The PTEN/PI3K/Akt and Wnt/β-catenin signaling pathways are involved in the inhibitory effect of resveratrol on human colon cancer cell proliferation

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Abstract. Colon cancer is one of the most common malignancies and the treatments for colon cancer have been developed substantially in the last decades, but there is still a great clinical need to explore new treatment regimens due to the undesirable prognosis. In this investigation, we demonstrated the anti-proliferative and apoptosis-inducing activities of resveratrol (Res) in human colon cancer cells, and the possible mechanisms underlying these effects. We used crystal violet staining, flow cytometry and western blotting to validate the anti-proliferative and apoptosis-inducing effects of Res on HCT116 cells. A xenograft tumor model was used to confirm the anti-proliferative effects of Res. We employed polymerase chain reaction, western blotting, recombinant adenovirus and luciferase reporter assay to explore the possible mechanism(s) of action. We found that Res inhibits significantly the proliferation and promotes apoptosis in HCT116 cells, as well as inhibits the xenograft tumor growth of colon cancer. Res upregulates the expression of phosphatase and tensin homolog (PTEN) and decreases the phosphorylation of Akt1/2. The exogenous expression of PTEN inhibits the PI3K/Akt signal and promotes the anti-proliferative effects of Res in HCT116 cells, while knockdown of PTEN increases PI3K/Akt signal but reduces the anti-proliferative function of Res. The protein and mRNA expression of β-catenin are all decreased by Res concentration-dependently. Thus, our findings strongly suggest that the anti-proliferative effects of Res in human colon cancer cells may be mediated by regulating separately the PTEN/PI3K/Akt and Wnt/β-catenin signaling.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies. The incidence of colon cancer shows an increasing trend with tendency for younger age (1). The treatments of colon cancer including surgery resection or radiotherapy combined with chemotherapy (2). However, these treatments improved patient survival with the decreasing of life quality. Thus, there is an urgent clinic need to develop new treatment regimens for colon cancer.

Natural products are widely accepted as validated agents for many conditions (3-6). Many herbs or their components have already been used clinically as potential candidates for anticancer drugs, including anthocyanidins, catechins, quercetin and genistein (7,8). A few studies and clinical epidemiologic studies indicated that red wine has apparent effect on reducing the risks of cardiovascular diseases and cancer (9,10). Polyphenolic compounds are present at high levels in red wine, which may mainly contribute to the benefits of red wine.

Resveratrol (3,5,4′-trihydroxystilbene, Res), as a natural polyphenolic compound mostly from beans and grapes, was discovered in red wine by Siemann and Creasy in 1992 (11), and can be used as inhibitor for platelet aggregation, cardioprotection and as anticancer agent (10,12). It has been reported also that Res can inhibit diet induced obesity (13) inhibiting proliferation and promoting apoptosis in various tumor cells, such as colon cancer, breast cancer and prostate cancer cells in vitro and in vivo (14-20). For colon cancer cells, the anti-proliferation and apoptotic inducing effects of Res have already been validated, but the mechanism underlying these activities remains unclear.

PI3K/Akt signaling plays a critical role in modulating cell survival and apoptosis (21), it has been found over-activated in many cancers reducing apoptosis and promoting prolif-
eration. The PI3K/Akt signaling was regulated by many other factors, one of the major negative regulators is phosphatase and tensin homolog (PTEN). PTEN was identified as a tumor suppressor (22,23), which is often deleted or mutated in a variety of cancers at high frequency (24,25). PTEN protein acts as a phosphatase specifically catalyzing the dephosphorylation of the 3-phosphate of the inositol ring in phosphatidylinositol (3,4,5)-trisphosphate (PIP3), leading to the biphosphate productPIP2. The dephosphorylation of PIP3 results in the inactivation of PI3K/Akt signal pathway because PIP3 is critical in activation of Akt (26).

Wnt/β-catenin signaling pathway is important for cell proliferation and differentiation, its aberrant activation is another major cause of colon cancer. The Wnt/β-catenin signaling can be upregulated by the phosphorylation of glycogen synthase 3 kinase β (GSK3β), an important negative regulator for Wnt/β-catenin signaling pathway, when PI3K/Akt signal is activated (27,28).

Herein, we investigated the possible molecular mechanism underlying the proliferation inhibitory and apoptosis inducing activities of Res in colon cancer cells. We found that Res exhibits prominent anti-proliferation and apoptosis inducing activity in HCT116 cells. Mechanistically, we demonstrated that Res can upregulate the expression of PTEN and inhibit the activation of PI3K/Akt signaling pathway, as well as to inhibit the Wnt/β-catenin signaling transduction, respectively. Our results support that Res can be used as anticancer agent alone or combined with other agents in colon cancer treatment.

Materials and methods

Reagents and cell culture. Resveratrol (Res) was purchased from Xi’an Haoxuan Biotechnology Co. Ltd., (Xi’an, China), and dissolved with dimethyl sulfoxide (DMSO). The aliquots were kept at -20˚C. VO-OHpic was from Sigma-Aldrich (St. Louis, MO, USA). HCT116 cell line was kindly provided by Dr Bert Vogelstein (Johns Hopkins Oncology Center; Baltimore, MD, USA). For in vivo experiment, Res was prepared with 0.5% carboxymethylcellulose sodium (CMC-Na) as suspension. All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Cells were maintained in the Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37˚C in 5% CO₂.

Crystal violet viability assay. Crystal violet assay was conducted as previously described (29). Experimentally, HCT116 cells were treated with indicated concentrations of Res. At 24, 48 or 72 h after treatment, cells were carefully washed with cold phosphate-buffered saline (PBS, 4˚C) and stained with 0.5% crystal violet formalin solution at room temperature for 20-30 min. The stained cells were washed with water and air dried for imaging and quantification. For quantification, the crystal violet was dissolved in 20% acetic acid at room temperature for 20 min with shaking. The absorbance at 570 nm was measured (30).

Flow cytometry analysis for cell cycle and apoptosis. Cells were seeded into 6-well plates. For cell cycle analysis, cells were treated with different concentrations of Res or solvent for 48 h. Then, cells were washed with PBS, collected and washed with cold (4˚C) 70% ethanol followed by washing with 50%, 30% ethanol and PBS; incubated with 1 ml of 20 mg/ml propidium iodide (PI) containing RNase (1 mg/ml) in PBS for 30 min followed by fluorescence activated cell sorting (FACS) assay. For apoptosis measure, cells were harvested after treated with different concentrations of Res for 48 h. Then, cells were washed with PBS (4˚C), followed by incubating with Annexin V-EGFP and PI as the kit procedures (KeyGen Biotech Co. Ltd., Nanjing, China). Then, the cells were subjected to FACS assay.

Western blot assay. Cells were seeded in 6-well plates and treated with different concentrations of Res or solvent. At the scheduled time point, cells were lysed and the lysate were denatured by boiling for 10 min. Total protein were separated by SDS-PAGE, transferred with polyvinylidene difluoride (PVDF) membrane, blocked in 10% skimmed milk and probed with antibody against the target proteins. Finally, the images of target bands were developed with SuperSignal West Pico Chemiluminescent substrate.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR). Cells were seeded in T25 flask and treated with different concentrations of Res. The total RNA was extracted with TRIzol reagents (Invitrogen, Carlsbad, CA, USA), followed by reverse transcriptional reaction to obtain the cDNA product. Finally, the cDNA products were used as PCR templates to detect the target gene expression. The primer sequences are available upon request.

Construction of recombinant adenoviruses for exogenous expression of PTEN, GFP and knockdown siRNA fragments for PTEN. Recombinant adenoviruses for exogenous expression of PTEN (Ad-PTEN) or GFP (Ad-GFP) were generated with the AdEasy system as previously described (31) as well as the recombinant adenoviruses expressing small interference RNA (siRNA) fragments for PTEN silence (Ad-siPTEN). Recombinant adenovirus expressing GFP was used as the vector control.

Xenograft tumor model of human colon cancer and histological evaluation. All animal experiments followed the guidelines of Institutional Animal Care and Use Committee of Chongqing Medical University (Chongqing, China). Athymic nude mice (female, 4-6 weeks old, 5/group) were from the animal center of Chongqing Medical University (Chongqing, China). HCT116 cells were cultured and resuspended in PBS for subcutaneous injection (1x10⁶/injection) into the flanks of the athymic nude mice. The mice were treated with Res (50 or 150 mg/kg) or the same volume of solvent through intragastric administration one week after cancer cell injection, once a day for four weeks. At the end of the 4th week, all nude mice were sacrificed, the tumor samples were retrieved and fixed in 10% formalin, and then embedded in paraffin. Serial sections of the embedded samples were stained with hematoxylin and eosin (H&E).
responsive elements reporter plasmid) using Lipofectamine (Invitrogen). After 12 h, cells were replated in 24-well plates and treated with different concentrations of Res or solvent, followed by crystal violet staining at the indicated time point. The crystal violet results were quantified as indicated in Materials and methods. *P<0.05 vs. control group; **P<0.01 vs. control group. (B) Res inhibits the proliferation of SW480 cells. This assay was carried out as described in (A). *P<0.05 vs. control group; **P<0.01 vs. control group. (C) Res arrests the cell cycle at G1 phase in HCT116 cells. (D) Res decreases the protein level of PCNA in HCT116 cells. Each condition was done in triplicate.

![Graph](image)

**Figure 1.** Resveratrol (Res) inhibits the proliferation of colon cancer cells. (A) Res inhibits the proliferation of HCT116 cells. Cells were seeded in 24-well plates and treated with different concentrations of Res or solvent, followed by crystal violet staining at the indicated time point. The crystal violet results were quantified as indicated in Materials and methods. *P<0.05 vs. control group; **P<0.01 vs. control group. (B) Res inhibits the proliferation of SW480 cells. This assay was carried out as described in (A). *P<0.05 vs. control group; **P<0.01 vs. control group. (C) Res arrests the cell cycle at G1 phase in HCT116 cells. (D) Res decreases the protein level of PCNA in HCT116 cells. Each condition was done in triplicate.

**Results**

Resveratrol inhibits the proliferation in colon cancer cells. It has been reported that Res inhibits the proliferation of various cancer cells and modulates the processes of cancer (32). To investigate whether Res can affect the proliferation of human
colon cancer, we analyzed the effect of Res on proliferation in human colon cancer cells. The results indicate that Res can inhibit the proliferation of HCT116 and SW480 cells concentration-dependently (Fig. 1A and B). Cell cycle analysis shows that Res can arrest the cell cycle at G1 phase in HCT116 cells (Fig. 1C). For further testing, we detected the effect of Res on the expression of proliferating cell nuclear antigen (PCNA) in HCT116 cells. The result shows that Res decreases the expression of PCNA concentration-dependently in HCT116 cells (Fig. 1D). These data demonstrate that Res is able to inhibit the proliferation of HCT116 cells.

Resveratrol induces apoptosis in colon cancer cells. Most anticancer agents have characteristics of apoptosis induction, so we tested whether Res could induce apoptosis in HCT116 cells. We employed Annexin V staining, FACS and western blotting to assay the effect of Res on apoptosis in HCT116 cells. The results show that Res can induce apoptosis clearly and concentration-dependently (Fig. 2A and B). Western blot assay results indicate that the protein level of caspase-3 increased substantially (Fig. 2C). These results strongly suggest that Res is a potent apoptosis inducer for human colon cancer cells.

Resveratrol inhibits tumor growth in a xenograft tumor model. The above evidence has proven that Res is a potent proliferation inhibitor agent for colon cancer cells. We next investigated the *in vivo* anticancer activity of Res with a xenograft tumor model for colon cancer. We injected $1 \times 10^6$ HCT116 cells into flanks of athymic nude mice. One week after injection, we treated the mice with intragastric administration of Res (50 or 150 mg/kg), once a day for four weeks.
The results show that tumor masses from mice treated with Res are smaller than those of the control group (Fig. 3A). The H&E staining results indicate that Res treated groups exhibited a decreased cellularity in tumor masses (Fig. 3B) suggesting that Res can inhibit tumor growth \textit{in vivo}, although it can not eliminated tumors completely.

\textbf{PTEN is involved in the anti-proliferation effect of resveratrol in colon cancer cells.} PTEN, as a tumor suppressor gene, has been found damaged or deficient in many cancer (33-35). It may be one of the targets for anticancer treatment. Thus, we investigated whether PTEN is involved in the anti-proliferation effect of Res in human colon cancer cells. The PCR and western blot results show that Res can induce the expression of PTEN concentration-dependently (Fig. 4A and B). The PTEN inhibitor attenuates the anti-proliferation effect of Res in HCT116 cells (Fig. 4C). Exogenous expression of PTEN potentiates the proliferation inhibitory effect of Res, while knockdown of PTEN inhibits this effect of Res in HCT116 cells (Fig. 4D). These results indicate that PTEN is involved in the anti-proliferation of Res in human colon cancer cells.

\textbf{Resveratrol downregulates PI3K/Akt signaling in HCT116 cells through upregulating PTEN.} One major function of PTEN is to negatively regulate PI3K/Akt signaling. The PI3K/Akt signaling pathway is one of the essential pathways for cell survival and proliferation, which is over-activated in many human cancers, such as breast, colon and prostate cancers (10,14). Thus, we investigated whether PI3K/Akt signaling is involved in the anti-proliferation effect of Res in HCT116 cells. The results indicated that Res has no apparent effect on the mRNA expression of Akt1 and Akt2 (Fig. 5A), but can reduce the phosphorylation level of Akt1/2 concentration-dependently (Fig. 5B). Res combined with adenovirus mediated exogenous expression of PTEN decreases the phosphorylation of Akt1/2 substantially (Fig. 5C), while knockdown of PTEN reverses the effect of Res on the phosphorylation of Akt1/2 (Fig. 5D). These results suggest that Res can inhibit PI3K/Akt signaling activation in HCT116 cells through the upregulation of PTEN.

\textbf{Resveratrol inhibits Wnt/\(\beta\)-catenin signaling transduction independent of PTEN/PI3K/Akt in HCT116 cells.} The abnormal activation of Wnt/\(\beta\)-catenin signaling is one of the major causes of cancer, which can be regulated by PTEN/PI3K/Akt signaling through the phosphorylation of GSK-3\(\beta\). HCT116 cells are predisposed to the mutation of \(\beta\)-catenin, thus, it can not be degradated by the degradative complex in the canonical Wnt signaling pathway. We measured whether Res could still decrease the level of \(\beta\)-catenin in HCT116 cells. We found that Res has no apparent effect on the total level of GSK-3\(\beta\), but decreases the phosphorylation of GSK-3\(\beta\) substantially, as well as the protein level of \(\beta\)-catenin (Fig. 6A). The \(\beta\)-catenin/Tcf4 reporter assay results indicate

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\caption{Resveratrol (Res) inhibits xenograft tumor growth of colon cancer. (A) Representative tumor masses from xenograft tumor assay. HCT116 cells were injected into the flanks of athymic nude mice (five mice per group, 1x10^6 cells per injection). Res was intragastrically administered one week after cancer cell injection (50 or 150 mg/kg), once a day for four weeks. The mice were sacrificed at the end of the fourth week after injection, and the tumor masses were harvested for histologic analysis. (B) Histological examination of the xenograft tumor masses. Retrieved tumor masses were fixed, embedded and subjected to hematoxylin and eosin staining. Representative images are shown.}
\end{figure}
that Res inhibited the reporter activity concentration-dependently (Fig. 6B). Moreover, the β-catenin mRNA expression was also decreased by Res (Fig. 6C). These results suggest that the canonical Wnt signaling pathway is involved in the anti-proliferation effect of Res in HCT116 cells.

Discussion

Colon cancer is one of the most frequently diagnosed malignancies, with high incidence in western countries (1). Although the treatment for colon cancer has advanced substantially, the prognosis is still more modest than had been hoped (36). There is a great clinical need to explore new treatment regimens for colon cancer. In this investigation, we demonstrated that Res has potent anti-proliferation activity in human colon cancer cells, and the anti-proliferation effect of Res may be mediated by inhibiting PI3K/Akt signaling through upregulating the expression of PTEN and blocking Wnt/β-catenin signaling transduction, respectively.

Res, as a natural polyphenolic compound, is found in the skin of red grapes and other fruits as well as in Japanese
knotweed roots (11). Several studies have proved that Res can inhibit proliferation and induce apoptosis of breast cancer cells, prostate cancer cells and colon cancer cells (10,13,14). No clinical trial has yet reported the cancer prevention effect of Res (37). Moreover, the bioavailability of Res is very low, so even a high dose of Res may not reach the sufficient concentration required for systemic treatment for cancers (38), however, this may be a benefit for digestive tract cancer treatment. Our in vitro results show that Res can inhibit the proliferation of HCT116 cells, even at the concentration of 20 µM. These data confirmed that Res has the potential to be an anticancer agent. Interestingly, our investigation indicates that Res shows no apparent proliferation inhibitory effect in HEK-293 cells, even at the concentration of 100 µM (data not shown). This result implies that the anti-proliferation effect of Res may be more specific to cancer cells.

The above studies, and our new results validate the proliferation inhibitory effect of Res on colon cancer cells, but the exact molecular mechanism underlying this remains unknown. Vanamala et al reported that Res could induce apoptosis through the suppression of Wnt pathway and activation of p53 signaling pathways in human colon cancer (39). The expression of MicroRNA-21 participates in the inhibition of prostate cancer growth and metastasis initialized by Res (16). Recent studies indicate that p38 and PI3K signaling pathways are involved in the anticancer activity of Res (40). Wnt, PI3K/Akt and p38 signaling pathways are all essential for cell proliferation and differentiation, and found to be aberrant in many cancers. However, the exact molecular mechanism of how Res regulates these signaling pathways remains unclear.

PTEN, a tumor suppressor often mutated or lost in many cancers, acts as a phosphatase to specifically catalyse PIP3 dephosphorylation at the 3-phosphate of the inositol ring and turn PIP3 to PIP2 through which it negatively regulates PI3K/Akt signaling. PI3K/Akt signaling has been associated with many cellular functions, including proliferation, differentiation, motility and survival. Previous studies indicated that PTEN may play an important role in early stages of sporadic colorectal carcinogenesis and reduced or lost PTEN expression is more frequent in colon cancer (25); colon cancer cells with high expression of PTEN is correlated with chemo-

Figure 5. Resveratrol (Res) inhibits PI3K/Akt signaling in HCT116 cells. (A) The effect of Res on the mRNA expression of Akt1 and Akt2. HCT116 cells were treated with different concentrations of Res or solvent, total RNA was harvested and RT-PCR performed to detect the expression of mRNA as described in Materials and methods. (B) Res decreases the phosphorylation of Akt1/2. HCT116 cells were treated with different concentrations of Res or solvent, combined with Ad-GFP. Total protein was harvested at the indicated time point, followed by western blot assay as described in Materials and methods. (C) Exogenous expression of PTEN enhances the inhibition of PI3K/Akt signaling induced by Res. HCT116 cells were treated with different concentrations of Res or solvent, combined with exogenous expression of PTEN (mediated by Ad-PTEN). Total protein was harvested at the indicated time point, followed by western blot assay as described in Materials and methods. (D) Knockdown of PTEN reverses the inhibition of PI3K/Akt signaling induced by Res. HCT116 cells were treated with different concentrations of Res or solvent, combined with recombinant adenovirus Ad-siPTEN. Total protein was harvested at the indicated time point, followed by western blot assay as described in Materials and methods. GAPDH was used as loading control. Each condition was done in triplicate.
Our results show that Res can upregulate the expression of PTEN in HCT116 cells. Either PTEN specific inhibitor or knockdown of PTEN can attenuate the anti-proliferation effect of Res in HCT116 cells, while exogenous expression of PTEN can potentiate the proliferation inhibitory effect of Res in these cells. These data indicate that PTEN may be important for the anti-proliferation effect of Res in HCT116 cells. Further analysis confirmed that the upregulated PTEN by Res is correlated with the inactivation of PI3K/Akt signaling by decreasing the phosphorylation of Akt1/2 in HCT116 cells. Therefore, our results suggest that PTEN/PI3K/Akt is involved in the proliferation inhibitory effect of Res in HCT116 cells.

Another major cause of colon cancer is the over activated Wnt/β-catenin signaling pathway. Most colon cancer cells are predisposed to the mutation of β-catenin or APC (42). Wnt signaling, including canonical and noncanonical Wnt signaling, plays an important role in embryogenesis and development. The β-catenin plays a pivotal role in canonical Wnt signaling. In the absence of Wnt proteins, Axin, GSK-3β and APC assemble as a complex to promote the proteolytic degradation of β-catenin. When Wnt proteins bind with the frizzled receptor, the degradation complex will be destroyed and the β-catenin can be accumulated in the cytoplasm and translocate to the nucleus. Eventually, β-catenin interacts with TCF/LEF transcription factors to regulate the downstream gene expression.

The mutation of APC, β-catenin or phosphorylation of GSK-3β cause β-catenin not to be degraded normally by the destruction complex and accumulate in the cytoplasm. The activation of PI3K/Akt can activate the canonical Wnt signaling through the phosphorylation of GSK-3β by the phosphorylated Akt1/2, blocking the formation of β-catenin destroying complex (28). Hence, the upregulation of PTEN may inhibit the canonical Wnt signaling by promoting the degradation of β-catenin. Although our results show that Res can decrease the phosphorylation of GSK-3β by PTEN/PI3K/Akt signaling. As it is predisposed to mutation of β-catenin in HCT116 cells, the β-catenin can not be degraded by the destruction complex in this colon cancer cells. With additional investigation, we unveiled that Res can inhibit the mRNA expression of β-catenin. Thus, these results suggest that Res can inhibit the Wnt/β-catenin signaling transduction, but it may not result from the upregulation of PTEN in HCT116 cells.

Taken together, Our data strongly suggest that Res can inhibit the proliferation and promote apoptosis in colon cancer cells. These activities of Res may be mediated by PI3K/Akt signaling through upregulating the expression of PTEN and reducing the Wnt/β-catenin signaling transduction through inhibiting the expression of β-catenin, respectively. However, the detailed molecular mechanism of how Res regu-
late the expression of PTEN and β-catenin need to be further deciphered.

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References


26. Franke TF, Kaplan DR, Cantley LC and Toker A: Direct regulation of the Akt proto-oncogene product by phosphati


28. Robertson BW and Chellaiah MA: Osteopontin induces beta


30. Stepanovici S, Vukovic D, Dakic I, Savic B and Svbatic


41. Liu HS, Pan CE, Yang W and Liu XM: Antitumor and immuno
