Resveratrol induces apoptosis of bladder cancer cells via miR-21 regulation of the Akt/Bcl-2 signaling pathway

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Abstract. Resveratrol, an edible polyphenolic phytoalexin present in grapes and red wine, has been reported to inhibit proliferation and induce apoptosis of bladder cancer cells. In the present study, the molecular mechanisms of resveratrol on human bladder cancer cell apoptosis was examined. The effect of resveratrol on the viability and apoptosis of T24 and 5637 cells was measured using an MTT assay and flow cytometric analysis, respectively. Next, the effect of resveratrol on miR-21 expression was detected by real-time PCR. The expression of phospho-Akt and Bcl-2 following treatment with resveratrol or the downregulation of miR-21 expression were also measured. Resveratrol induced the cytotoxicity and apoptosis of T24 and 5637 cells in a dose-dependent manner. Resveratrol decreased the expression of miR-21, the level of phospho-Akt and Bcl-2 protein expression. In addition, the downregulation of miR-21 expression inhibited the level of phospho-Akt and Bcl-2 expression. Insulin-like growth factor-1 was able to reverse the effect of the miR-21 inhibitor on Bcl-2 expression and apoptosis in T24 and 5637 cells. Notably, overexpression of miR-21 expression was able to restore the inhibition of Akt activity, downregulation of Bcl-2 expression and apoptosis induced by resveratrol. Collectively, data revealed that the effect of resveratrol on bladder cancer cell apoptosis was due to miR-21 regulation of the Akt/Bcl-2 signaling pathway.

Introduction

Bladder cancer is one of the most common types of malignancy worldwide, particularly in Europe and the United States (1). Due to serious side effects, conventional cisplatin-based chemotherapy is not suitable for all patients (2). Therefore, there is an urgent need for safe and effective drugs to treat bladder cancer. Resveratrol is an edible polyphenolic phytoalexin present in grapes and red wine and is able to prevent numerous age-associated diseases, including cardiovascular disease, Alzheimer's disease and cancer (3-5). It has been reported that resveratrol is important in inducing the cytotoxicity and apoptosis of bladder cancer cells (6,7).

MicroRNAs (miRNAs) are a naturally occurring class of non-coding RNAs involved in post-transcriptional gene regulation by binding to a target site in the 3'-untranslated region of target mRNAs (8). Several studies have indicated that altered microRNA expression contributes to carcinogenesis and is implicated in cancer cell proliferation and apoptosis. As a result, miRNAs may function as regulatory molecules and act as tumor suppressors or oncogenes (9). Among these microRNAs, miR-21 was highly expressed in various types of cancer, including breast, lung and pancreatic cancer (10,11). Functional studies have revealed that miR-21 is an oncogene, which is important in enhancing cell proliferation, promoting cell cycle progression and increasing anti-apoptotic activation in cancer cells (12). In numerous cancer cell lines, miR-21 was demonstrated to be the therapeutic target of resveratrol (13,14). Of note, miR-21 was significantly upregulated in advanced bladder cancer tissues and the overexpression of miR-21 promoted the proliferation of bladder cancer cell lines (15).

Akt (protein kinase B), a key signaling molecule in the phosphatidylinositol 3-kinase (PI3K) pathway, is important in the proliferation and survival of bladder cancer cells (16). Oka *et al* (17) reported that elevated levels of Akt protected bladder cancer cells from apoptosis. At present, drugs designed to specifically target Akt are being developed for clinical use to treat human bladder cancer. The Bcl-2 protein is also a key regulator of apoptosis and its tumorigenic potential is supported by the finding of overexpression of Bcl-2 in various types of tumor (18). In bladder cancer, the inhibition of Bcl-2 expression is able to reduce cell growth and sensitize cells to subsequent chemotherapy (19). In recent years, an increasing number of studies have focused on the regulation of Bcl-2 expression in tumor cells. As a result, the aim of the present study was to evaluate whether miR-21 regulatinon of the Akt/Bcl-2 signaling pathway is involved in the resveratrol-induced apoptosis of bladder cancer cells.

Materials and methods

Chemicals and reagents. Dulbecco's modified Eagle's medium (DMEM) and sodium pyruvate were purchased from Gibco-BRL (Rockville, MD, USA). Fetal bovine serum

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(FBS) was purchased from Gibco-BRL (Burlington, ON, USA). Resveratrol and dimethylthiazol-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant insulin-like growth factor-1 (IGF-1) was manufactured by R&D Systems (Minneapolis, MN, USA). The Annexin V-enhanced green fluorescent protein (EGFP)/propidium iodide (PI) apoptosis detection kit was obtained from KeyGen (Nanjing, China). The caspase-3 activity assay kits were obtained from Beyotime Institute of Biotechnology (Nantong, China). Rabbit polyclonal antibodies against phosphorylated Akt, total Akt and Bcl-2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibodies against β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The Detergent Compatible (DC) Protein Assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The miRNeasy Mini kit, the miScript Reverse Transcription kit and the miScript SYBR-Green PCR kit were purchased from Qiagen (Hilden, Germany).

Cell culture. The immortalized SV-HUC-1 normal human urothelial cell line and T24 and 5637 bladder cancer cell lines were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). These cells were cultured in DMEM supplemented with 10% FBS, 10 mM HEPES, 100 units penicillin/ml and 10 μg streptomycin/ml at 37˚C in a humidified atmosphere containing 95% air and 5% CO_2. Cultured cells were treated with resveratrol [dissolved in dimethylsulfoxide (DMSO)] in complete medium. To obtain reliable results, the final concentration of DMSO in the culture medium was maintained at <0.1%.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cell viability was determined using MTT assays. Briefly, the cells were seeded in 96-well dishes at $1x10^4$ cells per well, and treated with different concentrations of resveratrol (0, 10, 30 and 50 μmol/l) for 48 h. Then each well was supplemented with 10 μl MTT and incubated for 4 h at 37˚C. The medium was then removed and the resultant MTT formazan was solubilized in 150 μl DMSO. The optical density was measured at 490 nm using a microplate ELISA reader (Bio-Rad). The experiment was repeated three times and each experiment had six replicate wells.

Flow cytometric analysis. Flow cytometric analysis is based on the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface in early apoptotic cells (15). Briefly, cells were resuspended in a binding buffer. Next, Annexin V-EGFP and PI were added and the solution was incubated at room temperature for 15 min in the dark, followed by an assay on FACScan (Becton-Dickinson, Franklin Lakes, NJ, USA). The percentage of apoptosis was computed using Cell-Quest software (Becton-Dickinson).

Caspase-3 activity assays. Caspase-3 activity was analyzed using the caspase-3 activity assay kit according to the manufacturer's instructions. Cells were lysed and total cellular protein extracts were quantified using a protein-assay kit. Next, an equal quantity of total protein extract was incubated at 37˚C overnight with Ac-IETD-pNA for the caspase-3 assay. The release of pNA was estimated by determining the absorbance at 405 nm on a microplate ELISA reader (Bio-Rad). The relative activity of caspase-3 was calculated as follows: Caspase-3 activity = (mean experimental absorbance / mean control absorbance) x 100.

Quantitative PCR (qPCR) analysis of miRNA expression. Mature miRNAs of cultured SV-HUC-1, T24 and 5637 cells were isolated utilizing the miRNeasy Mini kit and reverse-transcribed with the miScript Reverse Transcription kit in accordance with the manufacturer's instructions. cDNA was subjected to quantitative PCR using an miScript SYBR-Green PCR kit. miRNA-specific quantitative PCR was performed using 3 ng cDNA per reaction. The relative quantities of miRNAs were calculated by calibration with U6 small nuclear RNA. All primers for miRNAs were purchased from Qiagen. Analysis and fold change were determined using the comparative threshold cycle (Ct) method. The change in miRNA expression was calculated as the fold-change, i.e. relative to the control.

Western blot analysis. T24 and 5637 cells were lysed with ice-cold lysis buffer containing: 50 mmol/l Tris-HCl (pH 7.4) 1% NP-40, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride and complete proteinase inhibitor mixture (one tablet per 10 ml; Roche Molecular Biochemicals, Indianapolis, IN, USA). The protein concentration in the cell lysate was quantified using the DC protein assay kit (Bio-Rad). Following protein content determination using a DC Protein Assay kit, western blot analysis was performed.

Transfection procedures. miR-21 was knocked down or overexpressed by transfection with an miRNA inhibitor or an miRNA mimic. The miR-21 mimic (5'-AACAUCAGUCUGAUAAGCUAUU-3'), miR-21 inhibitor (5'-UCAACAUCAGUCUGAUAAGCUA-3') and negative control (NC; 5'-CAGUACUUUUGUGUAGUACAA-3') were synthesized by Ribobio (Ribobio Co., Ltd, Guangzhou, Guangdong, China). All the oligonucleotides were transfected at a final concentration of 100 nM. T24 and 5637 cells were transfected with the miR-21 inhibitor or mimic using siPort Neo-FX (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed with statistical analysis software SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Statistical analyses were performed using either an analysis of variance or Student's t-test. Data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Resveratrol induces cytotoxicity and apoptosis in bladder cancer cells. The inhibitory effects of resveratrol on the growth of bladder cancer cells and normal human urothelial cells were evaluated using MTT assays. Resveratrol treatment inhibited the growth of T24 and 5637 cells in a dose-dependent manner (Fig. 1A). For instance, when T24 cells were treated with 10, 30 and 50 μmol/l resveratrol for 48 h, the inhibitory

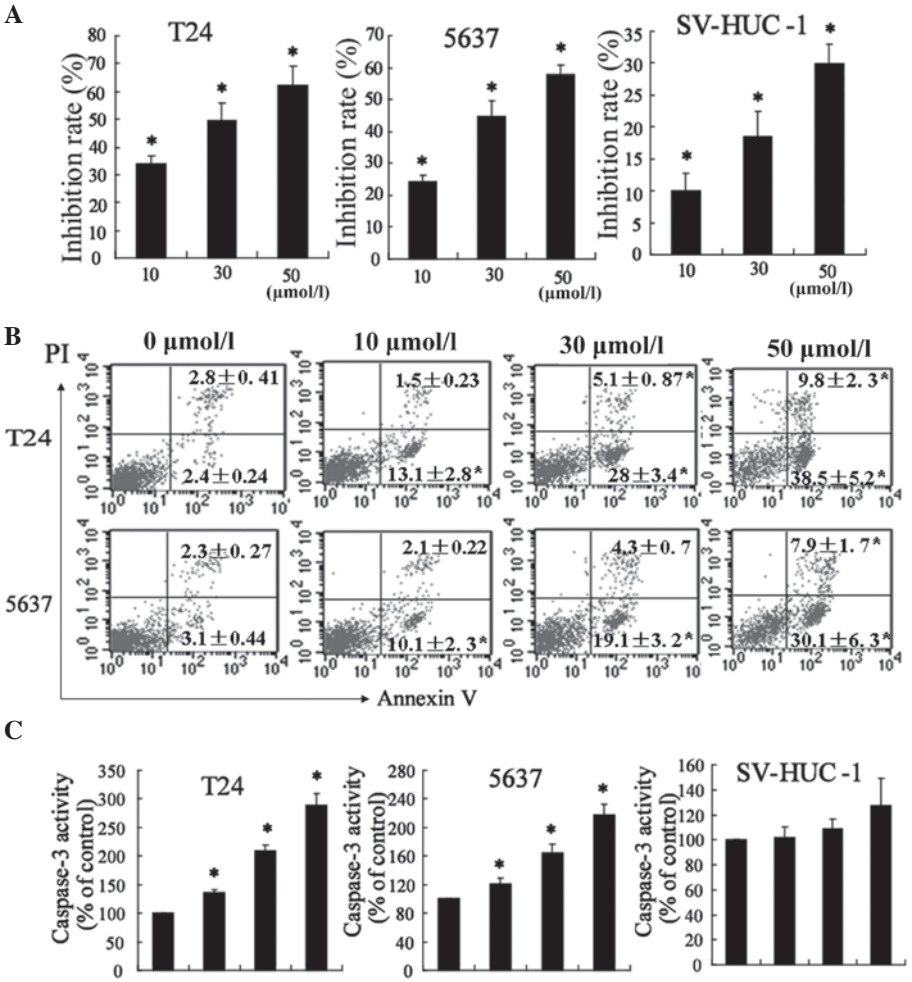

Figure 1. Resveratrol induced cytotoxicity and apoptosis in bladder cancer cells. (A) Exposure to various concentrations of resveratrol resulted in dose-dependent growth inhibition of T24, 5637 and SV-HUC-1 cells. (B) Flow cytometric analysis demonstrated that resveratrol induced apoptosis of T24 and 5637 cells in a dose-dependent manner. (C) Resveratrol stimulated the caspase-3 activity in T24 and 5637 cells in a dose-dependent manner. *P<0.05 vs. the control group. PI, propidium iodide.

rate of cell viability was 34.0, 49.4 and 62.3%, respectively, and the IC_{50} value 48 h after treatment with resveratrol was 26.75 μmol/l in T24 cells. Similar results were obtained in 5637 cells following treatment with different concentrations of resveratrol and the IC_{50} value following treatment with resveratrol was 35.58 μmol/l for 48 h. Normal human urothelial cells demonstrated greater resistance to the cytotoxic effect of resveratrol. In addition, the IC_{50} value at 48 h was 276 μmol/l for SV-HUC-1 cells.

Flow cytometric analysis demonstrated that the number of apoptotic T24 and 5637 cells significantly increased following treatment with resveratrol (Fig. 1B). The effects of resveratrol on the apoptosis of T24 and 5637 cells were dose-dependent (P<0.05). Activation of caspases is important in the execution of apoptosis (20). In order to determine whether resveratrol was able to alter the activity of caspases in bladder cancer cells, caspase-3 activity was assessed. As shown in Fig. 1C, resveratrol significantly increased caspase-3 activity in T24 and 5637 cells; however, had no effect on the activity in SV-HUC-1 cells.

Resveratrol inhibits miR-21 expression. To further identify the mechanism for resveratrol on the cytotoxicity and apoptosis of bladder cancer cells, qPCR was performed to detect miR-21 expression following treatment with resveratrol for 12 h. As shown in Fig. 2, resveratrol treatment significantly decreased the expression of miR-21 in a dose-dependent manner in the two cell lines (P<0.05).

Resveratrol decreases phosphorylation of Akt and the level of Bcl-2 protein. It has been reported that resveratrol is able to inhibit Akt activation in bladder cancer cells (7). Additionally, the effect of resveratrol on the PI3K/Akt pathway was examined by measuring the levels of phospho-Akt and total Akt. According to western blot analysis, the treatment of T24 and 5637 cells with resveratrol led to a dose-dependent decrease in the expression of phospho-Akt, while total Akt protein levels remained constant (Fig. 3A). Furthermore, resveratrol decreased Bcl-2 protein expression as shown in Fig. 3B.

Downregulation of miR-21 expression decreases phosphorylation of Akt and Bcl-2 expression. To determine whether miR-21 is able to regulate Akt activity and Bcl-2 expression in T24 and 5637 cells, the modulation of the levels of phosphorylation of Akt and Bcl-2 protein expression in cells transfected with the miR-21 inhibitor was investigated. The results from qPCR revealed that the miR-21 inhibitor

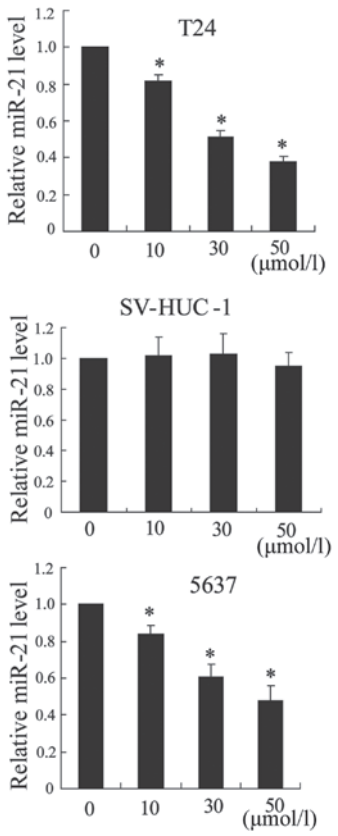

Figure 2. Resveratrol inhibits miR-21 expression. The expression of miR-21 was significantly decreased following treatment with different concentrations of resveratrol for 12 h in T24, 5637 and SV-HUC-1 cells. *P<0.05 vs. the control group. miR-21, microRNA-21.

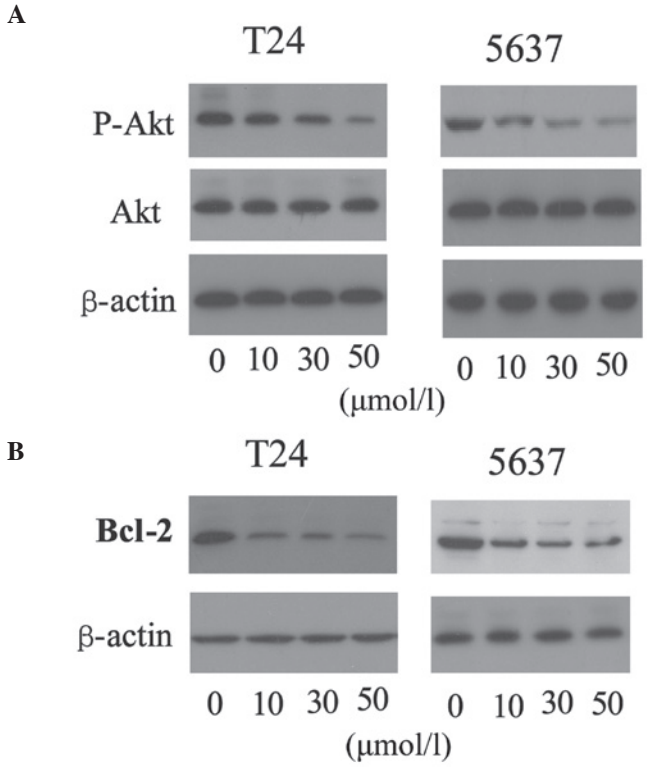

Figure 3. Resveratrol decreases the phosphorylation of Akt and the level of Bcl-2 protein. (A) Levels of Akt phosphorylation were significantly decreased following treatment with different concentrations of resveratrol for 24 h in T24 and 5637 cells. (B) Levels of the Bcl-2 protein were significantly decreased following treatment with different concentrations of resveratrol for 24 h in T24 and 5637 cells.

is able to significantly decrease the expression of miR-21 in T24 and 5637 cells (P<0.01; Fig. 4A), suggesting that the miR-21 inhibitor is efficiently introduced into the cells and acts to knock down miR-21 expression. Furthermore, the inhibition of miR-21 expression decreased the expression of phospho-Akt as shown in Fig. 4B. The Bcl-2 protein expression was also reduced by the miR-21 inhibitor, which was able to be reversed by IGF-1, a strong stimulator of Akt in bladder cancer cells (Fig. 4C) (21).

Downregulation of miR-21 expression increases apoptosis of bladder cancer cells. Flow cytometric analysis demonstrated that the number of apoptotic cells significantly increased following transfection of the miR-21 inhibitor (Fig. 5A). In addition, the miR-21 inhibitor significantly increased caspase-3 activity in T24 and 5637 cells, which was able to be reversed by IGF-1 (Fig. 5B).

Overexpression of miR-21 is able to reverse the effects of resveratrol on bladder cancer cells. In order to evaluate the role of miR-21 on the effect of resveratrol in bladder cancer cells, the cells were treated with resveratrol following transfection with the miR-21 mimic. As shown in Fig. 6A, the miR-21 mimic is able to significantly increase the expression of miR-21 in T24 and 5637 cells. Notably, the present study revealed that the overexpression of miR-21 was able to restore the decrease of phospho-Akt and Bcl-2 expression induced by resveratrol (Fig. 6B and C). In addition, caspase-3 activity significantly increased following treatment with 50 μmol/l resveratrol, which was able to be reversed by the miR-21 mimic (Fig. 6D and E).

Discussion

It has been reported that resveratrol has a strong inductive effect on the apoptosis of bladder cancer cells through the intrinsic mitochondrial-dependent pathway (6). The present study, aimed to further examine the molecular mechanisms of apoptosis induced by resveratrol in T24 and 5637 cells. Firstly, the effect of resveratrol supplementation on cytotoxicity and apoptosis in bladder cancer cells was evaluated and it was revealed that resveratrol significantly inhibited proliferation and induced apoptosis of these cells in a dose-dependent manner. Furthermore, the present study revealed that resveratrol was able to reduce miR-21 expression in T24 and 5637 cells and decrease Akt phosphorylation and Bcl-2 protein expression. Notably, overexpression of miR-21 was able to reverse the effect of resveratrol on bladder cancer cells.

Resveratrol has been demonstrated to be effective as a potential cancer chemoprevention agent against numerous types of tumor (22). As a natural product, resveratrol possesses anti-tumor activity with a low toxicity in normal cells (23). The results of the present study demonstrated that the IC_{50} value 48 h after treatment was 26.75 μmol/l for T24 cells, 35.58 μmol/l for 5637 cells but 276 μmol/l for SV-HUC-1 cells.

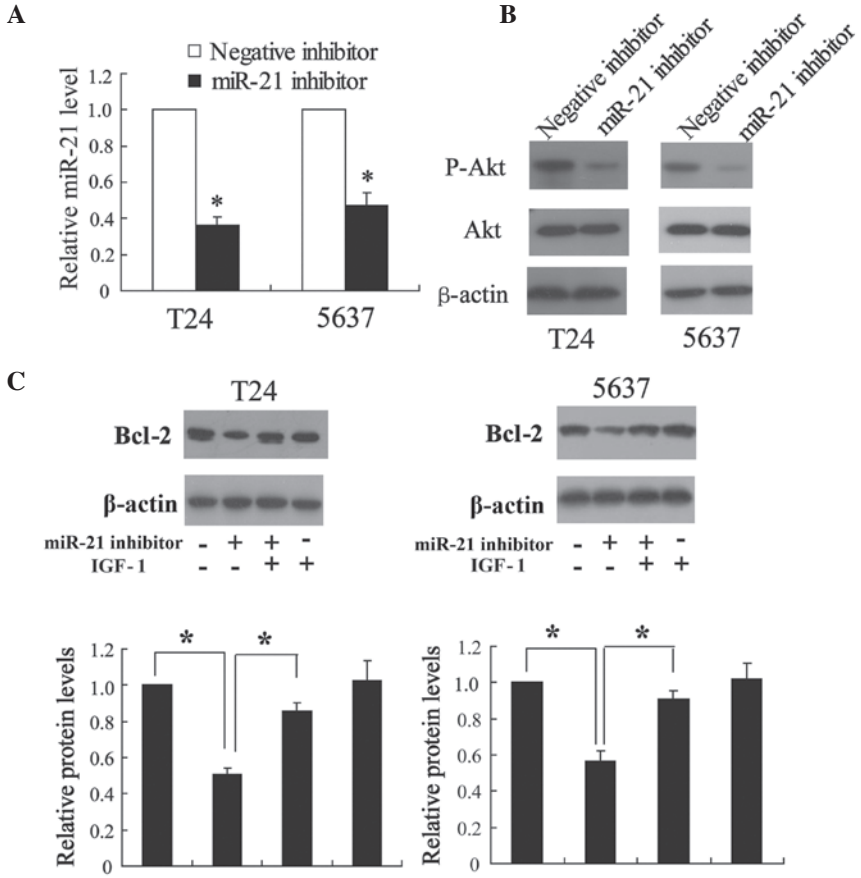

Figure 4. Downregulation of miR-21 expression decreases the phosphorylation of Akt and Bcl-2. (A) qPCR revealed that the miR-21 inhibitor significantly decreased the expression of miR-21. (B) Western blot analysis demonstrated that the transfection of the miR-21 inhibitor decreased the phosphorylation of Akt. The Bcl-2 protein levels were significantly decreased in T24 and 5637 cells treated with the miR-21 inhibitor, which was able to be reversed by IGF-1. (C) Fold changes of Bcl-2 protein levels were also determined. *P<0.05, compared with the respective control groups. miR-21, microRNA-21; IGF-1, insulin-like growth factor 1.

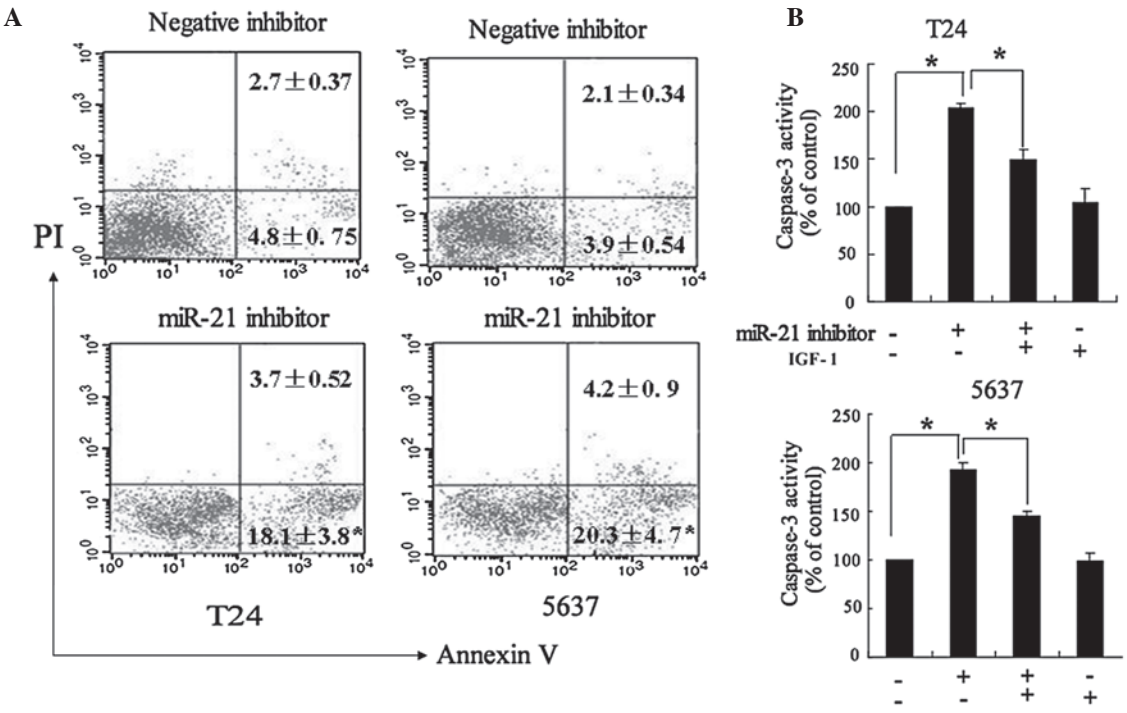

Figure 5. Downregulation of miR-21 expression increases apoptosis of bladder cancer cells. (A) Flow cytometric analysis demonstrated that the miR-21 inhibitor significantly increased apoptosis of T24 and 5637 cells. (B) The miR-21 inhibitor significantly increased caspase-3 activity in T24 and 5637 cells, which was able to be reversed by IGF-1. *P<0.05, compared with the respective control groups. miR-21, microRNA-21; PI, propidium iodide; IGF-1, insulin-like growth factor 1.

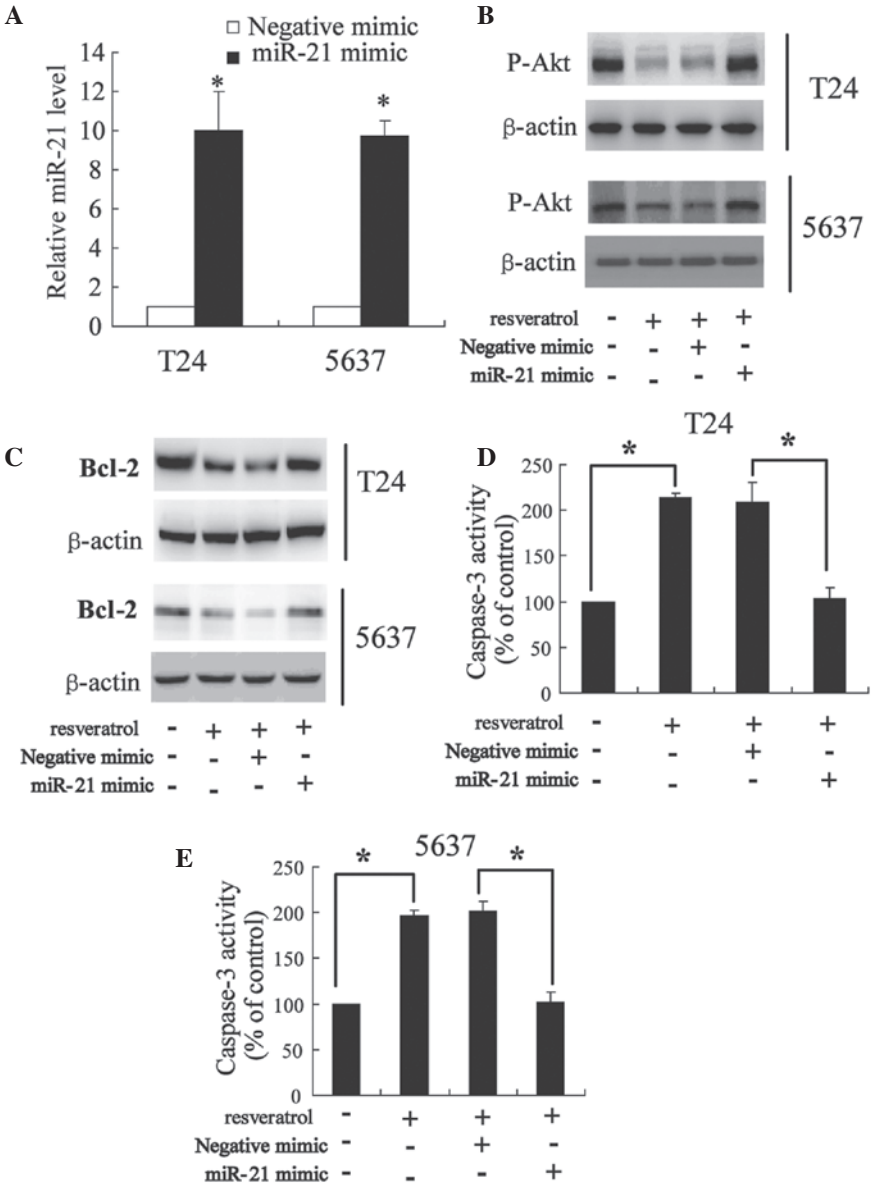

Figure 6. Overexpression of miR-21 is able to reverse the effect of resveratrol on bladder cancer cells. (A) miR-21 mimic was able to significantly increase the expression of miR-21 in T24 and 5637 cells. (B) miR-21 mimic was able to restore the inhibition of Akt phosphorylation induced by resveratrol. (C) miR-21 mimic was able to restore the inhibition of Bcl-2 expression induced by resveratrol. (D and E) miR-21 mimic was able to reverse the inhibition of caspase-3 activity in T24 and 5637 cells induced by resveratrol. *P<0.05, compared with the respective control groups. miR-21, microRNA-21.

These results indicated that resveratrol was able to specifically kill bladder cancer cells without exhibiting a cytotoxic effect on the normal cells.

To further clarify the mechanisms involved in the cytotoxicity and apoptosis of bladder cancer cells, the effect of resveratrol on the expression of miR-21 was investigated. Previous studies indicated that miR-21 was significantly upregulated in advanced bladder cancer tissues and the overexpression of miR-21 promoted the proliferation of bladder cancer cell lines (15). The present study revealed that resveratrol is able to inhibit the expression of miR-21 in T24 and 5637 cells, which indicated that miR-21 may be involved in resveratrol-induced apoptosis. The hypothesis that overexpression of miR-21 is able to reverse the effects of resveratrol on cytotoxicity and apoptosis was confirmed.

Akt is activated and regulates the process of proliferation and survival in bladder cancer cells (24). Inhibition of Akt activity is considered to be an effective strategy for bladder cancer treatment (25). Bcl-2 is also a direct participant in the apoptotic pathway and has tumorigenic potential (18). The present study demonstrated that resveratrol treatment resulted in dose-dependent inhibition of the increase in the levels of Akt phosphorylation and Bcl-2 protein expression, which was restored by downregulation of miR-21 expression. Our data also supported that miR-21 was important in the regulation of Akt activity and Bcl-2 expression in T24 and 5637 cells. The downregulation of miR-21 expression markedly decreased the phosphorylation of Akt and Bcl-2. The inhibition of Bcl-2 expression was significantly counteracted by treatment with an Akt stimulator of IGF-1. These results suggested that resveratrol may induce apoptosis by the regulation of the Akt/Bcl-2 signaling pathway by miR-21.

In conclusion, the present study established miR-21 as a novel target of resveratrol for mediating the cytotoxicity

and apoptosis of bladder cancer cells. The data support the hypothesis that resveratrol inhibits miR-21 expression, leading to the reduction of Akt activity, which results in a decrease in Bcl-2 expression. These data indicate that resveratrol may be a potent agent for the treatment of human bladder cancer.

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