Resveratrol inhibits human nasopharyngeal carcinoma cell growth via blocking pAkt/p70S6K signaling pathways

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Abstract. Resveratrol (*trans*-3,4',5-trihydroxystilbene) has been shown to exert potent anticancer effects on various types of cancer through its anti-proliferative, anti-angiogenic, antioxidant and pro-apoptotic functions. There is still a lack of experimental evidence regarding whether resveratrol has potential anticancer activity in human nasopharyngeal carcinoma (NPC) cells. The purpose of this study was to explore the anticancer activity of resveratrol in human NPC cells both *in vitro* and *in vivo*. Our results indicated that treatment with resveratrol led to a time- and dose-dependent decrease in cell proliferation in NPC cells. A dose-dependent increase in apoptosis in response to resveratrol treatment was also observed in NPC cells. Flow cytometric analysis showed that treatment of NPC cells with resveratrol led to cell cycle arrest at the S and G2/M phases. Mechanistically, resveratrol treatment downregulated the expression of Bcl-2 and hypoxia-inducible factor-1α (HIF-1α) proteins and upregulated the expression of caspase-3 protein. In addition, resveratrol treatment also significantly decreased the phosphorylation levels of Akt1, p70S6K and p-4E-BP-1 and the protein expression of several cyclins involved in cell cycle regulation. *In vivo* studies further showed that resveratrol was able to significantly inhibit the growth of NPC tumor xenografts in nude mice. Collectively, our findings suggest that resveratrol exerts potent anti-proliferative and pro-apoptotic effects on human NPC cells possibly through interfering with the pAkt1/p70S6K signaling pathways, thus it may potentially be developed as an effective agent for chemoprevention and chemotherapy of human NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy characterized by the distinctive ethnic and geographic distribution. The annual incidence of this malignant disease of head and neck is approximately 20-50 cases/100,000 people, with most cases reported in Southeast Asia, Southern China, the Middle East and Northern Africa (1). Each year, approximately 80,000 new cases of NPC are diagnosed across the world, and 50,000 individuals succumb to this malignancy (1,2). Accumulating evidence from epidemiology, clinical and pre-clinical studies suggests that multiple factors, including genetic and non-genetic factors such as genetic susceptibility, Epstein-Barr virus (EBV) infection, environmental and dietary factors, cooperatively contribute to the development of NPC (3). Due to the deep and silent anatomic location and atypical clinical symptoms and signs, NPC is often diagnosed at an advanced stage. Although the combination of radiotherapy and cisplatin-based chemotherapy has improved the 5-year survival rate of NPC patients, the overall prognosis, especially for advanced and metastatic NPC, remains poor due to the high recurrence and relapse rate (2,4,5). Therefore, the molecular and signaling mechanisms underlying NPC pathogenesis urgently need to be elucidated and novel molecular targets for effective prevention and treatment of NPC remain to be identified.

Resveratrol, a natural polyphenolic phytoalexin derived from the skin of grapes and other fruits, is known to have potent anti-inflammatory and antioxidant effects (6). A growing body of evidence from numerous *in vitro* and *in vivo* studies has shown that resveratrol is able to modulate the activation/function of various molecular targets and signaling pathways involved in cell proliferation, apoptosis/survival, cell cycle, inflammation and angiogenesis, which may constitute the foundation for its potent inhibitory effects on tumor growth (7-9). Despite mounting evidence that resveratrol has antitumor effects on various types of cancer, the effect of resveratrol on NPC growth and the corresponding molecular mechanisms remain largely elusive. In the present study, we showed that resveratrol potently inhibited the proliferation and induced apoptosis in CNE-1 and CNE-2Z cells, two established human NPC cell lines. The therapeutic effects of resveratrol on NPC growth were further confirmed in a xenografted CNE-2Z tumor model in nude mice. Our findings suggest that resveratrol may have the potential to become a chemopreventive and/or treatment chemotherapeutic agent for human NPC.

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Materials and methods

Reagents and antibodies. Resveratrol and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide were purchased from Sigma (St. Louis, MO, USA) and dissolved at a concentration of 100 mmol/l in 100% dimethyl sulf-oxide (DMSO) as a stock solution, stored at -20°C. The final DMSO concentration did not exceed 0.1% throughout the study. Annexin V-FITC and primary antibody for human hypoxia-inducible factor-1α (HIF-1α) was purchased from BD Transduction Laboratories (San Diego, CA, USA). Antibodies for p70S6K, p-4E-BP-1, caspase-3, Bcl-2, and β-actin were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA). Antibodies for cyclins A, B, D, E and cyclin-dependent kinases (CDK)/p34 were from New England Biolabs (Beverly, MA, USA). Horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Pierce (Rockford, IL, USA).

Cell culture. CNE-2Z, a poorly differentiated human NPC cell line, and CNE-1, a well differentiated human NPC cell line, were cultured in RPMI-1640 complete culture medium (Gibco, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS; Gibco), penicillin (100 U/ml) and strepto-mycin (100 μg/ml) at 37°C in a humidified incubator with 5% CO_2 (10).

Cell proliferation assay. The effect of resveratrol on the cell growth of CNE-1 and CNE-2Z cells was examined using the MTT assay. Cancer cells were plated in 96-well plates at 10^4 cells/well. Following pretreatment with different concentrations of resveratrol for 24 or 48 h, viable cells were determined using the MTT Assay kit (Chemicon, Temecula, CA, USA) according to the manufacturer's protocol. At indicated time-points, 20 μl of MTT solution (5 mg/ml in PBS) were added into each well and incubated for 4 h. Then, 100 μl of DMSO was added to dissolve the crystals. The plate was then left to stand for 10 min at room temperature and the corresponding absorbance was measured at 490 nm. Each experiment was performed in triplicate.

Flow cytometric analysis. For the apoptosis analysis, NPC cells were plated into 6-well plates (2×10^5 cells/well) and cultured for 24 h. Then cells were incubated with 50 μM resve-ratrol for 48 h. Subsequently, the trypsinized cells together with the medium were collected and centrifuged. The pelleted cells were washed and resuspended in PBS for flow cytometric analysis using the Annexin V-FITC/propidium iodide (PI) Apoptosis Detection kit according to the manufacturer's proto-cols (BD Biosciences).

For the cell cycle analysis, NPC cells were plated into 6-well plates (2×10^5 cells/well) and cultured for 24 h. Then cells were incubated with 50 μM resveratrol for 48 h. The cells were collected and washed with PBS 2 times, followed by resuspending in 1 ml PBS. The cells were fixed with ethanol (70%) for 2 h, centrifuged, washed with PBS, and resuspended in 1 ml of PI solution (0.1% Triton X-100, 200 μg/ml RNase, 20 μg/ml PI in 10 ml of PBS) and incubated at room tempera-ture for 30 min. Cell cycle distribution was analyzed by flow cytometry.

Western blot analysis. Treated and untreated NPC cells were lysed with RIPA lysis buffer containing 20 mmol/l β-mercaptoethanol, 250 μmol/l sodium orthovanadate, 1 mmol/l PMSF and complete protease inhibitor cocktail (Sigma), and incubated at 4°C for at least 1 h. The lysates were ultra-sonicated and centrifuged at 14,000 x g for 15 min. The supernatants were collected and stored at -70°C. Protein concentrations were determined by BCA methods. Protein (100 μg) was separated on 8-10% polyacrylamide-SDS gel and electroblotted onto nitrocellulose membranes (Bio-Rad). After blocking with TBS/5% skim milk, the membrane was incubated overnight at 4°C with specific primary antibodies (1:1,000-1:2,000) followed by incubation with HRP-conjugated secondary antibodies (1:5,000) for 1 h at room temperature, and signals were detected by ECL (10).

In vivo xenograft study. Six to eight-week-old female nude mice (BALB/c nu/nu) were purchased from the Animal Center of Southern Medical University (Guangzhou, China) and were housed in a sterile environment with a light/dark cycle of 12/12 h and were allowed free access to food and water for 1 week. All animal studies were performed following international guide-lines on animal welfare and were approved by the Institutional Animal Care and Use Committee (IACUC) of Guangdong Medical College. CNE-2Z (1×10^6) cells in 0.1 ml PBS were subcutaneously (s.c.) injected into the right hind flank of the mice. One week after inoculation of tumor cells (~100 mm³), the tumor-bearing mice were randomly divided into the control and resveratrol (20 mg/kg body weight) treatment groups (n=5/ group), whereby resveratrol dissolved in 200 μl of 10% DMSO in PBS was intraperitoneally (i.p.) injected into mice once a day for 3 weeks. Control animals were injected with the vehicle (10% DMSO in PBS). Tumor sizes were measured every other day with a caliper and were calculated as 1/2x length x width² ($V=LW^2/2$ mm³). At the end of the experiments, the mice were sacrificed and tumor tissues were excised for further analysis.

Statistical analysis. Data are expressed as the means ± SD and were analyzed with the commercially available statistical soft-ware package, SPSS 13.0 (SPSS). One-way ANOVA or Student's t-test was performed. $P<0.05$ was considered to indicate a statis-tically significant difference.

Results

Resveratrol inhibits proliferation of NPC cells. We first observed the effects of resveratrol on the proliferation of human NPC cells. CNE-1 and CNE-2Z cells were treated with increasing concentrations of resveratrol for 24 or 48 h followed by MTT assay. Our results indicated that resveratrol treatment led to a time- and dose-dependent reduction in the proliferation rate of both types of NPC cells, whereby at the concentrations of 50 and 100 μM, resveratrol exhibited more pronounced inhibitory effects on the proliferation of CNE-2Z cells than on that of CNE-1 cells (Fig. 1A and B). Additionally, we showed that resveratrol treatment inhibited colony forma-tion of CNE-2Z cells in a dose-dependent manner (Fig. 1C). These results suggest that resveratrol has the potential to inhibit the proliferation of NPC cells with different differen-tiation grades.
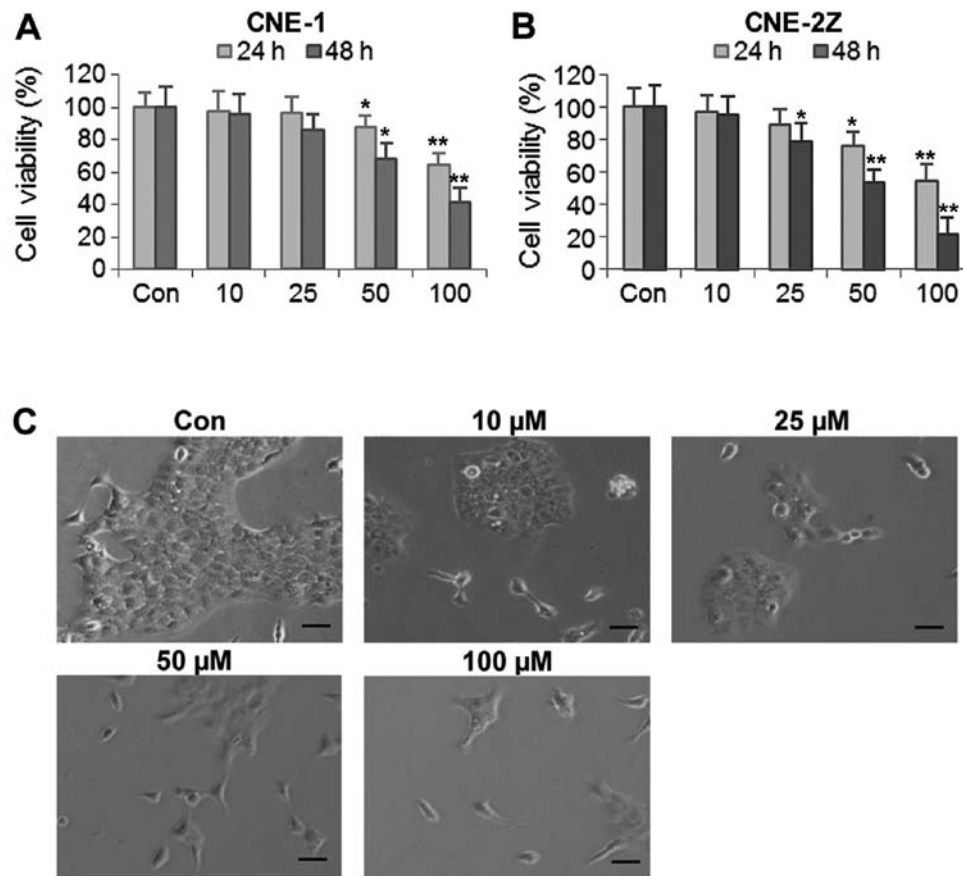
Figure 1. Resveratrol inhibits the proliferation of human NPC cells. (A and B) Human NPC cells, CNE-1 and CNE-2Z, were incubated with increasing concentrations of resveratrol (0-100 μM) for 24 or 48 h. Cell viability was determined by MTT assay. (C) CNE-2Z cells (1x10^4/well) were plated into 6-well plates and cultured in the absence or presence of different concentrations of resveratrol for 7 days. Cell morphology was observed and documented under an inverted microscope with a digital imaging system. Bars, 200 μm. Data represent means ± SD of 3 independent experiments. *P<0.05; **P<0.01.

Resveratrol induces apoptosis in NPC cells. Next, we investigated the effect of resveratrol on apoptosis in NPC cells. CNE-2Z cells were exposed to different concentrations of resveratrol for 48 h, followed by determination of apoptosis using the Annexin V-FITC/PI staining kit and flow cytometric analysis. Compared with controls, resveratrol treatment resulted in a dose-dependent induction of apoptosis of CNE-2Z cells (Fig. 2), suggesting that resveratrol potently induces apoptosis of NPC cells.

Potential mechanisms underlying resveratrol-induced apoptosis and inhibition of proliferation in NPC cells. We then explored the potential mechanisms underlying resveratrol-induced apoptosis and inhibition of proliferation in NPC cells. To this end, CNE-2Z cells were treated with different concentrations of resveratrol for 16 h (overnight), and the expression of several important genes involved in the regulation of apoptosis was determined by western blot analysis. Resveratrol treatment significantly inhibited the protein expression of 2 anti-apoptotic genes, Bcl-2 and HIF-1α (10,11), in CNE-2Z cells (Fig. 3A). On the other hand, resveratrol treatment led to a dose-dependent increase in the expression of caspase-3 which plays a critical role in apoptosis induction (10,11). Since the pAkt1/p70S6K/p-4E-BP-1 signaling pathway plays an essential role in the maintenance of proliferation and survival of cancer cells (12), we then explored whether resveratrol can block the

activation of this signaling pathway. Our results demonstrated that treatment of CNE-2Z cells with resveratrol led to dose-dependent decrease in the phosphorylated levels of pAkt1, p70S6K as well as p-4E-BP-1 (Fig. 3B). These findings suggest that resveratrol inhibits proliferation and induces apoptosis of NPC cells possibly by blocking PI3K/Akt signaling pathways.

Effects of resveratrol on cell cycle distribution in NPC cells. We then explored whether resveratrol had any effect on the cell cycle distribution of NPC cells. CNE-1 and CNE-2Z cells were exposed to 10 and 50 μM of resveratrol for 24 h and cell cycle distribution was analyzed with flow cytometry. Compared with controls, treatment of CNE-1 cells with resveratrol at 10 μM showed no obvious effects on the cell cycle distribution (P>0.05), while at 50 μM, resveratrol treatment significantly reduced the percentage of G1-phase cells (from 72.5±9.4 to 42.1±8.6%; P<0.01) and increased the percentage of S-phase cells (from 13.1±6.1 to 45.8±7.2%; P<0.05) (Fig. 4A, upper panel). Additionally, we showed that resveratrol exhibited stronger regulatory effects on cell cycle distribution in CNE-2Z cells. Compared with controls, treatment of CNE-2Z cells with resveratrol even at 10 μM led to a significant reduction in the percentage of G1-phase cells (from 70.3±8.3 to 52.6±6.5%; P<0.05) and a marked increase in the percentage of S-phase cells (from 12.8±4.1 to 32.3±7.8%; P<0.05), but had no obvious effects on the percentage of G2/M-phase cells (P>0.05)
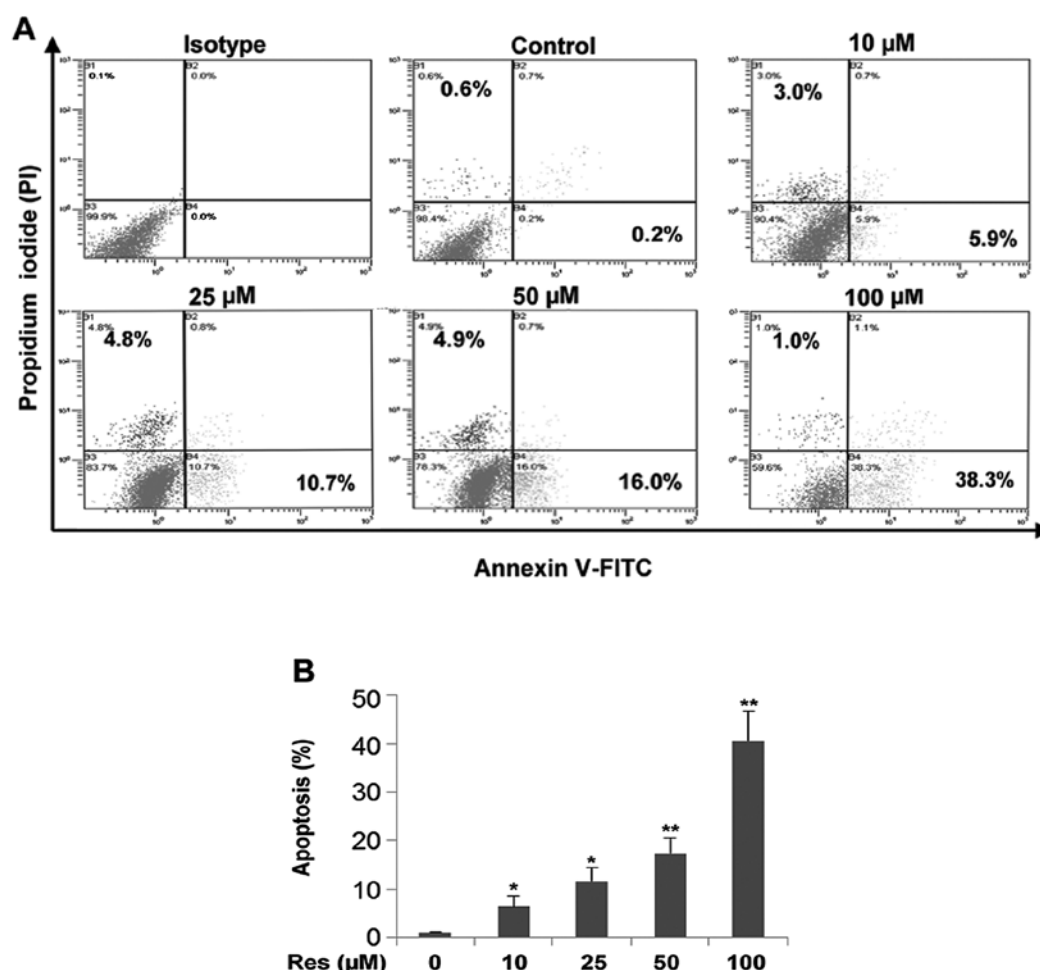
Figure 2. Resveratrol induces apoptosis in human NPC cells. CNE-2Z cells were incubated with increasing concentrations of resveratrol (0-100 μM) for 48 h. The cells were stained with Annexin V-FITC/propidium (PI) solution and subjected to flow cytometric analysis. (A) Representative histograms of flow cytometric analysis. (B) Data represent means ± SD of 3 independent experiments. *P<0.05; **P<0.01.
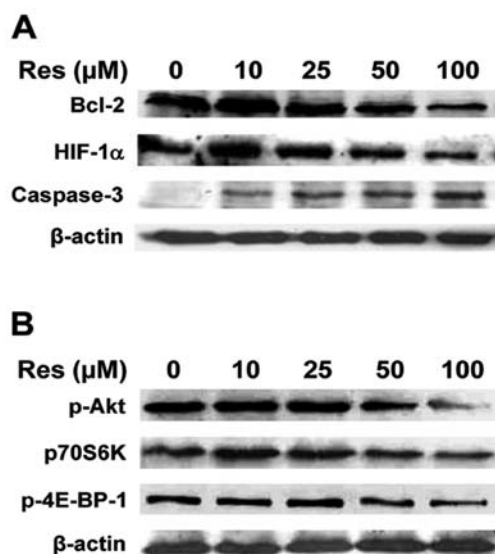

Figure 3. Effects of resveratrol on the expression of apoptosis regulatory genes and the pAkt/p70S6K signaling pathway. CNE-2Z cells were incubated with increasing concentrations of resveratrol (0-100 μM) for 16 h, and then whole cell lysates were prepared for western blot analysis. (A) Resveratrol induced changes in the expression of Bcl-2, HIF-1α and caspase-3 proteins in CNE-2Z cells. (B) Resveratrol induced changes in the phosphorylation of Akt1, p70S6K and p-4E-BP-1 in CNE-2Z cells. Data represent the results of 3 independent experiments.

(Fig. 4A, lower panel). At 50 μM, resveratrol treatment further decreased the percentage of G1-phase cells (36.2±7.9%; P<0.01) but simultaneously increased the percentage of cells at both the S-phase (40.5±8.2%; P<0.01) and G2/M-phases (16.6±5.2 to 25.7±6.9%; P<0.05) (Fig. 4A, lower panel). These findings suggest that resveratrol treatment was able to arrest the cell cycle progression of NPC cells at the S- and G2/M-phases.

To further explore the molecular mechanisms involved in resveratrol-induced alterations in cell cycle distribution of NPC cells, CNE-2Z cells were treated with increasing concentrations of resveratrol for 24 h. Then, the protein expression of several cyclins involved in cell cycle regulation was determined by western blot analysis. We found that resveratrol treatment markedly inhibited the expression of cyclin A, B, D and E, and CDK/p34 proteins in CNE-2Z cells, thus further confirming the effects of resveratrol on cell cycle progression in NPC cells.

In vivo effects of resveratrol on NPC growth. To further validate the anticancer effect of resveratrol on NPC, *in vivo* studies on xenografts of CNE-2Z cells in nude mice were performed. Our results showed that i.p. administration of resveratrol (20 mg/kg body weight) significantly suppressed the growth of xenografted tumors from Day 12 after resveratrol administration (P<0.05) (Fig. 5A and B). Histological analysis by H&E
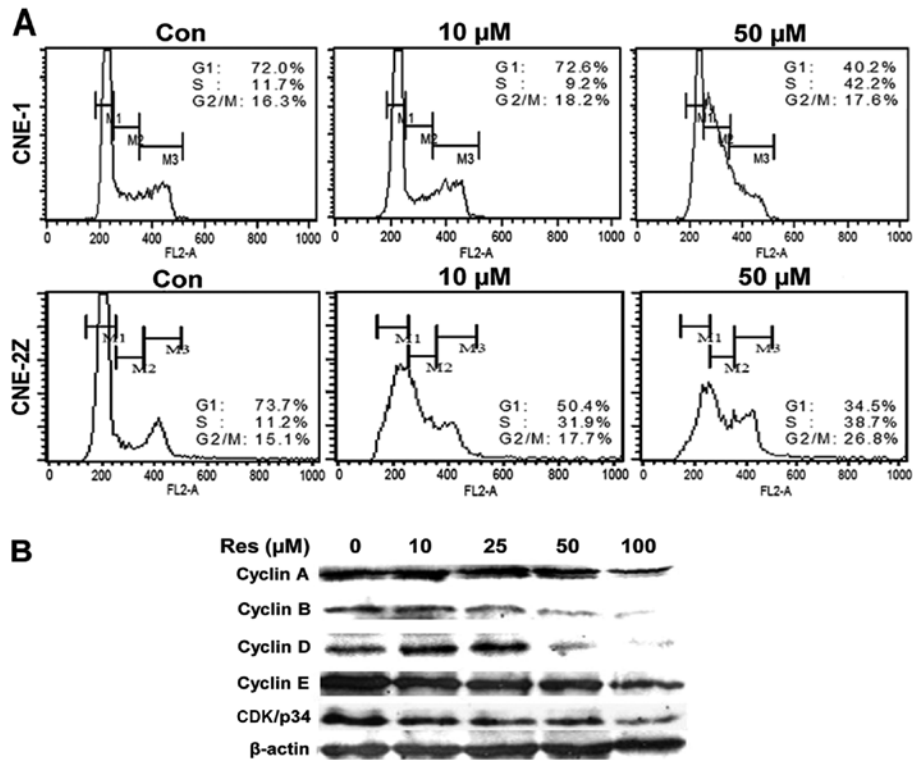
Figure 4. Effects of resveratrol on cell cycle progression in human NPC cells. CNE-1 and CNE2Z cells were exposed to increasing concentrations of resveratrol (0-100 μM) for 48 h, and then collected and prepared for flow cytometric analysis of cell cycle distribution. (A) Representative histograms of flow cytometric analysis. (B) CNE-2Z cells were incubated with increasing concentrations of resveratrol (0-100 μM) for 16 h, and the expression of cyclins A, B, D, E and CDK/p34 proteins was determined by western blot analysis. Data represent the results of 3 independent experiments.
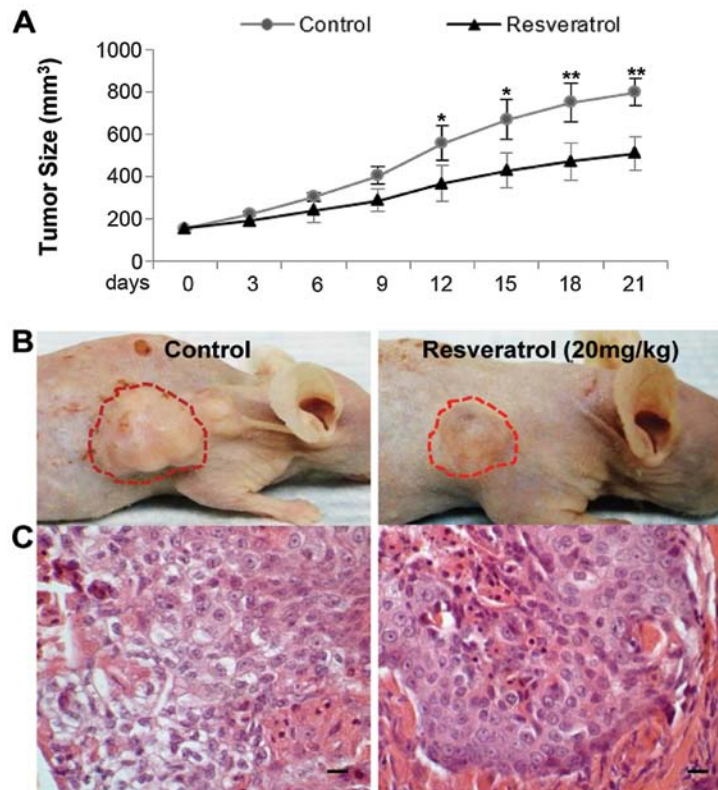

Figure 5. Effects of resveratrol on NPC xenografts in nude mice. CNE-2Z (1x10^6) cells in 0.1 ml PBS were subcutaneously (s.c.) injected into the right hind flank of the mice. One week after inoculation of tumor cells (~100 mm^3), the tumor-bearing mice were randomly divided into the control and resveratrol (20 mg/kg body weight) treatment groups (n=5/group). Resveratrol was intraperitoneally (i.p.) injected into mice once a day for 3 weeks. Control animals were injected with the vehicle (10% DMSO in PBS). (A) Tumor growth curve. Tumor sizes were measured every other day with a caliper and were calculated as 1/2x length x width^2 (V=LW^2/2 mm^3). (B) Representative pictures of grown tumors 3 weeks after resveratrol treatment. (C) Representative H&E staining images of paraffin-embedded tumor sections. Bars, 100 μm. Data represent means $\pm$ SD of 3 independent experiments. *P<0.05; **P<0.01.

staining indicated that resveratrol-treated tumors appeared to be more organized with fewer cellular components and more collagenous structures (Fig. 5C). Collectively, these results provide substantial evidence that resveratrol possesses potent anticancer effects on human NPC.

Discussion

A growing body of evidence has shown that resveratrol possesses multiple beneficial effects on health, such as cardio-protective and neuroprotective, anti-aging, anti-inflammatory, antioxidant, and anticancer activities (8,9). In recent years, the anticancer activities of resveratrol have been indicated in numerous *in vitro* and *in vivo* studies with various types of cancer and have also been tested in clinical trials (13). It appears that the multiple anticancer activities of resveratrol involve all the hallmarks of cancer (14), including cancer cell proliferation/survival (15), apoptosis (16-18), cell cycle altera-tion (19-21), angiogenesis (22), metastasis (23), inflammation (23), resistance to chemo- and radio-therapies (24,25), and cancer metabolisms (26). In the present study, we demonstrated that resveratrol can also potently inhibit the growth and induce apoptosis in human NPC cells (Figs. 1, 2 and 5).

At the molecular level, resveratrol-mediated multiple anticancer activities may be due to the wide spectrum of modulatory effects on multiple signaling pathways and downstream transcriptional factors of resveratrol (8,27). The signaling pathways targeted by resveratrol include, but are not limited to, MAPK signaling pathways such as extracellular signal-regulated protein kinases (ERKs), c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), and p38 kinases (28,29), PI-3K/Akt/p70S6K signaling pathways (17,22,30), signal transducer and activator of transcription (STAT)-3 (20,31) and Wnt/β-catenin (32) signaling pathways. Furthermore, resveratrol has been shown to inhibit numerous transcriptional factors that are downstream of these signaling pathways, including, but not limited to, NF-κB, activator protein-1 (AP-1) and HIF-1α (27). Herein, we demonstrated that resveratrol can significantly inhibit the activation of pAkt/p70S6K/p-4E-BP-1 signaling pathways as well as the expression of HIF-1α in NPC cells (Fig. 3), which contribute to its anticancer activities in human NPC.

Previous studies have shown that resveratrol inhibits proliferation of cells by inhibiting cell-cycle progression at different stages depending on the cell contexts, whereby the mechanisms may involve the downregulation of the cyclins/cyclin-dependent kinases (CDKs) and the upregulation of CDK inhibitors. For instance, Notas *et al* (19) reported that resveratrol exerts its anti-proliferative effect on HepG2 hepato-cellular carcinoma cells by inducing cell cycle arrest at the G1 and G2/M-phases. Studies by Joe *et al* (21) showed that resve-ratrol inhibits the growth of several human cancer cell lines by inducing S-phase arrest. Casanova *et al* (24) showed that resveratrol chemosensitizes breast cancer cells to melphalan by inducing cell cycle arrest at S-phase. In addition, numerous studies have shown that resveratrol can induce apoptosis in various types of cancer cells by downregulating anti-apoptotic proteins such as Bcl-2, Bcl-xL, survivin, inhibitor of apoptosis proteins (IAPs) (16,19), and by upregulating pro-apoptotic proteins such as p53 (16,28,29), Bax/Bad and activating

caspases (16). In this study, we demonstrated that treatment of human NPC cells with resveratrol led to the arrest of cell cycle progression at S- and G2/M-phases, which may be attributed to the downregulation of the expression of several cyclins and CDKs (Fig. 4). Resveratrol treatment also significantly inhib-ited the expression of Bcl-2 and promoted the expression of caspase-3, which may contribute to the induction of apoptosis in human NPC cells (Figs. 2 and 3).

To date, only three *in vitro* studies have suggested the potential inhibitory effects of resveratrol on human NPC cells (33-35). The underlying molecular mechanisms remain largely unknown, and there is also a lack of *in vivo* evidence to support the anticancer activity in human NPC. In the present study, we further demonstrated that resveratrol possesses potent anticancer activities in human NPC both *in vitro* and *in vivo*, whereby resveratrol-mediated anticancer activities in human NPC may be attributed to its inhibitory effects on cancer cell proliferation as well as to its pro-apoptotic func-tions. However, more studies are warranted to further explore whether resveratrol can also affect hallmarks of cancer other than proliferation and survival/apoptosis in human NPC. Findings from these studies will lead to the identification of novel molecular targets of resveratrol for efficacious chemo-prevention and chemotherapy of human NPC.

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