Abstract. Colon cancer is a common type of cancer worldwide and accounts for a significant number of cancer-related deaths. Although surgical techniques and treatment strategies for colon cancer have advanced over the past two decades, the prognosis has not improved considerably. Resveratrol, a natural stilbene compound, possesses antioxidant, cardioprotective and anti-cancer properties. However, the role of resveratrol in colon cancer has not been fully elucidated. The present study demonstrated that resveratrol inhibited cell proliferation and colony growth in DLD1 and HCT15 colon cancer cells, but did not affect normal colon epithelial cells. The resveratrol-mediated inhibition of cell proliferation correlated with an induction of apoptosis and with G1 phase cell cycle arrest in colon cancer cells. Additionally, resveratrol treatment decreased the protein expression levels of cyclin D1, cyclin E2 and BCL2 apoptosis regulator, while it increased BCL2 associated X and tumor protein p53, all of which are involved in the regulation of cell cycle and apoptosis. Notably, the results obtained from in silico computational screening identified AKT serine/threonine kinase 1 (AKT1) and AKT2 as novel targets of resveratrol. Computational docking suggested that there are three or four possible hydrogen bonds in the active pocket of AKT1 and AKT2 that contribute to the mode of action of resveratrol. The present study confirmed that resveratrol bound to AKT1 and AKT2 with a pull-down assay. Furthermore, knockdown of AKT1 and AKT2 inhibited cell proliferation and colony growth, by attenuating cell cycle progression and increasing apoptosis in colon cancer cells, effects that were similar to those caused by resveratrol treatment. Taken together, the present results suggest that the targeting effects of resveratrol to AKT1 and AKT2 may be a potent strategy for chemoprevention or therapy for colon cancer.

Introduction

Colon cancer is the third most common malignancy and the fourth leading cause of cancer-related deaths worldwide (1,2). In China, colon cancer is the 4-5th most commonly diagnosed type of cancer in men and women. Despite improvements in colon cancer diagnosis and therapy, many patients are still diagnosed at late stages, and the 5-year survival rate is only ~30%. Owing to its aggressive nature and poor response to chemotherapy, colon cancer remains a challenging disease to treat. Therefore, the identification and development of more effective drugs to prevent or treat colon cancer is urgently needed.

Over recent years, there has been increasing interest in natural compounds derived from plants that show potential preventative effects against cancer development but have minor side effects on normal cells or organs (3). Resveratrol (3,5,4′-trihydroxystilbene) is a natural compound derived from plants that can be found in large amounts in the skin of grapes and tomatoes as well as in red wine (4). It can also be extracted from the traditional Chinese plant Polygonum cuspidatum (5). Resveratrol has exhibited promising anticancer activity against breast cancer, ovarian cancer, leukemia, prostate cancer, liver cancer, melanoma, head and neck squamous cell carcinoma, thyroid cancer, and bladder cancer, via regulating...
cancer-related gene expression or cancer-associated signaling pathways (5-10). The anticarcinogenic action of resveratrol is associated to its ability to neutralize reactive oxygen species (ROS) and to modulate cellular processes, such as apoptosis and cancer cell proliferation and differentiation (11). Resveratrol can regulate the levels of ROS. Several papers have demonstrated that resveratrol increases ROS levels via extracellular signal-regulated kinase (ERK)1/2 inhibition and mitogen-activated protein kinase (MAPK) activation to induce human diffuse large B cell lymphoma cell apoptosis in vivo and in vitro (12). Resveratrol also regulates the activation of the mitochondrial enzyme system and the caspase cascade; upregulates the expression of cyclin-dependent kinase inhibitors, tumor suppressor genes, and death-induced cytokines and their receptors; and downregulates the expression of survival proteins associated with the development of chemoresistance, including survivin, cellular FLICE inhibitory protein (cFLIP), cellular inhibitor of apoptosis proteins (cIAPs), and antiapoptotic proteins BCL2 apoptosis regulator (Bcl2) and BCL2 extra-large (Bcl-XL). Resveratrol activates AMP-activated protein kinase (AMPK) and inhibits the MAPK and phosphoinositide 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) signaling pathways (13). It has also been reported that during the cancer promotion phase, resveratrol reduces the transcription and activity of cytochrome p450 (14). Several reports have illustrated that resveratrol inhibits the proliferation of colon cancer cells, induces colon cancer cell apoptosis and G2 phase arrest and modulates gene expression through its effects on p21 and BCL2 associated X (Bax) (10,15-17). However, the exact molecular mechanism requires further investigation.

AKT is a proto-oncogene belonging to the serine/threonine kinase family that regulates a large number of downstream mediators and ultimately controls critical cell survival and metabolic processes (18). In addition, it promotes cell cycle progression and inhibits apoptosis. AKT is highly activated in 60-70% of human colon cancers (19). The AKT signaling pathway is upregulated in numerous cancer types and is involved in cancer cell proliferation, survival, and metabolism (20). Two FDA-approved anticancer drugs, everolimus and temsirolimus, have employed targeted inhibition of AKT in the clinic and are currently in early clinical trials for various types of cancer (21,22). Targeting AKT signaling, from the perspective of finding novel molecular targets for cancer therapy, has resulted in the discovery of candidates and their therapeutic inhibitors in crucial pathways. MK-2206, an AKT kinase inhibitor, has shown promising preclinical anticancer activity (23,24) and entered phase II clinical trials for metastatic breast cancer and colorectal cancer in 2017. The crucial roles of AKT kinase have rendered it an attractive target for developing therapeutic cancer drugs (25). It has been reported that the AKT1 interaction with N-ribosylidyhydronicotinamide-quinone reductase (NQO2) is a target of resveratrol (26).

Signal transducer and activator of transcription (STAT)3 is an important transcription factor that translocates to the nucleus to regulate the expression of essential pro-inflammatory factors, such as matrix metallopeptidases, heat shock protein (HSP)70 and HSP90, and it is also downstream of various tyrosine kinase receptor signaling pathways that are involved in angiogenesis (27). Several reports have focused on the role of AKT phosphorylation and STAT3 translocation. Resveratrol can inhibit cell proliferation and promote cell apoptosis via the STAT3 signaling pathway (28,29).

The present study investigated the anticancer activity of resveratrol in colon cancer cells. The results demonstrated that resveratrol inhibited cell growth of colorectal cancer by inhibiting AKT and its downstream signaling targets, and that AKT served as an upstream regulator of STAT3.

Materials and methods

Reagents. Resveratrol was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Antibodies against phosphorylated (p-) STAT3 (Tyr705; cat. no. 9145), STAT3 (cat. no. 9139), p53 (cat. no. 48818), Bcl2 (cat. no. 15071), Bax (cat. no. 5023), AKT1 (cat. no. 75692), AKT2 (cat. no. 2964), cyclin D1 (cat. no. 2978) and cyclin E2 (cat. no. 4132) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). An AKT1/2 (cat. no. sc-1619) antibody was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Active AKT1/2 recombinant protein was purchased from SignalChem (Richmond, BC, USA). RPMI-1640, fetal bovine serum (FBS) and Basal Medium Eagle (BME) were obtained from Biological Industries (Kibbutz Beit-Haemek, Israel). F-12K medium was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell culture. The human colon cancer cell lines DLD1 and HCT15 were purchased from the American Type Culture Collection (Manassas, VA, USA). The human colonic epithelial cell (HCEC) line was kindly provided by Dr Jerry W Shay, University of Texas Southwestern Medical Center, Dallas, Texas (30). DLD1 and HCT15 cells were cultured in RPMI-1640 medium/10% FBS. HCECs were cultured in F-12K medium/10% FBS. All cells were cytogenetically tested and authenticated prior to freezing and were cultured with antibiotics at 37°C in a 5% CO2 incubator for a maximum of 10 passages.

MTS assay. Cells at a density of 1x10³ or 1x10⁴ per well were seeded in 96-well plates in a final volume of 100 µl per well for analysis of proliferation or cytotoxicity, respectively. Cells were cultured overnight and were then treated with different concentrations of resveratrol and incubated for various times, as indicated. Cell Titer96 Aqueous MTS reagent (20 µl; Promega Corporation, Madison, WI, USA) was added to each well and the cells were incubated for an additional 1 h at 37°C. The absorbance was then measured at 492 nm with a spectrophotometer (Multiskan; Thermo Fisher Scientific, Inc.).

Anchorage-independent cell growth assay. DLD1 or HCT15 cells (8x10³ per well) were suspended in 1 ml of BME/10% FBS, and 0.33% agar with various concentrations of resveratrol and plated on a layer of solidified BME, 10% FBS, and 0.5% agar with the same concentration of resveratrol as the suspension. The cultures were maintained at 37°C in an incubator with 5% CO2 for 1-2 weeks. The colonies were photographed and counted with Image-Pro Plus software (v.6.2; Media Cybernetics, Rockville, MD, USA).
**Flow cytometry for analysis of apoptosis and cell cycle progression.** DLD1 and HCT15 cells were seeded in 6-well plates and treated with different concentrations of resveratrol (10, 20, 30 or 40 µM) for 72 h. The cells were harvested and stained with Annexin V and propidium iodide prior to flow cytometry analysis, data were analyzed by CellQuest Pro 6.0 (both from BD Biosciences, San Jose, CA, USA). For cell cycle detection, cells were fixed with ice-cold 70% ethanol overnight at -20°C. The cells were stained with propidium iodide, the cell cycle phase was determined by flow cytometry and data were analyzed by ModFit LTÔ 4.0.5 (Verity Software House, Inc., Topsham, ME, USA).

**Western blot analysis.** DLD1 and HCT15 cells were treated with various concentrations of resveratrol for 72 h and were then lysed with RIPA buffer (50 mM Tris base, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and 0.1% SDS; dissolved in 400 ml water and adjusted to pH 7.4) supplemented with 1 mM PMSF. Protein concentration was determined with a bicinchoninic acid protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein was loaded (50 µg/lane) and separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore Corp., Burlington, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature. The blots were then probed with the appropriate primary antibodies (1:1,000) overnight at 4°C and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000; cat. no. SC-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Protein bands were visualized with a chemiluminescence reagent (GE Healthcare Biosciences, Pittsburgh, PA, USA).

**Computational docking model.** To further confirm that resveratrol can bind AKT1 and AKT2, a *in silico* docking assay was performed using the Schrödinger Suite 2017 software program (Schrödinger LLC, Cambridge, MA, USA). AKT1 (PDB ID, 3OCB) and AKT2 (PDB ID, 3D0E) crystal structures were first obtained from the Protein Data Bank and were then prepared under the standard procedures of the Protein Preparation Wizard (Schrödinger Suite 2017). Hydrogen atoms were added consistent with a pH of 7, and all water molecules were removed. The ATP-binding site-based receptor grid of AKT1/2 was generated for docking. Resveratrol was prepared for docking with the default parameters using the LigPrep program. Then, the docking of resveratrol with AKT1 and AKT2 was accomplished with the default parameters under the extra precision mode of the program Glide. Finally, the best-docked representative structures were obtained.

**In vitro pull-down assay.** Resveratrol-Sepharose 4B beads (Amersham Pharmacia Biotech; GE Healthcare, Chicago, IL, USA) were prepared following the manufacturer's instructions. DLD1 and HCT15 cell lysates (500 µg) were incubated with Resveratrol-Sepharose 4B beads with rocking overnight at 4°C. Following incubation, the beads were washed 3 times with buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 0.01% NP-40 and 0.2 mM PMSF). Bound proteins were analyzed by western blotting.

**Preparation of AKT1/2 knockdown cells.** The viral vectors and the packaging vectors (pMD2G, psPAX2, Mock, shAKT1 and shAKT2) were obtained from Sigma-Aldrich (Merck KGaA). Several shRNA sequences targeting AKT1 and AKT2 were tested. Then, all plasmids were transfected into 293T cells, and viral supernatant fractions were collected after 48 h. DLD1 and HCT15 cells were infected with mock or shAKT1 and shAKT2 virus particles for 24 h. Cells were selected with puromycin to obtain both AKT1- and AKT2-silenced cell lines (shAKT1/2). The appropriate experiments were performed with these cells until the control cells (without infection) completely died (usually 2-3 days) in the puromycin medium.

**Statistical analysis.** All quantitative data are presented as the mean values ± standard deviation. Each experiment was repeated at least three times. Data were analyzed with SPSS 19.0 software (IBM Corporation, Armonk, NY, USA). Statistically significant differences were determined using one-way analysis of variance, and multiple comparisons between groups were conducted using the Dunnett’s test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Resveratrol suppresses the cell proliferation and colony growth of colon cancers.** The chemical structure of resveratrol is shown in Fig. 1A. Resveratrol is a natural compound and has few side effects, giving it an important advantage. First, normal HCECs were treated with resveratrol. The results demonstrated that resveratrol had no toxicity in the normal HCECs until the 40 µM concentration (Fig. 1B). However, resveratrol significantly decreased the growth of the DLD1 and HCT15 colon cancer cells when they were exposed to different concentrations (Fig. 1C). Furthermore, colony growth was also inhibited by treatment with resveratrol in a dose-dependent manner (Fig. 1D). These results demonstrated that resveratrol might be a potential treatment for colon cancer.

**Resveratrol induces cell apoptosis and cell cycle arrest at the G1 phase.** Next, further analyses were conducted to determine whether resveratrol could lead to the inhibition of cancer cell growth by regulating cell cycle progression and apoptosis. The results of Annexin V staining revealed that resveratrol treatment resulted in a significant increase in the apoptosis rate in both cell lines, DLD1 and HCT15 (Fig. 2A). Cell cycle analysis revealed that resveratrol significantly induced cell cycle arrest at the G1 phase (Fig. 2B). The effects of resveratrol were further verified by examining the expression levels of cell apoptosis and cell cycle biomarkers, using western blot analysis. The results revealed that resveratrol treatment decreased cyclin D1 and cyclin E1 (Fig. 2C), increased p53, and decreased Bcl2 protein expression levels in a dose-dependent manner (Fig. 2D).

**Resveratrol binds with AKT1 and AKT2 kinases.** To elucidate the underlying mechanism of resveratrol's effects, potential targets of resveratrol were screened by Schrödinger software (release 2017) (31). The results predicted that resveratrol may bind with AKT1 and AKT2. To elucidate the potential binding site, an *in silico* docking study was conducted...
using the induced fit docking module in the Schrödinger software. The results indicated that resveratrol interacts with the ATP-binding pockets of AKT1 (Fig. 3A-a and b) and AKT2 (Fig. 3A-c and d). For the binding of resveratrol with AKT1, the carbonyl oxygen of resveratrol interacts with the residues Glu228, Ala230 and Glu234 of AKT1 individually through hydrogen bonds (Fig. 3A-b). For the binding of resveratrol with AKT2, the carbonyl oxygen of resveratrol interacts...
with the residues Glu230, Ala232, Glu279 and Asp293 of AKT2 individually through hydrogen bonds (Fig. 3A–d). Thus, AKT1/2 activity might be dependent upon the presence of these residues. These computational results also indicated that resveratrol may elicit ATP-competitive inhibitory effects on the AKT1/2 kinase activity. To confirm the docking model, an in vitro pull-down assay was performed.

The results demonstrated that Sepharose beads conjugated to resveratrol bound with AKT1 and AKT2 in both the DLD1 and the HCT15 cell lysates, but the Sepharose beads alone did not bind with AKT1 and AKT2 (Fig. 3B). Additionally, the expression of p-STAT3 (Tyr705), which is a downstream target, was suppressed by treatment with resveratrol in a dose-dependent manner (Fig. 3C). The total protein levels of STAT3 were not affected by treatment with resveratrol (Fig. 3C). These results illustrate that the AKT signaling...
pathway is involved in the inhibitory effect of resveratrol in colon cancer cells.

**AKT1/2 knockdown inhibits colon cancer cell proliferation and colony formation.** Because of the crucial role of AKT in colon cancer, the present study examined whether knocking down AKT expression would produce an antitumor effect in colon cancer cells. After AKT1 and AKT2 were knocked down with short hairpin RNA (shRNA) in DLD1 and HCT15 colon cancer cells, the expression of AKT1 and AKT2 was markedly decreased (Fig. 4A). AKT1/2 knockdown markedly decreased the growth of both cell lines at 48 and 72 h (Fig. 4B). In addition,
AKT1/2 knockdown exhibited a significant inhibitory effect on anchorage-independent colony growth by decreasing the number and size of colonies compared to the number and size of the colonies in the mock group (Fig. 4C).

Next, it was examined whether silencing AKT1/2 could affect cancer cell apoptosis or cell cycle progression. Upon AKT1/2 knockdown, the number of cells in the G1 phase of the cell cycle increased, indicating that the cells suffered cell cycle...
Figure 5. Effects of AKT1/2 knockdown on cellular processes. (A) Effect of AKT1/2 knockdown on DLD1 and HCT15 cell cycle progression and (B) apoptosis. *P<0.05 and ***P<0.001 compared with mock-treated cells. (C) Representative images from western blot analysis showing changes in the levels of cyclin D1 and cyclin E2 (cell cycle markers), (D) Bax and Bcl2 (apoptosis markers), and (E) p-STAT3 and STAT3 (downstream signaling targets), following AKT1/2 knockdown. AKT, AKT serine/threonine kinase; Bax, BCL2 associated X; Bcl2, BCL2 apoptosis regulator; STAT3, signal transducer and activator of transcription 3; p-, phosphorylated; shRNA, short hairpin RNA.
a natural compound, resveratrol may have fewer side effects and lower toxicity to normal colon epithelial cells. Indeed, the present results demonstrated that resveratrol treatment had no effect in the viability of the normal HCEC cell line.

Multiple critical protein-encoding genes and pathways are believed to be responsible for tumorigenesis. Colorectal tumors contain a median of 76 mutations, and 15 of these affect candidate cancer genes. Increased understanding of the genetic and genomic changes in colorectal cancer has helped to direct therapies and predict responses. Genetic and epigenetic errors in signal transduction pathways result in malignant transformations and have thus emerged as key candidates for targeted molecular therapies. Over the past 10 years, the number of targeted agents used in various malignancies has increased dramatically. Currently, there are seven FDA-approved targeted agents for colorectal cancer, with many more in development and in clinical trials. The addition of targeted therapies over the past 10 years has improved OS in colorectal cancer by between 20 and 24 months. Resveratrol is a naturally occurring phytochemical that is produced by plants. Resveratrol has been demonstrated to have diverse biological properties, including anti-inflammatory, anti-oxidant, antiviral, neuroprotective, antifungal and anticaner properties (32,33). Resveratrol has been reported to inhibit cancer cell growth via the AKT signaling pathway (34). The PI3K/AKT pathway is an important endogenous protective mechanism that can prevent cell death and cell damage and is also associated with cancer progression and involved in drug resistance (35). AKT activation may lead to cell survival and can also inhibit apoptosis via the phosphorylation of BCL2 associated agonist of cell death (Bad) and caspase-9. STAT3 is an important transcription factor, and resveratrol can inhibit colon cancer cell proliferation via the STAT3 signaling pathway (36). Therefore, inhibition of this signaling pathway may be significant for cancer therapy. The computational docking results in the present study revealed that resveratrol interacted with the ATP-binding pockets of AKT1 and AKT2. In vitro pull-down assays confirmed that Sepharose beads conjugated to resveratrol bound AKT1 and AKT2 in colon cancer cell lysates. In addition, silencing AKT1/2 inhibited cell growth, increased apoptosis and induced G1 phase arrest. Several phase I clinical trials have investigated the potential of resveratrol for the treatment of patients with colon cancer (https://clinicaltrials.gov), performed by Nguyen et al (37), Patel et al (38) and Howells et al (39). The small sample size and the possible confounding effect of medications limited the conclusions reached; no definitive conclusions were obtained from any single trial. However, a set of well-designed and performed trials may provide more information, and this is a long lasting process.

In summary, the present study revealed that resveratrol inhibited colon cancer cell proliferation and colony formation, and induced cell apoptosis and G1 phase arrest. The mechanism of the anticancer effects of resveratrol was demonstrated to occur, at least partially, via inhibiting the AKT/STAT3 signaling pathway (Fig. 6). Taken together, the present results suggest that resveratrol may be a promising chemopreventive or therapeutic drug for colon cancer. Although resveratrol has a clear inhibitory effect on colon cancer cell proliferation and growth in vitro and ex vivo, these effects have not been...
confirmed yet in animal models and humans. Further research and clinical trials are warranted to fully elucidate the effects of resveratrol on human cancer.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

DL, SL and ZG designed the study. GW and GJ performed the kinase and pull down assays. KY, ZZ, LB and YG analyzed the data. NL, WD, BC, YL and XC performed all other experiments. DL and GJ wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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