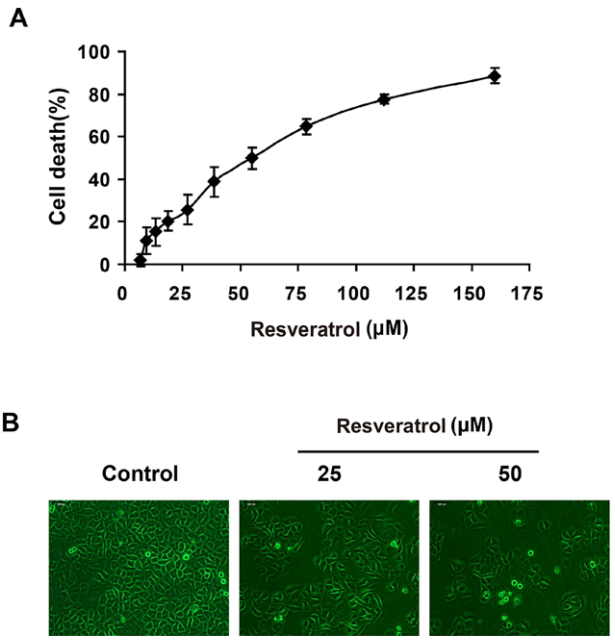


**FIGURE 1** Resveratrol inhibits the viability of SGC-7901 cells. A, SGC-7901 cells were treated with the different concentrations of resveratrol for 24 h. Cell viability was estimated by MTT assay. B, SGC-7901 cells were treated with different concentrations of resveratrol for 24 h and then observed under inverted light microscope

### 3.2 | Resveratrol induces apoptosis in SGC-7901 cells

To identify apoptosis induced by resveratrol, we first examined the biochemical events induced by resveratrol in SGC-7901 cells. As shown in Figure 2A, significant alterations of the nucleus were observed in using DAPI staining after treatment for 24 h. The cells presented the morphological features of apoptosis, such as bright nuclear condensation and the apoptotic body, which appeared with the increasing concentration of resveratrol (Figure 2A). Then resveratrol-induced apoptosis was further studied by Annexin-V/PI staining assay. As shown in Figure 2B, after treated with 25 and 50 $\mu$M resveratrol for 24 h, the early and median apoptotic cells (right lower section of fluorocytogram) were determined to be 16.8% and 21.6%, respectively, whereas the control was 6.5% only. Meanwhile, the late apoptotic and necrotic cells (right upper section of fluorocytogram) were determined to be 3.4% and 4.5%, and the control was 1.2%. Annexin V/PI staining assay demonstrated that the percentage of apoptotic cells increased with increasing concentrations of resveratrol. When cells were treated with 50 $\mu$M resveratrol for 24 h, the apoptotic rate reached to 26.1%. Furthermore, caspase-mediated PARP cleavage and caspase-3 cleavage showed that resveratrol-induced apoptosis was a concentration-dependent process (Figure 2C). These results suggested that apoptosis induction of resveratrol was involved in its antitumor activity.

### 3.3 | Resveratrol induces of MMP collapse of SGC-7901 cells

Next, we detected the changes of the mitochondrion. The green-fluorescent JC-1 probe existed as a monomer at low membrane potential but formed red-fluorescent "J-aggregates" at higher potentials. Thus, the ratio of red-to-green JC-1 fluorescence only depended on the membrane potential. As shown in Figure 3A, with the increase of drug concentration, the red fluorescence was attenuated whereas the green fluorescence was enhanced. The results demonstrated that the mitochondrial membrane potential of cells dissipated after resveratrol treatment for 24 h. Percentage of cells of MMP lost reached 15.7% when cells were incubated with 50 $\mu$M resveratrol for 24 h, as determined by FACSCalibur flow cytometer assay (Figure 3B). These

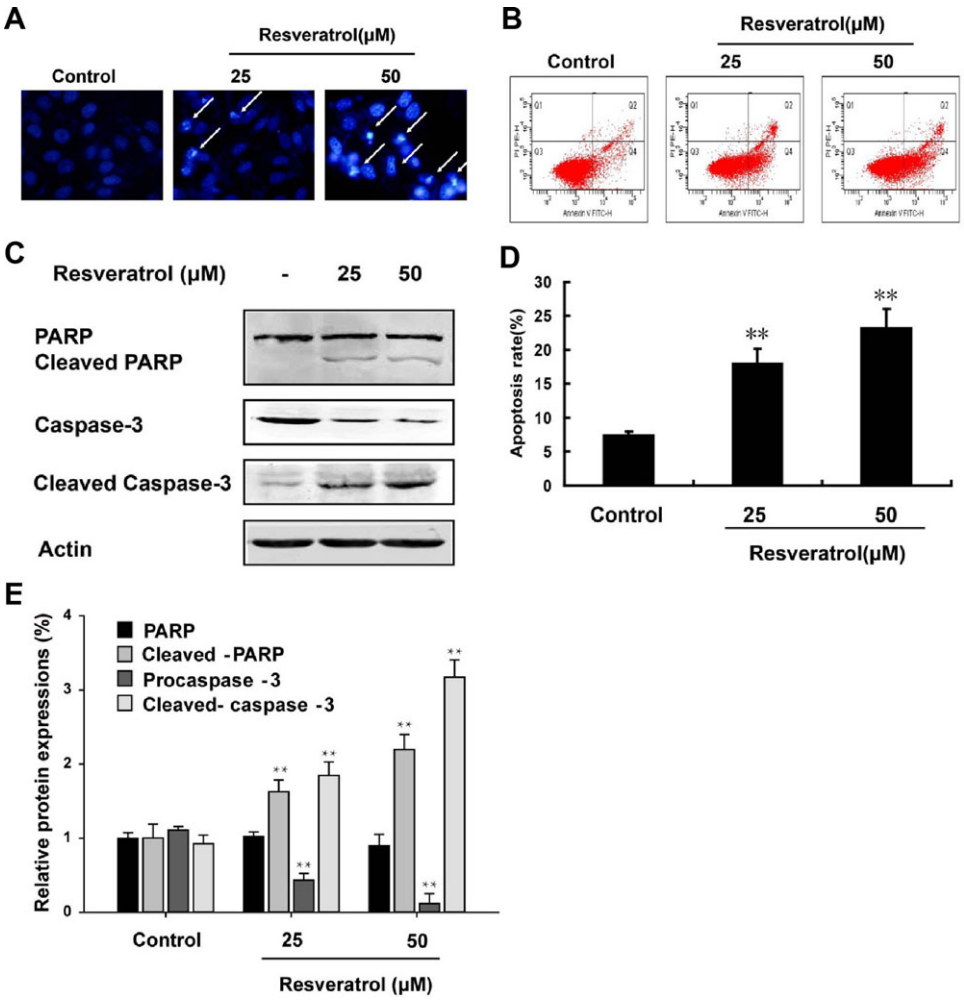


**FIGURE 2** Resveratrol induces apoptosis in SGC-7901 cells. A, DAPI staining of the SGC-7901 cells in the DMSO, resveratrol (25 $\mu$M), and resveratrol (50 $\mu$M) for 24 h. B, Meanwhile, annexin V/PI staining were used to detect the apoptotic cells after incubating with resveratrol for 24 h. Results were given as means ± SEM of three independent experiments. C, Cells were treated with indicated concentrations of resveratrol for 24 h. The levels of PARP and caspase-3 were assessed by Western blot analysis

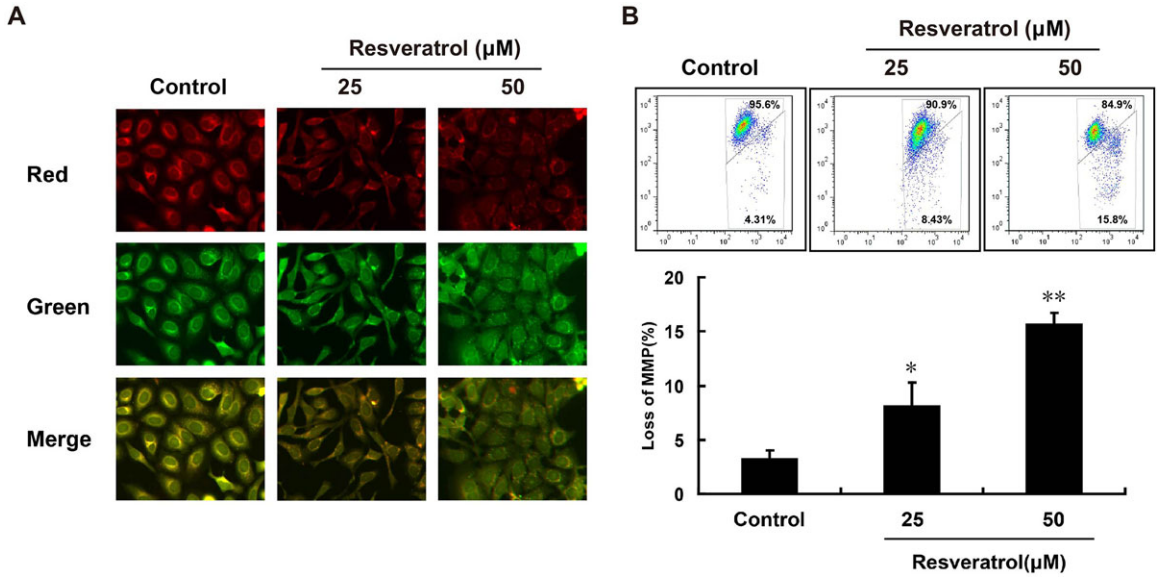


**FIGURE 3** Resveratrol induces of MMP collapse of SGC-7901 cells. A, Mitochondrial transmembrane potential was determined by Mito PT apoptosis detection kit. After cells were treated with indicated concentration of resveratrol for 24 h, changes of mitochondrial membrane potential of cells were detected with JC-1 using laser scanning confocal microscope and flow cytometer (B), respectively. Results were given as means ± SEM of three independent experiments

results suggested that the mitochondrion pathway may be involved in resveratrol-induced apoptosis.

### 3.4 | Mitochondrion pathway involved in resveratrol-induced apoptosis

We further examined the involvement of caspases-9 in resveratrol mediated apoptosis, which was involved in mitochondrial pathway.[21] The result showed that the level of pro-caspase-9 decreased after treatment for 24 h (Figure 4A). Meanwhile, the apoptotic protein Bax increased while Bcl-2 decreased, so the ratio of Bax/Bcl-2, which was crucial for the activation of mitochondrial apoptotic pathway increased in the cells treated with resveratrol. Moreover, cytochrome *c* released from mitochondrial to the cytosol were observed after resveratrol treatment for 24 h (Figure 4B). These results demonstrated that mitochondrion pathway was involved in resveratrol induced apoptosis in SGC-7901 cells.

### 3.5 | Resveratrol induced Bcl-2 mitochondrial translocation *in vivo*

Immunofluorescence analysis was used to detect the subcellular localization of Bcl-2 and mitochondria *in vivo*. SGC-7901 cells were transplanted in BALB/C nude mice and 50 mg/kg of resveratrol was administrated by intraperitoneal injection. Twenty mg/kg 5-Fu was used as the positive control. After 21 days, tumor samples were collected and studied by immunohistochemical staining. The results clearly showed that resveratrol significantly inhibited the growth of SGC-7901 tumors (Figure 5A–C). Compared with resveratrol, 5-Fu showed much greater cytotocity as evidenced by declined body weight (Figure 5A). The photographs of confocal microscopy demonstrated that Bcl-2 in cytoplasm distributed more widely than that in the control group (Figure 5D).

### 3.6 | Resveratrol suppressed the tumor growth in mouse xenograft model

The anticancer effect of resveratrol was explored in xenograft tumor model. SGC-7901 cells were transplanted in BALB/C nude mice and 50 mg/kg of resveratrol was administrated by intraperitoneal injection. Fifty mg/kg 5-Fu was used as the positive control. After 21 days, tumor samples were collected and studied by immunohistochemical staining. The results clearly showed that resveratrol significantly inhibited the growth of SGC-7901 tumors (Figure 5A and B). Compared with resveratrol, 5-Fu showed much greater cytotocity as evidenced by declined body weight (Figure 5C). Western blotting analysis was carried out to further determin cleaved caspase-3, which were highly induced by resveratrol, leading to apoptosis in tumor tissues and cell death eventually (Figure 5D). Moreover, apoptosis of tumor tissues in different groups were also determined by TUNEL assay shows that apoptosis index of resveratrol more widely than that in the control group. Immunohistochemical analysis indicated that Bcl-2 expression in tumor sections was decreased after treatment with resveratrol (Figure 5E).

## 4 | DISCUSSION

Resveratrol is the member of phytoalexin family, which is low molecular weight and secondary metabolites produced by plants as a defensive mechanism against certain stresses. Studies showed that resveratrol exerted its anticancer effect in several cancers by various mechanisms, such as inducing apoptosis,[6] suppressing invasion and metastasis,[8] increasing antioxidant capacity[24] and sensitizing to chemotherapy-triggered apoptosis.[25] Mitochondria are a key pharmacological target in all cancer cells, because the structure and

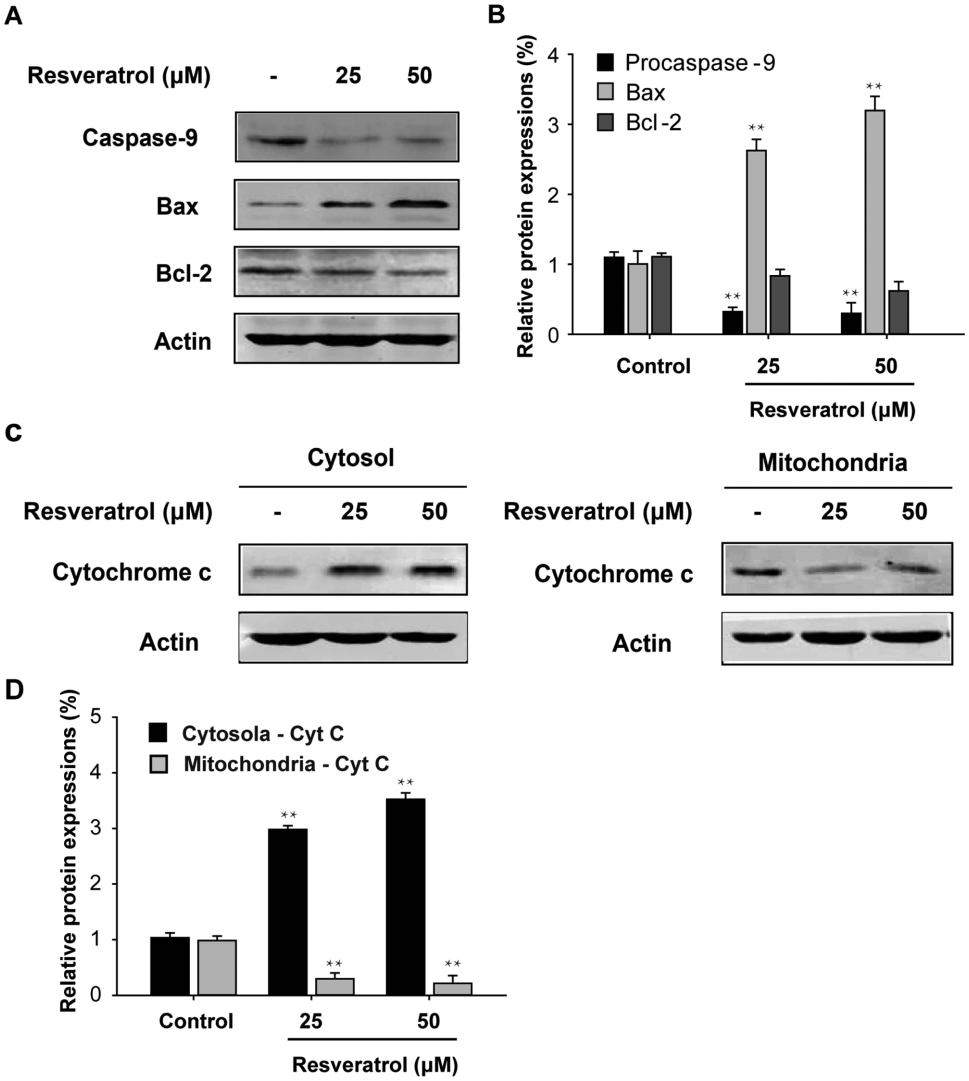


**FIGURE 4** Mitochondrion pathway involved in resveratrol-induced apoptosis. A, SGC-7901 cells were treated with indicated concentrations of resveratrol for 24 h. The levels of pro-caspase-9, Bax and Bcl-2 were assessed by Western blot analysis. B, Western blotting analysis of cytochrome *c* in the cytosolic fraction and mitochondrial fraction respectively. The fractionation of the mitochondrial protein and cytosolic protein were performed according to the cytochrome *c* release apoptosis assay kit instruction. Cytochrome *c* was analyzed by western blotting analysis with cytochrome *c* antibody

function of this organelle is different between healthy and malignant cells. The mitochondria in tumor cells maintains in hypermetabolic status, which means the mitochondrial pathway is a complex course with central gateway controllers and the Bcl-2 family of proteins. A critical regulation of the cellular apoptosis through the MPTP is the balance between pro- and anti-apoptotic Bcl-2 family proteins, so the drugs aiming at mitochondria are supposed to having a tumor selectivity.

The aim of this study was to investigate the inhibitory effect of resveratrol on human gastric carcinoma SGC-7901 cells and the underlying mechanism. In a series of systematic studies, we have characterized the mechanisms by which resveratrol exerts the inhibitory effect on SGC-7901 cells through inducing apoptosis. Our results showed that resveratrol significantly inhibited the proliferation of SGC-701 cells in a concentration-dependent manners with $IC_{50}$ at 48.28 ±5.6 $\mu$M for 24-hour treatment (Figure 1A). DAPI staining and Annexin V/PI staining assay showed that resveratrol induced apoptosis in SGC-7901 cells (Figure 2A and B).

Caspases family plays an important role in the regulation of apoptosis, and activation of caspases is a hallmark of promoting apoptosis in response to death-inducing signals originated from cell surface receptors, mitochondria, or endoplasmic reticulum.[26] In particular, activation of caspase-3 plays the central role in the initiation of apoptosis.[27] This enzyme has substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD) and cleaves poly(ADP-ribose) polymerase (PARP). It has been shown that activation of caspase-3 requires the activation of initiator caspases such as caspase-8 or -9 in responses to the proapoptotic signals.[28] Caspase-8 is believed to be activated through the engagement of certain members of tumor necrosis factor with death receptors on cell surface, whereas involvement of mitochondria has been found in caspase-9 activation. In our study, the event was very clear, particularly after 24 hours of resveratrol treatment cleaved

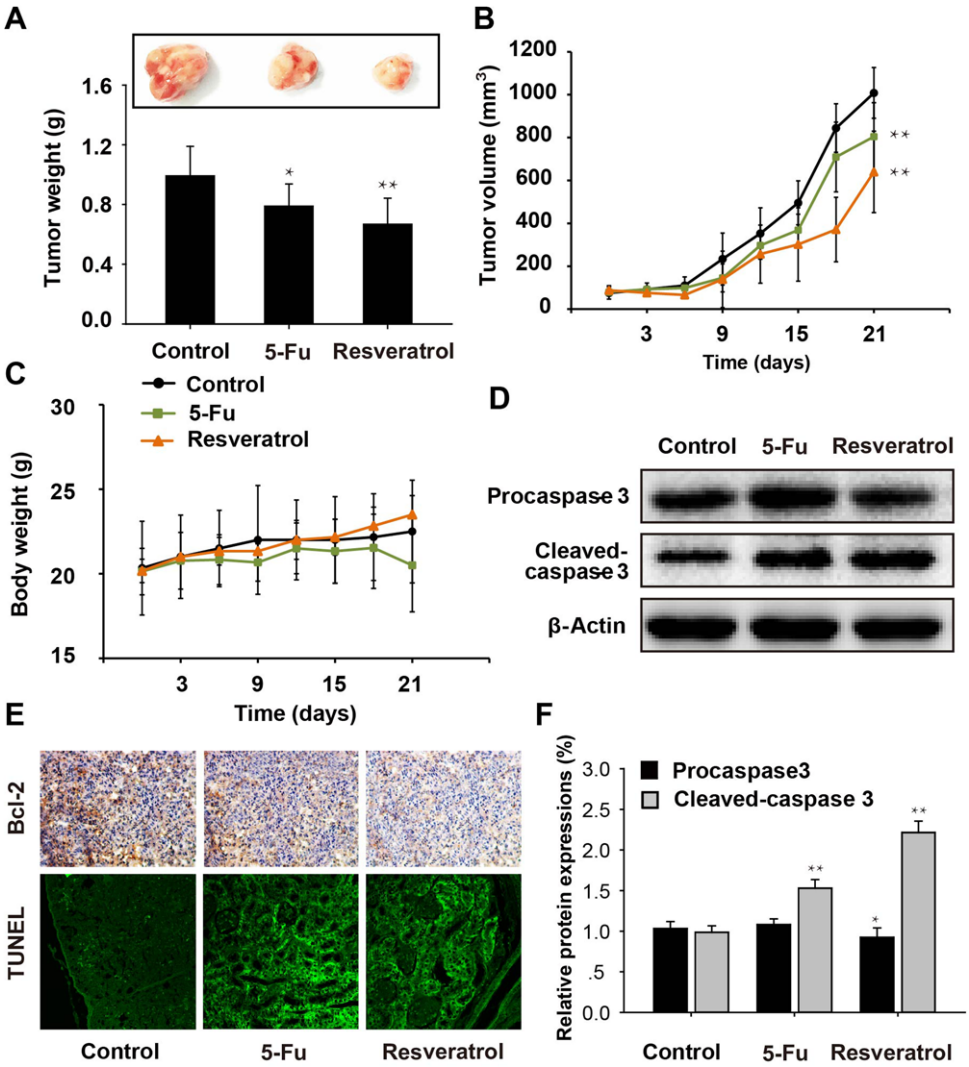


**FIGURE 5** The effects of resveratrol treatment on the tumorigenicity of SGC-7901 cells *in vivo*. (A) Mice were treated with resveratrol or 5-Fu, as described in the Materials and Methods once every 3 days for 21 days. Tumors were excised from the animals after treatment. Tumor mass were weighed. (B) Tumor volumes were measured and calculated once every 3 days. (C) Body weight was measured every 3 days. (D) Caspase-3 expression in tumor xenograft tissues were detected by Western blotting. (E) The immunohistochemical analysis and TUNEL assay were used to analyze Bcl-2 level and apoptosis in tumor tissue isolated from mice treated under various conditions (× 200). Each histogram represented the mean ± SD of six mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistically significant differences versus control group

PARP were observed and caspase-3 and caspase-9 were all activated in the cells (Figures 2C and 4A). Our results indicated that mitochondrial pathways were involved in resveratrol-induced apoptosis.

Mitochondrial membrane damage, the release of cytochrome *c* from mitochondria and the activation of caspase-9 have all been observed during apoptosis induced by various cellular stresses.[29,30] At the same time, loss of MMP, accumulation of ROS and decline of GSH are observed with the release of cytochrome *c* and AIF from mitochondria to the cytosol. Our results showed that the mitochondrial membrane potential of cells dissipated after resveratrol treatment for 24 h in SGC-7901 cells (Figure 3A and B). Meanwhile, the release of cytochrome *c* from mitochondria were observed (Figure 4B). These results demonstrated that resveratrol induced apoptosis of SGC-7901 cells by mitochondrial pathways.

In summary, our study demonstrated that resveratrol inhibited the growth of human gastric carcinoma SGC-7901 cells and apoptosis induction by mitochondrial pathways was an important mechanism. These results provided more basis for resveratrol acting as anti-tumor agents in cancer therapy.

## ACKNOWLEDGMENTS

This project was supported by Project of Jiang-su Province Health and Family Planning Commission (H2017035), Project of Jiang-su Province Department (BK20161598), the Project of Science and Technology of Nanjing Science Committee (201605006), Project of Jiang-su Province Health and Family Planning Commission (H201510) and Project of Jiangsu Cancer Hospital (ZK201603).

## CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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**How to cite this article:** Yang Y, Huang X, Chen S, et al. Resveratrol induced apoptosis in human gastric carcinoma SGC-7901 cells via activation of mitochondrial pathway. *Asia Pac J Clin Oncol*. 2018;1–8. https://doi.org/10.1111/ajco.12841