# Sulforaphane Inhibits Proliferation and Apoptosis of Colorectal Cancer Cells by Down-Regulating the Cyclooxygenase-2/Protein Kinase B/Glycogen Synthase Kinase-3 Beta Signaling Pathway

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**To determine the effects of sulforaphane on the proliferation and apoptosis of colorectal cancer cell line HCT116 and the levels of proteins related to the cyclooxygenase-2/protein kinase B/glycogen synthase kinase-3 beta signal transduction. Colorectal cancer HCT116 cell lines were cultured *in vitro* and assigned into sulforaphane (30, 60 and 120 μM) groups, simple cisplatin group and cisplatin+sulforaphane group. The relative survival rate and apoptosis rate of HCT116 cells were determined by cell counting kit-8 assay and flow cytometry, respectively. Besides, the expression levels of proteins survivin, caspase-3, cyclooxygenase-2, protein kinase B, phosphorylated-protein kinase B, glycogen synthase kinase-3 beta and phosphorylated-glycogen synthase kinase-3 beta in HCT116 cells were measured through Western blotting. Compared with that in control group, the relative survival rate of cells in 60 and 120 μM sulforaphane groups, simple cisplatin group and cisplatin+sulforaphane group declined significantly ($p<0.05$). Sulforaphane can exert its anti-tumor effect by inducing apoptosis and suppressing proliferation of HCT116 cells and its mechanism may be associated with inhibition on the activation of the cyclooxygenase-2/protein kinase B/glycogen synthase kinase-3 beta signaling pathway.**

**Key words: Sulforaphane, colorectal cancer, cyclooxygenase-2, protein kinase B, glycogen synthase kinase-3**

Sulforaphane, also known as "raphanin", is an isothiocyanate derivative extensively existing in broccoli, cauliflower, cabbage, radish, mustard and other cruciferous plants[1]. Pharmacological studies have revealed that sulforaphane has anti-tumor, antioxidant, anti-inflammatory and immune regulation effects, showing good pharmacological activities in gastric cancer, colon cancer and lung cancer[2,3]. According to a study, the occurrence and development of tumor are closely associated with the high expression of Cyclooxygenase-2 (COX-2) and the expression of COX-2 is significantly increased in colorectal cancer cells[4]. Early studies have also suggested that highly expressed COX-2 can also induce the Protein Kinase B (Akt)/Glycogen Synthase Kinase-3 beta (GSK3β) signal transduction and this signal transduction is also involved in biological processes such as proliferation, invasion and migration of colorectal cancer cells[5,6]. However, there have been few studies on whether sulforaphane inhibits the proliferation of colorectal cancer SW480 cells by regulating the activation of the COX-2/Akt/GSK3β signaling pathway. Hence, this study aimed to observe the effects of sulforaphane on the proliferation and apoptosis of colorectal cancer SW480 cells and on the COX-2/Akt/GSK3β signaling pathway. Human colorectal cancer SW480 cell lines were purchased from Wuhan Buffalo Biological Co., Ltd., and Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) and trypsin were sourced from Gibco. Cell Counting Kit-8 (CCK-8) was bought from Solarbio, Radioimmunoprecipitation Assay (RIPA) lysate was provided by Zhejiang Tianhang

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Biotechnology Co., Ltd., and Bicinchoninic Acid (BCA) protein assay kit and Annexin V-Fluorescein Isothiocyante (FITC) apoptosis detection kit were purchased from Beijing 4A Biotech Co., Ltd. In addition, rabbit anti-mouse survivin, caspase-3, COX-2, Akt, phosphorylated (p)-Akt, GSK3β, p-GSK3β and β-actin antibodies were sourced from CST (USA), goat anti-rabbit Immunoglobulin G (IgG) secondary antibody was provided by Shanghai Guge Biological Technology Co., Ltd., and Enhanced Chemiluminescence (ECL) assay kit was bought from Advansta. Bio-Rad 550 microplate reader (BIO-RAD, USA), Amersham Imager 600 (GE Healthcare, USA) and FACSCalibur flow cytometer (Becton, Dickinson and Company, USA) were prepared. SW480 cells were cultured in DMEM containing 10 % FBS and 1 % antibiotics under 5 % carbon dioxide at 37°. When the cells reached about 90 % confluence, they were subcultured, specifically as follows: The cells were washed with Phosphate-Buffered Saline (PBS) twice, digested with trypsin and then added with culture medium to terminate digestion after the cells became round; later, the cells were transferred into a sterile Eppendorf (EP) tube and centrifuged at 1000 r/min for 5 min; after the original culture medium was discarded, the cells were added with fresh culture medium for resuspension, followed by subsequent experiment. SW480 cells in logarithmic growth phase were inoculated onto a 96-well plate, with $1\times10^5$ cells per well. After overnight culture, the cells were treated with different concentrations (0, 30, 60 and 120 μM) of sulforaphane, cisplatin (3 μM), or cisplatin+sulforaphane (30, 60 and 120 μM) for 24 h. Later, the original culture medium was removed and 20 μl of CCK-8 reagent was added into each well. Then, the cells were further cultured in a constant temperature incubator for 2 h. Subsequently, the cell Absorbance (A) at 450 nm was measured using a microplate reader and the relative survival rate of cells (%) was calculated as: $A_{experimental\ group}/A_{control\ group}\times100$ %. SW480 cells in logarithmic growth phase were inoculated onto a 6-well plate, with $2\times10^6$ cells per well. After 24 h of culture, the cells were treated with different concentrations (0, 30, 60 and 120 μM) of sulforaphane, cisplatin (3 μM), or cisplatin+60 μM sulforaphane for 24 h, followed by digestion and centrifugation. Then the cells were collected, washed twice with PBS, centrifuged and then added with 1×binding buffer to prepare cell suspension. Thereafter, the cell suspension was placed into a flow tube, added with Annexin V-FITC (5 μl) and Propidium Iodide (PI) (5 μl). After mixing evenly, the cells were incubated in dark at room temperature for 15 min and then added with 1×binding buffer and fully mixed. Later, apoptosis was detected using a flow cytometer. The drug intervention was conducted as mentioned above. After the cells were collected and fully lysed on ice for 30 min, the concentration of total protein was determined by BCA method. Next, 50 μg of the sample was loaded in each well for gel electrophoresis (constant voltage of 70 V, 3 h). After the proteins were completely separated, they were transferred onto a membrane (constant current of 275 mA, 70 min). Later, the protein bands were blocked with 5 % skimmed milk for 1 h, incubated with primary antibodies (survivin, caspase-3, COX-2, Akt, p-Akt, GSK3β, p-GSK3β and β-actin, dilution ratio of 1:1000) at 4° overnight and then the membrane was washed 3 times with Tris-Buffered Saline-Tween 20 (TBST), followed by incubation with second antibodies (dilution ratio of 1:3000) at room temperature for 90 min and washing with TBST for 3 times. After ECL development and exposure in a dark room, the gray-scale values of the proteins were analyzed using the ImageJ software. Statistical Package for the Social Sciences (SPSS) 26.0 software was utilized for statistical analysis. The measurement data in line with normal distribution were expressed as mean±standard deviation. After variance analysis, Least Significant Difference (LSD)-t test was performed for comparisons between groups. $p<0.05$ indicated that a difference was statistically significant. No significant difference was found in the survival rate of SW480 cells between the low-dose (30 μM) sulforaphane group and the control group ($p>0.05$). However, the survival rate in the other sulforaphane groups, cisplatin group and cisplatin+sulforaphane groups was significantly lower than that in control group ($p<0.05$). In addition, the survival rate in cisplatin+sulforaphane group was significantly lower than that in cisplatin group ($p<0.05$) and sulforaphane groups with the same dose of sulforaphane ($p<0.05$) (fig. 1). The apoptosis rate of SW480 cells in control group, low-, medium- and high-dose sulforaphane groups, cisplatin group and cisplatin+medium-dose sulforaphane group were (0.78 %±0.11 %), (1.23 %±0.11 %), (2.78 %±0.12 %), (3.89 %±0.12 %), (5.54 %±0.43 %) and (7.89 %±0.45 %), respectively. It can be seen that the apoptosis rate of SW480 cells was significantly higher in sulforaphane groups (with different doses of sulforaphane), cisplatin group and cisplatin+medium-dose sulforaphane group than that in control group ($p<0.05$) and it rose with the increase of sulforaphane concentration. In addition, the early apoptosis rate in cisplatin+medium-dose sulforaphane

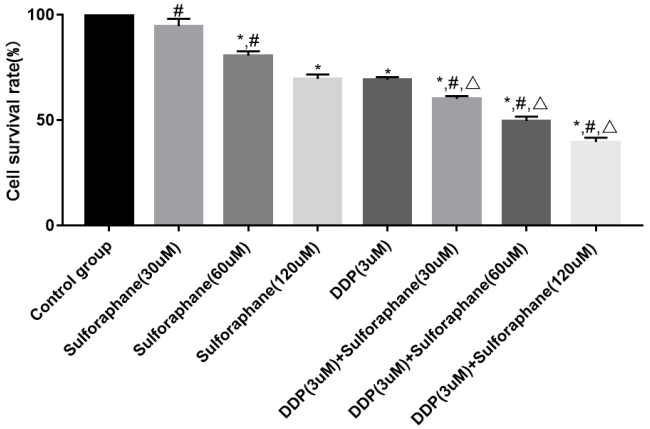


**Fig. 1: Effect of sulforaphane on survival rate of SW480 cells determined by CCK-8 assay, *p<0.05 *vs.* control group; #p<0.05 *vs.* cisplatin group; △p<0.05: cisplatin+sulforaphane group *vs.* sulforaphane groups with the same dose of sulforaphane**

group was significantly higher than that in sulforaphane groups (with different doses of sulforaphane) ($p<0.05$) (fig. 2). The expression level of survivin was significantly lower, while the expression level of caspase-3 was significantly higher in sulforaphane groups, cisplatin group and cisplatin+sulforaphane groups than that in control group ($p<0.05$). With the increase of sulforaphane dose, the expression level of survivin declined significantly, while the expression level of caspase-3 rose significantly, showing statistically significant differences among groups ($p<0.05$). Moreover, the expression level of survivin was significantly lower, while the expression level of caspase-3 was significantly higher in cisplatin group than that in sulforaphane groups ($p<0.05$). The expression level of survivin in cisplatin+sulforaphane groups was significantly higher than that in sulforaphane groups and cisplatin group ($p<0.05$) (fig. 3). The expression levels of COX-2, p-Akt and p-GSK3β in sulforaphane groups, cisplatin group and cisplatin+sulforaphane groups were significantly lower than those in control group ($p<0.05$). With the increase of sulforaphane dose, the expression levels of COX-2, p-Akt and p-GSK3β were declined significantly, showing statistically significant differences among groups ($p<0.05$). In addition, the expression levels of COX-2, p-Akt and p-GSK3β were significantly lower in cisplatin group than those in sulforaphane group ($p<0.05$) and significantly lower in cisplatin+sulforaphane groups than those in sulforaphane groups and cisplatin group ($p<0.05$) (fig. 4). Sulforaphane, derived from cruciferous plants, not only has strong anti-tumor activity[7,8], but also shows good pharmacological effects on digestive diseases[9,10]. However, whether sulforaphane has potential value for adjuvant treatment of colon cancer needs further investigation. In this study, therefore, human colon cancer SW480 cells were adopted the *in vitro* research model and the effects of sulforaphane on the proliferation and apoptosis of SW480 cells were observed, whose mechanism was preliminarily discussed. Since the pathogenesis of tumor is not only associated with abnormal proliferation and differentiation of cancer cells, but also with inhibition of apoptosis, blocking the proliferation and inducing

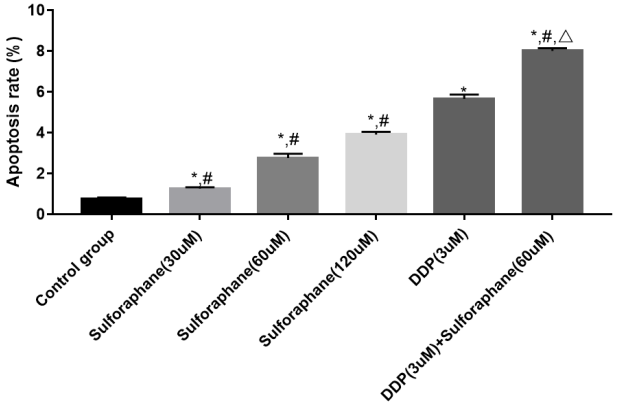


**Fig. 2: Effect of sulforaphane on apoptosis rate of SW480 cells, *p<0.05 *vs.* control group; #p<0.05 *vs.* cisplatin group; △p<0.05: cisplatin+sulforaphane group *vs.* sulforaphane groups with the same dose of sulforaphane**

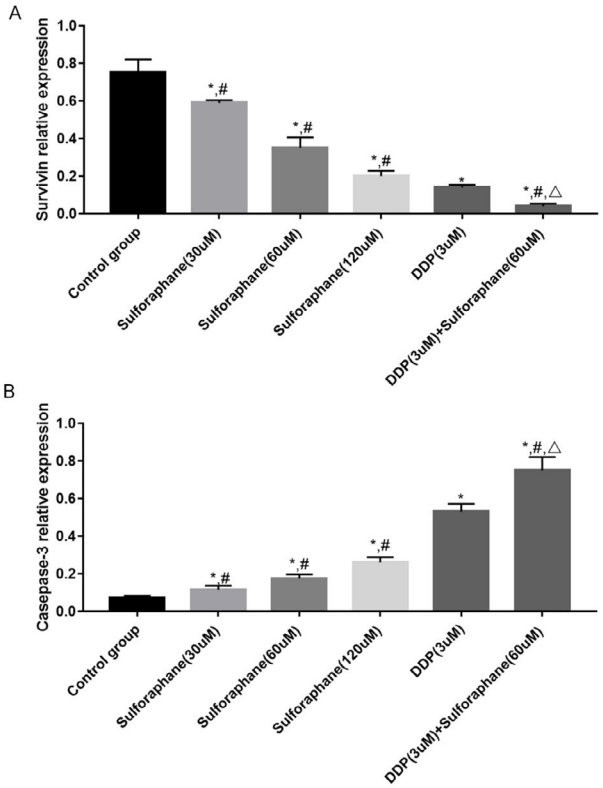


**Fig. 3: Effect of sulforaphane on protein expressions of (A) Survivin and (B) Caspase-3 in SW480 cells, *p<0.05 *vs.* control group; #p<0.05 *vs.* cisplatin group; △p<0.05: cisplatin+sulforaphane group *vs.* sulforaphane groups with the same dose of sulforaphane**

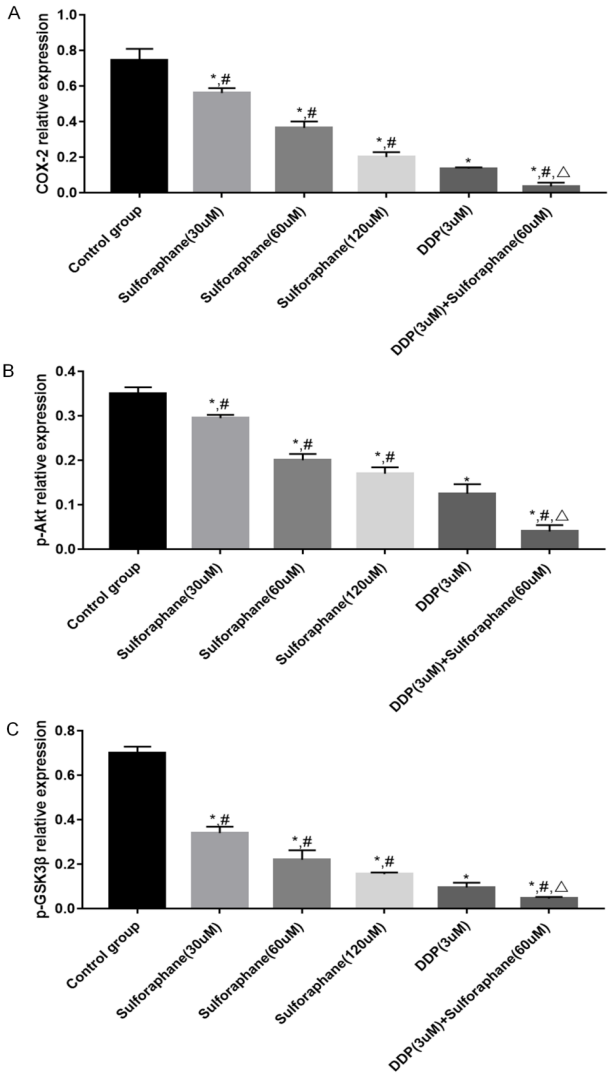


**Fig. 4: Effect of sulforaphane on expression levels of (A) COX-2; (B) p-Akt and (C) p-GSK3β in SW480 cells, *p<0.05 *vs.* control group; #p<0.05 *vs.* cisplatin group; △p<0.05: cisplatin+sulforaphane group *vs.* sulforaphane groups with the same dose of sulforaphane**

apoptosis of tumor cells may be the major means to prevent and treat cancer. Previous studies have proved that[11,12] sulforaphane can exert an anti-tumor effect through inducing apoptosis of cancer cells, suppressing DNA synthesis in the cells and boosting cell immunity. Apoptosis is a gene-regulated autonomous programmed cell death. Such a biological effect can be modulated by the activation of various genes and the apoptosis pathways include death receptor pathway and mitochondrial pathway, both of which can lead to the activation of the effector caspase-3, resulting in endonuclease activation and thereby inducing apoptosis[13-15]. Caspase is the major protein that regulates apoptosis, while survivin is a protein which inhibits apoptosis mainly through suppressing the activities of caspase-3 and caspase-7 and the expression levels of the above proteins are closely associated with the proliferation and apoptosis of human colorectal cancer cells[16,17]. In this study, the proliferation and apoptosis of SW480 cells were taken as the starting point to further investigate the molecular mechanism of sulforaphane. The results of CCK-8 assay demonstrated that sulforaphane suppressed the proliferation of SW480 cells in a concentration-dependent manner. Nevertheless, the inhibitory effect of different doses of sulforaphane on the growth of SW480 cells was less obvious than that of cisplatin alone and cisplatin+sulforaphane and the combination of cisplatin and sulforaphane showed a synergistic effect. Apoptosis detection results revealed that different doses of sulforaphane, cisplatin alone and cisplatin+sulforaphane could induce early apoptosis of SW480 cells and the early apoptosis rate was significantly positively correlated with the dose of sulforaphane. In addition, the apoptosis rate in cisplatin+sulforaphane groups was higher than that in sulforaphane groups and cisplatin group and the combination of cisplatin+sulforaphane had a synergistic effect. The results of Western blotting manifested that sulforaphane, cisplatin and the combination of cisplatin and sulforaphane could dramatically reduce the protein expression of survivin and induce the protein expression of caspase-3. COX-2 is highly expressed in human colorectal cancer cells. Early studies have proved that COX-2 can induce phosphorylation of Akt, thus promoting phosphorylation of GSK3β and the activation of this signal transduction can modulate biological processes such as cell proliferation, adhesion, differentiation and apoptosis[18-20]. In this study, it was found that different doses of sulforaphane, cisplatin and cisplatin+sulforaphane could remarkably repress the expressions of COX-2, p-Akt and p-GSK3β, which were positively correlated with the dose of sulforaphane. Moreover, the inhibitory effect of cisplatin+sulforaphane was more obvious than that of sulforaphane alone and cisplatin alone and the combination of cisplatin+sulforaphane displayed a synergistic effect. In summary, sulforaphane shows anti-cancer activity through inducing apoptosis and suppressing proliferation of colorectal cancer cells. Such a biological effect may be related to the suppression of the COX-2/Akt/GSK3β signal transduction, up-regulation of the protein expression of caspase-3 and down-regulation of the protein expression of survivin.

## Authors' contributions:

Yongjun Zhu and Haijun Hu have contributed equally to this work.

## Conflict of interests:

The authors declared no conflicts of interest.

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