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Catalpol promotes cellular apoptosis in human HCT116 colorectal cancer cells via microRNA-200 and the downregulation of PI3K-Akt signaling pathway

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Abstract. Catalpol is an effective active ingredient that functions as a diuretic and laxative, and exhibits blood sugar-lowering, liver protective, anti-aging and anticancer effects. In traditional Chinese medicine, catalpol is believed to be Yin nourishing. The anticancer effect of catalpol on human HCT116 colorectal cancer cells were investigated and the mechanism of action was evaluated. Cellular viability was detected using an MTT assay. Caspase-3 and caspase-9 activity, cellular apoptosis and nucleic morphology were analyzed using caspase-3 and caspase-9 activity assay kits, flow cytometric assays and DAPI staining assay, respectively. Western blot analysis was used to measure the protein expressions of phosphatidylinositol 3-kinase (PI3K), phosphorylated-protein kinase B (p-Akt) and Akt. Expression of microRNA-200 was detected using the reverse transcription-quantitative polymerase chain reaction. HCT116 cells were incubated with PI3K inhibitors in order to analyze the effect of catalpol on cell proliferation. Catalpol was able to inhibit HCT116 cell proliferation. Furthermore, catalpol induced apoptosis in HCT116 cells, which depended on the increased activities of caspase-3 and -9. In addition, catalpol reduced the expression of PI3K, p-Akt and Akt in HCT116 cells. However, downregulation of PI3K/Akt decreased the viability of HCT116 cells following treatment with catalpol and enhanced microRNA-200 expression. Catalpol promoted cellular apoptosis in human HCT116 colorectal cancer cells through upregulation of microRNA-200 expression, which depended on a downregulation of the phosphatase and tensin homolog/PI3K-Akt signaling pathway.

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Introduction

In recent years, the incidence of colorectal cancer presents an increasing trend (1). According to epidemiological statistics, there are significant differences between colon cancer and rectal cancer in progression and etiology (2). The incidence of colon cancer in Shanghai has increased by 78% over ~20 years, while the incidence of rectal cancer has increased by 6% over the same period. Analysis has demonstrated that the Westernization in lifestyle and diet is associated with the incidence of colorectal cancer (3).

Increasing evidence has demonstrated that microRNAs exhibit endogenous regulatory functions, which serve a regulatory role in ontogeny, cellular proliferation, apoptosis and differentiation, viral replication, reproduction, and tumors to a certain degree. A large number of experiments have demonstrated that the specifically expressed microRNAs are involved in the regulation of cancer development. Recent studies have identified that MDA-MB-231 and BT-549 breast cancer stromal cells, epithelial cadherin transcription factors mediated by microRNA-200 family are upregulated, which is directly associated with ZEB1 translation and indirectly associated with the increased acetylation of histone H3 (4,5).

In certain diseases, embryo prototype mutation of phosphatase and tensin homolog (PTEN) is >80%, of which the substrate is a lipid generated by phosphatidylinositol 3-kinase (PI3K) and requires protein kinase B (Akt) activation (6,7). PTEN regulates Akt activity by controlling activated phosphatidylinositol (3,4,5)-triphosphate (PIP3). Therefore, PTEN mutations lead to the loss of the ability to regulate Akt and uncontrolled cellular proliferation, which causes cancerization. PTEN is able to dephosphorylate PIP3, antagonize PI3K activity and reduce the concentration of PIP3 within the cells, thereby inhibiting the activation of Akt, through which PTEN regulates cellular activity (8). In addition, protein phosphatase activity is associated with tumors. Fish oil suppressed cell growth of colorectal cancer by regulating PTEN and nuclear factor-κB signaling (9). Debroy *et al* (10) suggested

that anti-microRNA-221 enhanced the radiosensitivity of colorectal cancer cells by upregulating PTEN. Peroxisome proliferator-activated receptor γ induced apoptosis of colorectal cancer cells by upregulating PTEN and inhibiting PI3K activity (11). Isayev *et al* (12) reported that ribonuclease inhibitor suppresses proliferation and metastasis in colorectal cancer cells by inhibiting the PI3K/Akt signaling pathway.

Catalpol is one of the primary active ingredients in rehmannia, which functions as a diuretic and laxative, and exhibits blood sugar-lowering, liver protective, anti-aging and anticancer effects (13-16). In traditional Chinese medicine, catalpol is believed to be Yin nourishing. Previous studies have observed that catalpol may protect neurons from cytotoxic damage, reducing neuronal apoptosis following cerebral ischemia (17-19). The aim of the present study was to observe the effects of catalpol in colorectal cancer cells, and to investigate its mechanism and determine its therapeutic value in treating colorectal cancer.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MTT was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Caspase-3 and caspase-9 activity assay kits, and the bicinchoninic acid (BCA) protein assay kit were purchased from Beyotime Institute of Biotechnology (Haimen, China). The Annexin V-fluorescein isothiocyanate (V-FITC)/propidium iodide (PI) Apoptosis Detection kit was purchased from BestBio Co. (Shanghai, China). Catalpol was purchased from Sigma-Aldrich (Merck KGaA).

Cell culture. The human colorectal cancer HCT116 cell line was purchased from Union of Basic Medical Cell Center (Beijing, China). HCT116 cells were cultured in DMEM containing 10% FBS with 100 U/ml penicillin and 100 U/ml streptomycin, and cultured at 37˚C in an atmosphere containing 5% CO_2.

Cell viability assays. HCT116 cells ($2x10^4$ cells/well) were seeded in 96-well plates and cell viability was detected using MTT. HCT116 cells were cultured with various concentrations of catalpol (0, 25, 50 and 100 μg/ml). Following treatment for 24, 48, and 72 h, 20 μl MTT solution (0.5 mg/ml) was added into each well and cells were incubated at 37˚C for 4 h. The culture medium of each well was subsequently removed and 150 μl dimethyl sulfoxide was added into each well at room temperature whilst being shaken for 20 min. Absorbance was measured at 570 nm using a Bio-Rad ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Specific PI3K inhibitors LY294002 and wortmannin were provided by Calbiochem (Merck KGaA). The specific PI3K inhibitors LY294002 (5 μM) was incubated at 37˚C for 1 h prior to the detection in the wells.

Caspase-3 and caspase-9 activities. HCT116 cells were seeded ($1x10^6$ cells/well) in 6-well plates, and the activities of caspase-3 and -9 were determined using caspase-3 and caspase-9 activity assay kits. Following treatment for 48 h with catalpol (0, 25, 50 and 100 μg/ml), HCT116 cells were evaluated for hydrolysis of the peptide substrate Ac-IETD-pNA by caspase-3 and -9, resulting in the release of a pNA moiety. Absorbance values were measured with a microplate reader (Bio-Rad Laboratories, Inc.) at 405 nm. The activities of caspase-3 and -9 were expressed as nmol pNA/mg total protein.

Flow cytometric assays for Annexin V-FITC/PI. HCT116 cells were seeded ($1x10^6$ cells/well) in 6-well plates and the apoptosis of HCT116 cells was measured using the Annexin V-FITC/PI Apoptosis Detection kit (cat no. 556570; BD Biosciences, San Jose, CA, USA) according to the manufacturer' protocol. Following treatment for 48 h with catalpol (0, 25, 50 and 100 μg/ml), HCT116 cells were washed twice with ice-cold PBS and subsequently added to 1X binding buffer. A total of 5 μl V-FITC was added into each well for 30 min in the dark. Subsequently, 10 μl PI was added into each well in the dark. Apoptosis in HCT116 cells was analyzed using flow cytometry (FACSCalibur; BD Biosciences).

DAPI staining assay. HCT116 cells were seeded ($1x10^6$ cells/well) in 6-well plates and nucleic morphology was tested using a DAPI staining assay. Following treatment for 48 h with catalpol (0, 25, 50 and 100 μg/ml), PBS was used to wash HCT116 cells, and 0.5 ml paraformaldehyde (4%) was added to each well and cultivated for 30 min at 4˚C. HCT116 cells were washed twice with PBS, and sodium citrate (0.1%) was subsequently added containing 0.1% Triton X-100 and incubated for 10 min on ice. DAPI was added to HCT116 cells and incubated for 15 min at 4˚C in the dark. Nucleic morphology was viewed under ultraviolet light. HCT116 cells were observed and images were captured using florescence microscopy (Zeiss Axio Observer A1; Carl Zeiss AG, Oberkochen, Germany) at 340 nm.

Western blot analysis. HCT116 cells were seeded ($1x10^6$ cells/well) in 6-well plates, and the expressions of PTEN, PI3K, phosphorylated (p)-Akt and Akt protein were detected via western blot analysis. Following treatment for 48 h with catalpol (0, 25, 50 and 100 μg/ml), HCT116 cells were washed twice with ice-cold PBS and lyzed for 30 min on ice in cell-lysis buffer (cat no. FNN0021; Thermo Fisher Scientific, Inc.). Protein concentration was determined using a BCA protein assay kit. Protein samples (20 μg/well) were resolved using 10% SDS-PAGE. Separated protein was subsequently transferred onto polyvinylidene difluoride membranes (PVDF) membrane for 2 h at 60 V. The membranes were blocked with 5% non-fat milk powder in TBS-Tween-20 (TBST) buffer overnight at 4˚C. The PVDF was incubated with anti-PI3K (1:1,000; cat no. GW21071; Sigma-Aldrich; Merck KGaA), anti-Akt (1:1,000; cat no. P0024-1), anti-p-Akt (1:1,000; cat no. BM1612) (both from Boster Biological Technology, Pleasanton, CA, USA) and anti-β-actin (1:500; cat no. BM0627; Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 2 h at room temperature. Following three washes with TBST twice for 30 mins, the membrane was incubated with anti-immunoglobulin G secondary antibody (1:500; cat no. BA1054; Wuhan Boster Biological Technology, Ltd.) was added prior to incubation at room temperature for 2 h with shaking. The bands were detected using the Enhanced

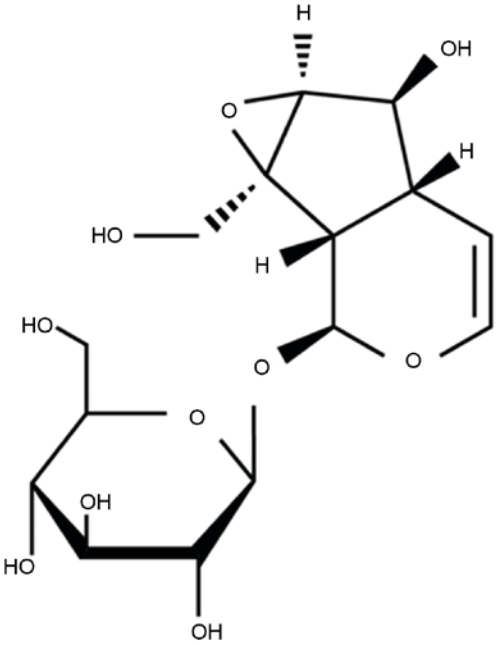

Figure 1. The chemical structure of catalpol.

Chemiluminescence Prime western blotting kit (GE Healthcare Life Sciences, Little Chalfont, UK).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of microRNA-200. Total RNA was extracted from HCT116 cells using TRIzol (Tiangen Biotech Co., Ltd., Beijing, China). MicroRNA-200 was performed using the High Capacity cDNA Reverse Transcription kit with the ABI 7500 qPCR system (both from Takara Bio, Inc., Tokyo, Japan), according to the manufacturer's protocol. The qPCR was performed according to the manufacturer's protocol (cat no. 1725085; Bio-Rad Laboratories, Inc.). The PCR cycling conditions consisted of 93˚C for 3 min, then 10 cycles at: 94˚C for 15 sec, 55˚C for 15 sec and 72˚C for 30 sec; then 20 cycles at: 89˚C for 15 sec, 55˚C for 15 sec and 72˚C for 30 sec; and an extension cycle at 72°C for 10 min. All primers used are purchased from Sangon Biotech (Shanghai, China). The forward and reverse primers for microRNA-200 were 5'-TGCATCATTACCAGGCAGTATTAGA-3' and 5'-CCTCTTACCTCAGTTACAATTTATA-3', respectively. The forward and reverse primers for U6 were 5'-CGCTTCGGCACATATACTA-3' and 5'-CGCTTCACGAATTTGCGTGTCA-3', respectively. The results were quantified using the $2^{-\Delta\Delta Cq}$ method (20).

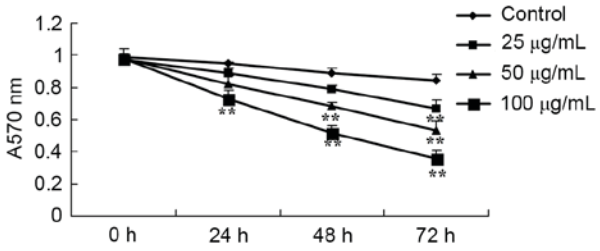

Figure 2. Effect of catalpol on cell viability. HCT116 cells were cultured with various concentrations (0, 25, 50 and 100 μg/ml) of catalpol. Following treatment for 24, 48, and 72 h, 20 μl of MTT solution was added into each well and the cells were incubated at 37˚C for 4 h. **P<0.01 vs. the control group at the respective time point. A, absorbance.

Statistical analysis. Experiments were repeated at least three times and were analyzed using SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Differences were tested using the Student's unpaired t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of catalpol on cellular viability. The chemical structure of catalpol is indicated in Fig. 1. To investigate the anticancer effect of catalpol on the viability of human colorectal cancer cells, HCT116 cells were treated with catalpol at different concentrations (0, 25, 50 and 100 μg/ml) for 24, 48 and 72 h, respectively. The absorbances of HCT116 cells were detected using the MTT assay. As presented in Fig. 2, the viability of HCT116 cells was inhibited by treatment with catalpol in a dose- and time-dependent manner. These data suggest that catalpol markedly inhibits human colorectal cancer cell viability.

Effects of catalpol on caspase-3 and -9 activities in HCT116 cells. To investigate the activities of caspase-3 and -9 in human colorectal cancer cells, HCT116 cells were treated with different doses of catalpol (0, 25, 50 and 100 μg/ml). When the HCT116 cells were treated for 48 h, a significant increase in the activities of caspase-3 and -9, following treatment with 50 and 100 μg/ml catalpol, was observed compared with the respective controls (Fig. 3).

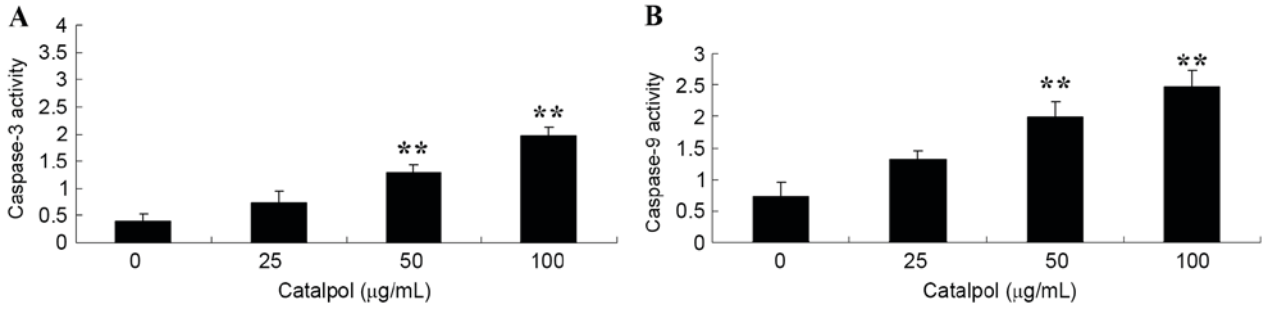

Figure 3. Effects of catalpol on caspase-3 and caspase-9 activity in HCT116 cells. HCT116 cells were seeded (1x10^6 cells/well) in 6-well plates and treated with catalpol (0, 25, 50 and 100 μg/ml) for 48 h. (A) Caspase-3 and (B) -9 activity was subsequently determined. **P<0.01 vs. the control group.

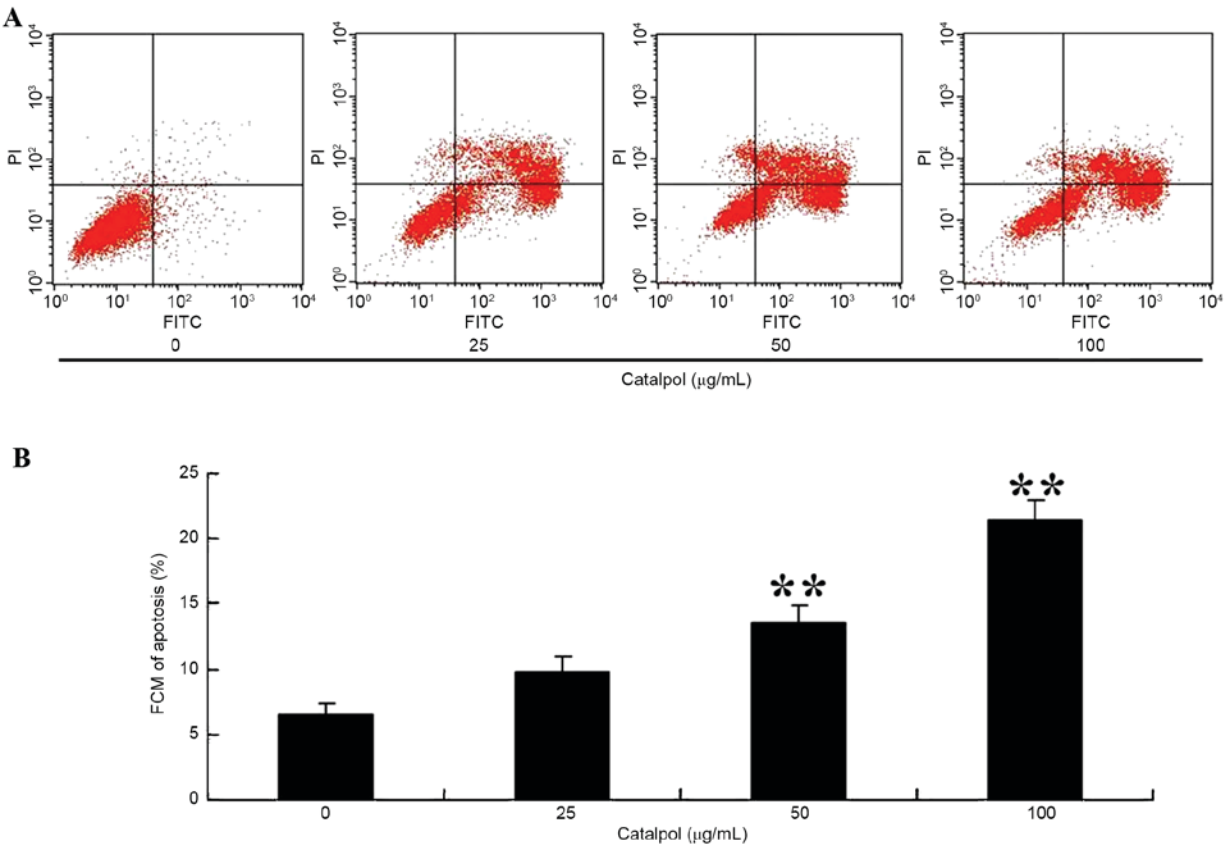

Figure 4. Catalpol induces apoptosis in HCT116 cells. HCT116 cells were seeded (1x10^6 cells/well) in 6-well plates and treated with catalpol (0, 25, 50 and 100 μg/ml) for 48 h. (A) Apoptosis was subsequently evaluated. (B) Quantification of apoptotic data. **P<0.01 vs. the control group. PI, propidium iodide; FITC, fluorescein isothiocyanate; FCM, flow cytometry.

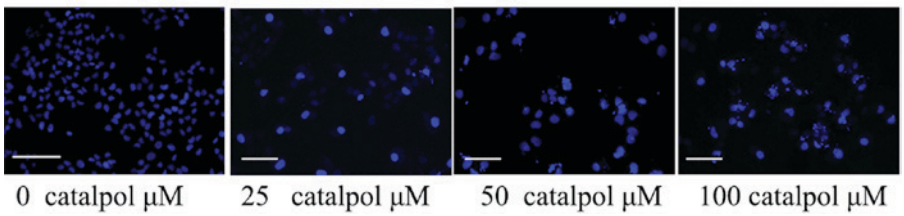

Figure 5. Effects of catalpol on the nucleic morphology of HCT116 cells. HCT116 cells were seeded (1x10^6 cells/well) in 6-well plates and treated with catalpol (0, 25, 50 and 100 μg/ml) for 48 h. The nucleic morphology of cells was investigated by DAPI staining (scale bar, 100 μm in the first image; scale bar, 200 μm in all other images).

Effects of catalpol-induced apoptosis in HCT116 cell. To observe apoptosis in HCT116 cells following treatment with catalpol, the apoptosis ratio was measured using a flow cytometric assay. As presented in Fig. 4A and B, the apoptosis percentage was increased following 48 h treatment with 50 or 100 μg/ml catalpol.

Effects of catalpol on the nucleic morphology of HCT116 cells. The anticancer effect of catalpol on the nucleic morphology of HCT116 cells was assessed. As presented in Fig. 5, catalpol (50 or 100 μg/ml) influenced the nucleic morphology of HCT116 cells and accelerated HCT116 cell nucleic apoptosis compared with the control group.

Effects of catalpol on the PI3K-Akt signaling pathway. To further investigate the mechanism of catalpol on the viability and apoptosis of human colorectal cancer HCT116 cells, the expression of PI3K and p-Akt protein was evaluated. Following treatment with catalpol (0, 25, 50 or 100 μg/ml) for 48 h, the expressions of PI3K and p-Akt proteins were analyzed by western blotting. The protein expression of PI3K and p-Akt was markedly decreased compared with the control group (Fig. 6).

Effects of catalpol on microRNA-200 expression of HCT116 cell. To further investigate the mechanism of catalpol on proliferation and apoptosis in human colorectal cancer HCT116 cells, microRNA-200 expression was evaluated in HCT116 cells. As demonstrated in Fig. 7, the expression of microRNA-200 was increased following treatment with catalpol (0, 25, 50 and 100 μg/ml) for 48 h compared with the control group.

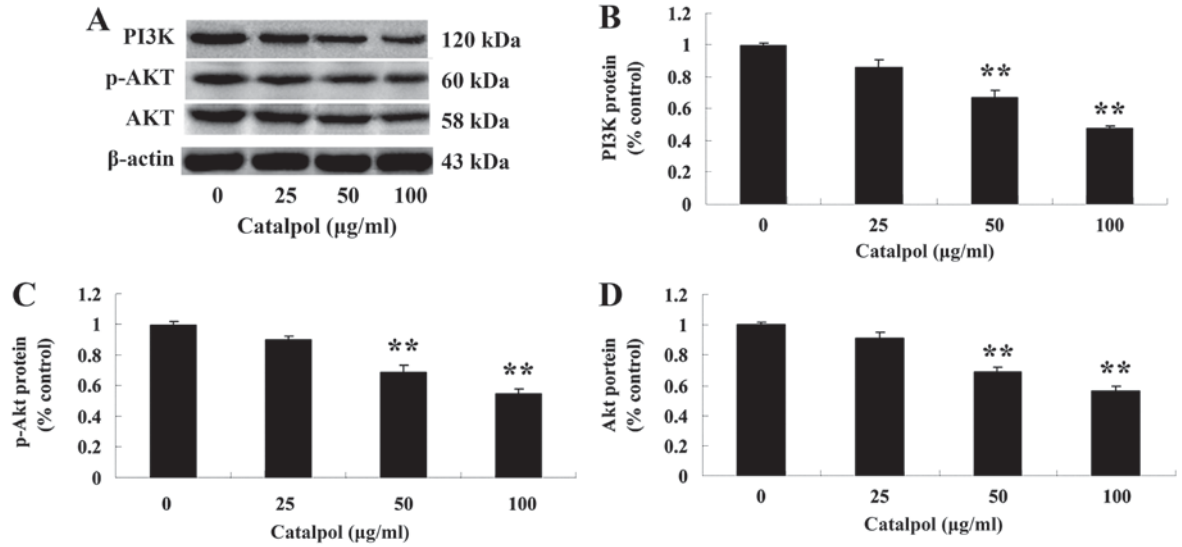

Figure 6. Effects of catalpol on the PI3K-Akt signaling pathway. HCT116 cells were seeded (1x10^6 cells/well) in 6-well plates and treated with catalpol (0, 25, 50 and 100 μg/ml) for 48 h. (A) The protein expressions of PI3K, Akt and p-Akt were measured by western blotting. Quantification of the (B) PI3K, (C) p-Akt and (D) Akt western blotting results. **P<0.01 vs. the control group. PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; p, phosphorylated.

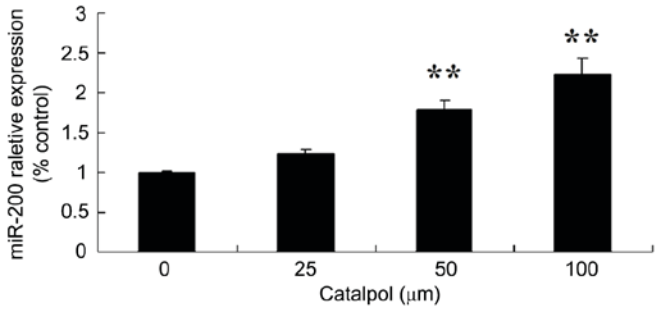

Figure 7. Effects of catalpol on miR-200 expression in HCT116 cells. HCT116 cells were seeded (1x10^6 cells/well) in 6-well plates and treated with catalpol (0, 25, 50 and 100 μg/ml) for 48 h. Total RNA was extracted from HCT116 cell using TRIzol and miR-200 expression was quantitatively analyzed. **P<0.01 vs. the control group. miR, microRNA.

Downregulation of PI3K-Akt following treatment with catalpol and the effect on cellular viability. To investigate the mechanism of catalpol on viability and apoptosis in human colorectal cancer HCT116 cells, PI3K inhibitor (LY294002, 5 μM) was added to HCT116 cells. The results indicated that the PI3K inhibitor inhibited the PI3K-Akt signaling pathway by suppressing PI3K and p-Akt protein expression in HCT116 cells compared with the catalpol-treated (50 μg/ml) group (Fig. 8A-D). In addition, downregulation of PI3K-Akt further decreased cell viability in HCT116 cells compared with the catalpol-treated (50 μg/ml) group (Fig. 8E).

Effect of downregulation of PI3K-Akt on microRNA-200 expression in HCT116 cells. To verify the mechanism of catalpol on the viability and apoptosis of human colorectal cancer HCT116 cells, microRNA-200 expression in HCT116 cells was detected following downregulation of PI3K-Akt. As presented in Fig. 9, the downregulation of PI3K-Akt enhanced microRNA-200 expression in HCT116 cells compared with the catalpol-treated (50 μg/ml) group.

Discussion

Colorectal cancer is the most common malignant tumor and it is estimated there were 1.167 million new cases in 2007 worldwide, ranking third among all malignant tumors; 603,000 succumbed to colorectal cancer in the same year (21,22). Currently, colorectal cancer remains a major threat to human health. In the present study, the results demonstrated that the cell viability of human colorectal cancer cells was inhibited by treatment with catalpol. In addition, the treatment of catalpol increased the activities of caspase-3 and -9, increased the apoptosis ratio, and accelerated cell nucleic apoptosis in human colorectal cancer HCT116 cells. Lee *et al* (23) demonstrated that catalpol inhibited the proliferation of ovarian cancer A2780 cells, human epidermoid carcinoma, human rhabdomyosarcoma and transgenic murine L-cells (17).

Increasing evidence has demonstrated that the expression of microRNAs in certain malignant human tissues is altered, including in lung, liver, colon, nasopharyngeal, ovarian and breast cancer. The regulatory role of microRNAs in gastric cancer has also been proven in an increasing number of experiments. In human colon cancer cell lines, microRNA-200 expression is increased and the expression of microRNA-200b decreased following treatment with 5-fluorouracil. MicroRNA-200 inhibits tyrosine-protein phosphatase non-receptor type 12, which inactivates oncogenes including tyrosine-protein kinase ABL1, proto-oncogene tyrosine-protein kinase Src and GTPase Ras. A previous study demonstrated that microRNA-200 exhibits a regulatory role in the proliferation of gastric cancer MGC-803 cells and that overexpression of microRNA-200 may inhibit the proliferation of gastric cancer MGC-803 cells (24-27). Gao *et al* (28) reported that catalpol suppresses proliferation and facilitates apoptosis in OVCAR-3 ovarian cancer cells through activation of microRNA-200 and down-regulating matrix metalloproteinase-2 expression. In the

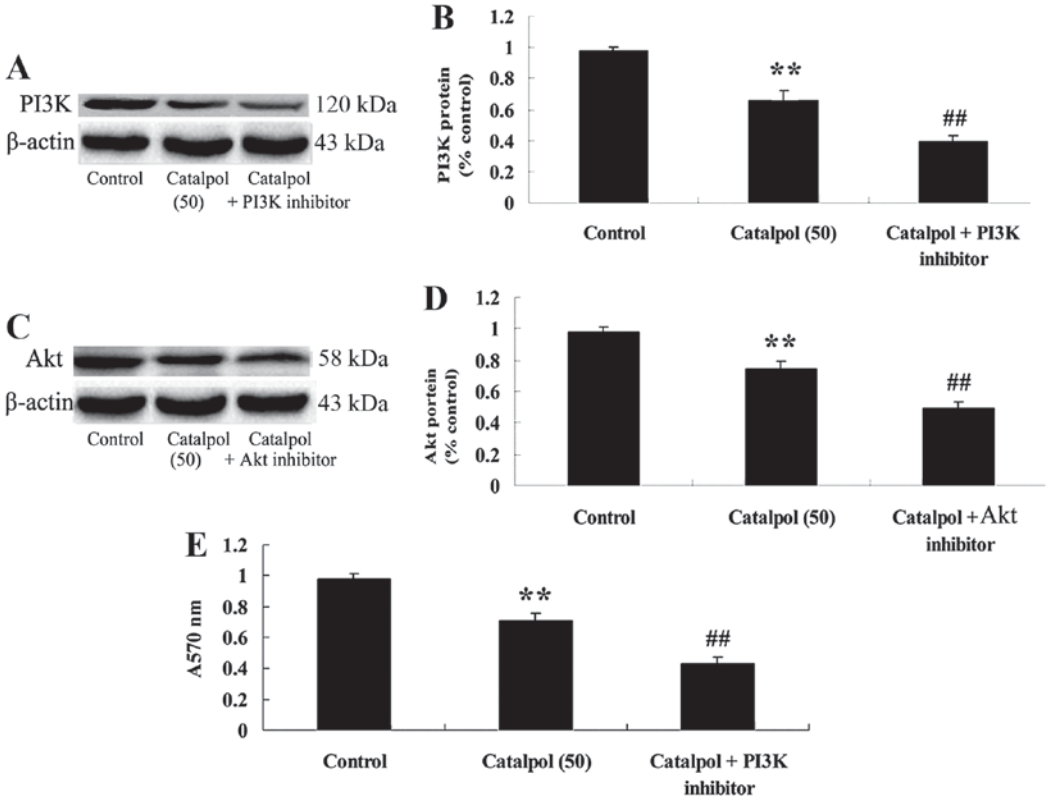

Figure 8. Effect of PI3K downregulation of on cell viability following treatment with catalpol. (A) Western blotting of PI3K following PI3K inhibition. (B) Quantification of PI3K western blotting results. (C) Western blotting of Akt following PI3K inhibition. (D) Quantification of Akt western blotting results. (E) HCT116 cell viability assay following PI3K inhibition. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the catalpol-treated group. PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B.

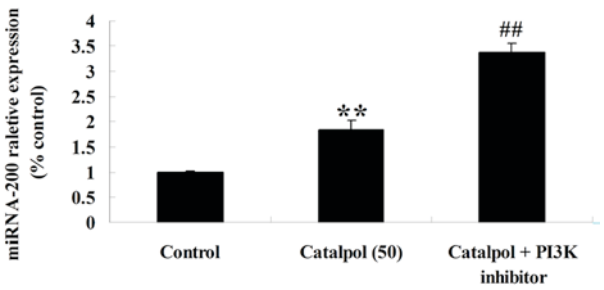

Figure 9. Effect of phosphatidylinositol 3-kinase downregulation on microRNA-200 expression in HCT116 cells treated with 50 μg/ml catalpol. miRNA-200 expression was quantitatively analyzed. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the catalpol-treated (50 μg/ml) group. miRNA, microRNA-200.

present study, similar results were obtained; treatment with catalpol also promoted the expression of microRNA-200 in HCT116 cells.

The PI3K/Akt signal transduction pathway serves an important role in cell proliferation, in which Akt is a downstream target protein of PI3K, and continuous activation of the pathway is associated with tumor development (29). The PI3K/Akt signaling pathway is regulated by a variety of cytokines, in which negative regulator molecules, including PTEN, form primary components (30). In the present study, it was observed that catalpol reduced the expressions of PI3K and p-Akt in HCT116 cells. A PI3K inhibitor decreased the viability of human colorectal cancer HCT116 cell following treatment with catalpol and increased the expression of microRNA-200 in HCT116 cells. Chamnanphon *et al* (18) reported that catalpol protected oligodendrocyte survival and oligodendrocyte progenitor differentiation through the Akt signaling pathway in rats. Sukasem *et al* (31) demonstrated that catalpol decreased peroxynitrite formation and consequently exerts cardioprotective effects through the PI3K/Akt signaling pathway in ischemic/reperfusion rats.

In conclusion, catalpol may be used as a natural anticancer drug in human colorectal cancer HCT116 cells. The present study suggests that, administration of catalpol reduced cell viability, increased the activities of caspase-3 and -9, increased cellular and nucleic apoptosis, suppressed the protein expression of components of the PI3K-Akt signaling pathway, and promoted the expression of microRNA-200 in HCT116 cells. In addition, the present study investigated whether downregulation of the PI3K-Akt signaling pathway was due to the effect of catalpol on HCT116 cells. However, treatment with a PI3K inhibitor augmented the effect of catalpol on cell viability and increased the expression of microRNA-200 in HCT116 cells. Therefore, catalpol promotes apoptosis in human colorectal cancer cells through the promotion of microRNA-200 and the downregulation of the PI3K-Akt signaling pathway. These results suggest that catalpol is a promising seed for novel types of anti-tumor agents; however, their molecular targets require further clarification.

Acknowledgements

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