Resveratrol inhibits the proliferation of human melanoma cells by inducing G1/S cell cycle arrest and apoptosis

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Abstract. Resveratrol (Res), a natural plant extract, is an effective inducer of cell apoptosis and cell cycle arrest in multiple carcinoma cell types, which has been demonstrated by its ability to inhibit the proliferation of multiple human tumor cells *in vitro*. Although Res possesses chemopreventive properties against several types of cancer, the molecular mechanism underlying its anticancer activity remains to be fully elucidated. The present study demonstrated that Res induced cell cycle arrest and inhibited the proliferation of human melanoma A375 (IC50=23 μM after 48 h; P<0.05) and SK-MEL-31 (IC50=15 μM after 48 h; P<0.05) cells. Western blot analysis demonstrated that Res induced the apoptosis of human melanoma A375 and SK-MEL-31 cells by upregulating the expression of Bcl-2-associated X protein and B-cell lymphoma 2, possibly via the p53 pathway and activation of caspase-9 and caspase-3.

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Introduction

Resveratrol (Res; $C_{14}H_{12}O_3$; PubChem, CID: 445154; Fig. 1A) is primarily found in the skin of grapes and has been demonstrated to exhibit health-promoting benefits to the coronary, neurological, hepatic and cardiovascular systems (1-3). In addition, Res inhibits the proliferation of tumor cells of different etiologies (4,5). The biological properties of Res have been described in detail (6-9). The predominant form of resveratrol in plants is the glycosylated (3-O-β-D-glucosides) form (also termed the piceid form). It has traditionally been used as an anti-inflammatory agent. Other beneficial properties of resveratrol include antioxidant effects, cardioprotection and increased longevity. The antitumor activities of Res are mediated through several cell signaling pathways, including cell cycle arrest, suppression of tumor cell proliferation, induction of apoptosis and differentiation, angiogenesis and the inhibition of invasion, adhesion and metastasis (10). Skin cancer is one of the major causes of cancer-associated mortality worldwide. In addition, human cutaneous malignant melanoma is an aggressive cause of mortality, which exhibits a rising trend every year (9). In order to inhibit the development of cancer, pharmacological or natural chemopreventive and chemotherapeutic agents are commonly used (12).

p53 is a frequent target for mutation in various types of human tumor. It functions as a cell nucleus phosphate protein, which responds to various types and levels of stress arising from apoptosis, cell cycle arrest, senescence, DNA repair and cell metabolism (13-15). A previous study demonstrated that gambogic acid, an efficient apoptosis inducer, was able to repress the expression of B-cell lymphoma 2 (Bcl-2) via increasing the level of p53 in MCF-7 cells (16). Although previous studies have described intracellular changes leading to cell cycle arrest or apoptosis in response to Res treatment, the precise mechanisms underlying Res-regulated tumor growth remain to be fully elucidated (20-22). In the present study, the anti-cancer effects of Res in human melanoma A375 and SK-MEL-31 cells were assessed. The results demonstrated for the first time, to the best of our

knowledge, that the regulation of caspase and p53 proteins was involved in the induction of apoptosis and cell cycle arrest by Res.

Materials and methods

Cell culture. Human melanoma A375 and SK-MEL-31 cells, obtained from the Affiliated Hospital of Guangdong Medical College (Guangdong, China), were grown as a suspension culture in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) 100 U/ml penicillin and 100 U/ml streptomycin. They were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C.

Reagents. Res was purchased from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal human anti-rabbit antibodies to caspase-9, caspase-3, Bcl-2, Bcl-2-associated X protein (Bax), p53 and GAPDH were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA) and goat anti-rabbit IgG-horseradish peroxidase (EarthOx Life Sciences, Millbrae, CA, USA) was used as a secondary antibody.

Cell viability assays. The cell densities were adjusted to $2x10^4$ cells/100 μl. The cells were seeded into a 96-well plate and treated with 10, 50 and 100 μM Res for 24 h. The cell viability was then assessed using a Cell Titer 96 AQueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

Analysis of cell apoptosis and cell cycle arrest. Cells were pretreated with 10, 50 and 100 μM Res for 48 h. Cells were quantified using a Cell Cycle Analysis kit (Beyotime Institute of Biotechnology) and a PE Annexin V Apoptosis Detection kit I (BD Biosciences, Franklin Lakes, NJ, USA), respectively. Analysis was preformed using flow cytometry (FACSCalibur; Beckton Dickinson, Franklin Lakes, NJ, USA) and then analyzed using ModFit and CellQuest software (BD Biosciences).

Western blot analysis. Cells were lysed in lysis buffer (100 mM Tris-hydrochloride, 4% pH 6.8 (m/v) sodium dodecyl sulfonate, 20% (v/v), glycerol, 200 mM mercapto-ethanol, 1 mM phenylmethyl sulfonylfluoride and 1 g/ml aprotinin). The total protein concentration in the supernatants was detected using a BCA Protein assay kit (Beyotime Institute of Biotechnology). Proteins were then transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Detection was performed using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. The data were analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). All results are expressed as the mean ± standard deviation from triplicate experiments performed in a parallel manner, unless otherwise indicated.

Results

Res induces growth inhibition, proliferation and cell apoptosis in A375 and SK-MEL-31 cells. In the Res-treated cells, certain

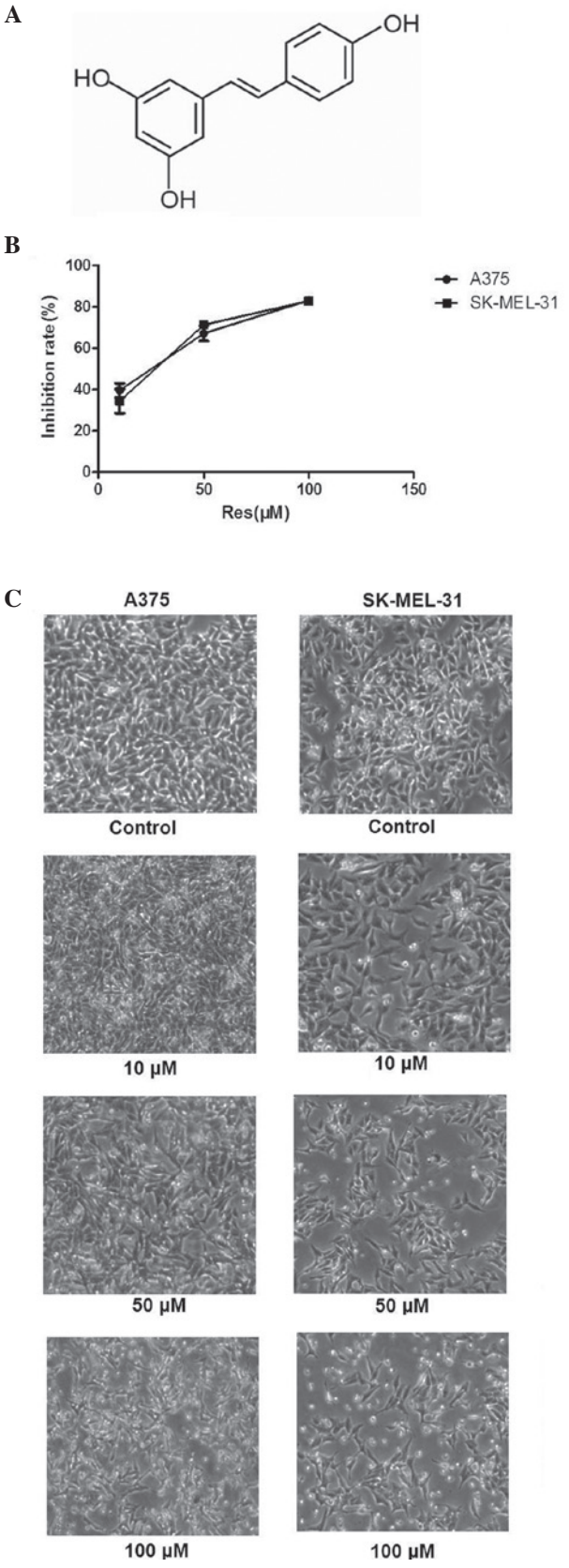

Figure 1. Res induces the loss of cell viability in A375 and SK-MEL-31 cells. (A) Chemical structure of Res. (B) Cells were incubated for 24 h with increasing concentrations of Res. Cell viability was measured using a 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide assay. (C) Res-induced inhibitory cell proliferation in A375 and SK-MEL-31 cells with different concentrations for 48 h, which was visualized using a microscope (magnification, x100). Res, resveratrol.

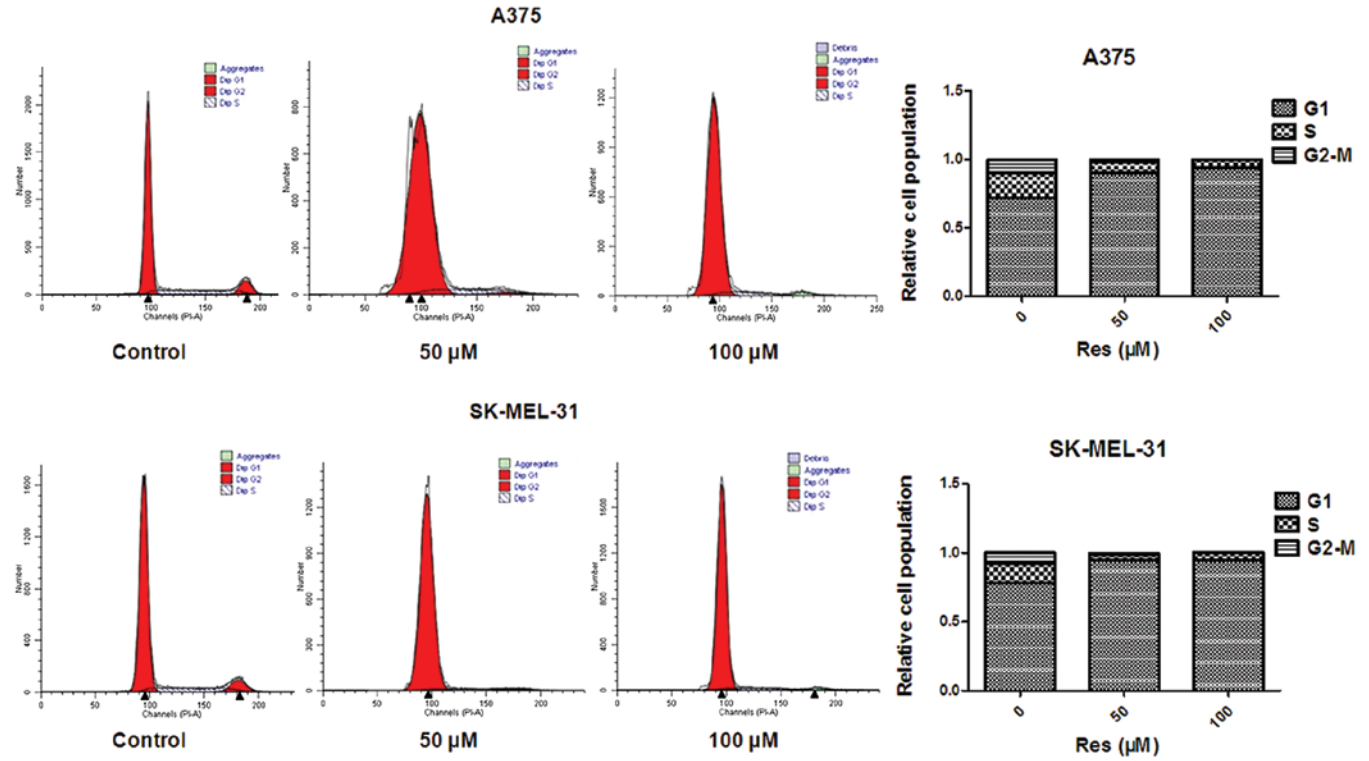

Figure 2. Res induces cell cycle arrest in A375 and SK-MEL-31 cells. Cells incubated for 48 h with increasing Res concentrations were analyzed by flow cytometric analysis. The data in the graphs are expressed as the mean ± standard deviation from three independent experiments. Res, resveratrol.

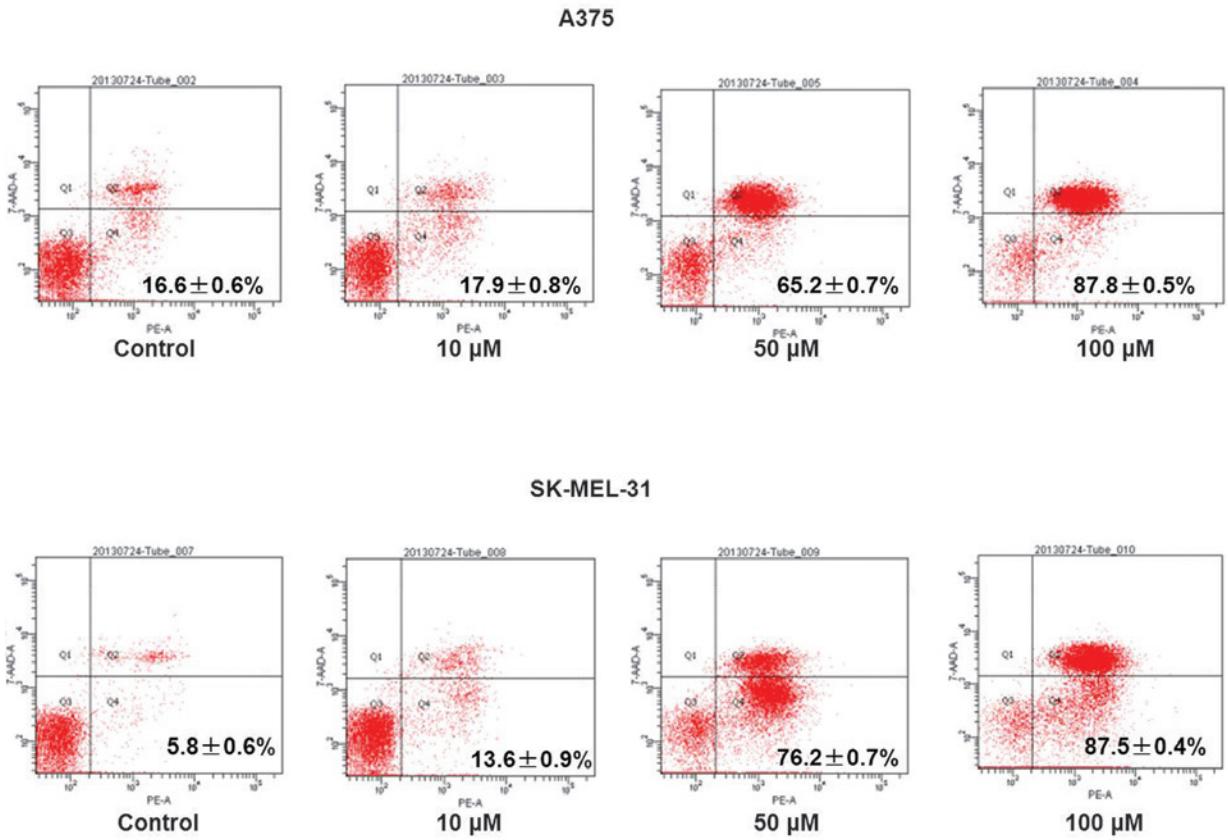

Figure 3. Res induces apoptosis in A375 and SK-MEL-31 cells. Cells were treated with Res for 48 h and stained with propidium iodide and annexin V, followed by flow cytometric analysis. Res, resveratrol.

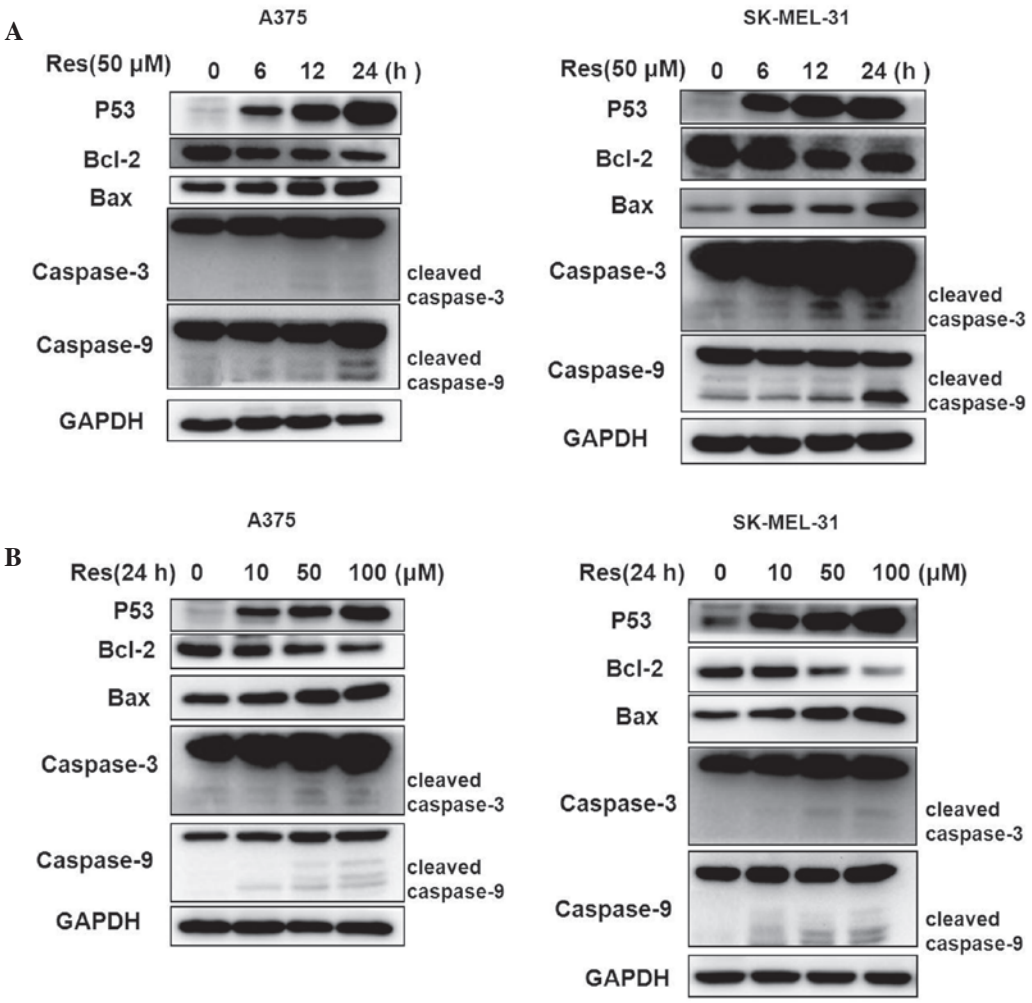

Figure 4. Cell cycle and apoptosis-associated protein analysis. Cells were treated with Res for (A) increasing time periods and at (B) increasing concentrations. Western blot analysis was performed for detection of protein expression. Res, resveratrol; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

cells became round and floating and a marked reduction in cell viability was observed over the experimental period in a concentration-dependent manner (Fig. 1B and C). Following treatment with 50 and 100 μM Res, inhibition of the cell cycle was observed in the A375 and SK-MEL-31 cells (Fig. 2). The population of cells in the G1 phase increased in A375 (78-93%) and SK-MEL-31 (78-94%) cells, coinciding with a reduction in cells in the S phase, when compared with the vehicle-treated control. This result demonstrated that the primary growth inhibitory effect of Res was due to inhibition of the cell cycle at the G1 phase. Cell apoptosis was detected using flow cytometry. Data revealed that Res induced cell apoptosis in a concentration-dependent manner (Fig. 3). In addition, the Q2 and Q4 cell population in A375 (16.6-87.8%) and SK-MEL-31 (5.8-87.5%) cells increased compared with the vehicle-treated control (Fig. 3).

Res induces the expression of cell cycle and apoptotic-related proteins in A375 and SK-MEL-31 cells. Western blot analysis demonstrated that cell cycle and apoptotic-related proteins altered following treatment with Res (Fig. 4). The Bcl-2 protein was downregulated, however, the apoptotic proteins of p53, Bax, caspase-3 and caspase-9 were markedly upregulated in a concentration- and time-dependent manner.

Discussion

Res, a natural plant polyphenol compound, has been extensively investigated for several years due to its various potential health-promoting benefits (8,20-23). In the present study, the effects of Res in human melanoma A375 and SK-MEL-31 cell lines was investigated. The inhibitory and apoptotic-promoting effects of Res on the growth of two human melanoma cells were determined, these cells have different genetic aberrations and acquired growth aggressiveness. The results from the cell viability and apoptotic rate assay demonstrated that the anti-cancer properties of Res in A375 and SK-MEL-31 cells were almost identical. The cell viability, G1 phase cell-cycle arrest and apoptotic rates increased in a concentration-dependent manner. Subsequently, the cell cycle and apoptotic-related proteins p53, Bcl-2, Bax, caspase-3 and caspase-9 were analyzed. The protein expression of Bcl-2 reduced, however, the protein expression of p53, Bax, caspase-3 and caspase-9 were markedly enhanced compared with the control. Cleaved caspase-9, cleaved caspase-3 and the ratio of Bax and Bcl-2 increased gradually in a concentration- and time-dependent manner. Caspase-9 and caspase-3 activation is a crucial step in apoptotic cell death (24,25) and the increased expression of cleaved caspase-9 and cleaved caspase-3 may be considered as

a marker of apoptosis. The apoptosis-inducing effect is more dependent on the balance of Bcl-2 and Bax than on the quantity of Bcl-2 alone, which is important in cell proliferation. The steady state of cell survival is decided by the balance of Bcl-2 and Bax expression (26,27). Previous studies have demonstrated that the apoptotic inducer, p53, induces cell growth arrest, apoptosis and senescence in response to different stimuli and this was associated with cancer cell metastasis (28-31). The increased expression level of the Bax/Bcl-2 protein ratio in cells treated with Res suggested that the p53 and Bax/Bcl-2 proteins are important in Res-induced apoptosis.

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