Clinics in Oncology

Research Article
Published: 08 Apr, 2019

Grape Seed Proanthocyanidins Alters the Characteristics of Colon Cancer Cells Regarding an Akt Signaling

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Abstract

Objective: To investigate the mechanism of Grape Seed Proanthocyanidins (GSPs) on colon cancer cells.

Methods: SW480 and SW620 cells were treated with GSPs and characterized by evaluating cell morphology, cell viability, cell cycle and apoptosis respectively. The expression and activation of some important molecules were explored by PCR and Western blot respectively.

Results: GSPs suppressed the proliferation and altered the morphology of both SW480 and SW620 cells significantly for long term treatment. GSPs caused arrest of SW480 and SW620 cell cycle in G0/G1 phase and facilitated apoptosis. Cyclin D1 and CDK4 as well as some molecules in Akt signaling pathway were identified to be down-regulated by GSPs. Furthermore, GSPs also suppressed the activation of Akt and some downstream molecules.

Keywords: Grape seed proanthocyanidins; Colorectal cancer; Apoptosis

Introduction

Colorectal Cancer (CRC) is one of the most common malignancies [1]. With the changes in diet and lifestyle, the incidence of colorectal cancer in China has been increasing significantly. Although the level have been continuously improved regard of radiotherapy and chemotherapy techniques and surgical procedures, serious adverse reactions will accompany in the course of treatment, and furthermore, little benefit can be obtained for the patients with high metastasis and recurrence of cancer [2].

Tabung et al. [3] showed that dietary patterns can influence the body's inflammatory state by which increases the risk of colorectal cancer. Intake of more pro-inflammatory diets such as red meat, processed meat, and animal internal organs increases the risk of colorectal cancer in men and women. Liu L et al. [4] showed that diet-related inflammation may promote the development of colorectal cancer by inhibiting adaptive anti-tumor immune responses. Apparently, dietary patterns affect systemic and local intestinal inflammation, which is related to inflammatory bowel cancer. Chronic inflammation interferes with adaptive immune response, which is closely related to tumorigenesis.

Plant compounds that are widely found in nature have a good chemopreventive and therapeutic effect on tumors. GSPs (Grape Seed Proanthocyanidins), a type of polyphenols that are prevalent in nature and found in our daily diets such as fruits, vegetables, nuts, seeds, etc. [5], had been reported to have a tumor control efficacy. Given the components in our food, obviously, GSPs can be an idea drug for the prevention and treatment of colon cancer. GSPs are flavonoid polyphenols extracted from grape seeds, mainly dimers of catechins, epicatechins or catechins and epicatechins, as well as some trimers. It is reported that GSPs have some characteristics, which are antioxidant, scavenging free radicals [6], protection of cardiovascular [7], anti-inflammatory, anti-tumor [8-10] and other effects.

Our results showed that GSPs could significantly alter the morphology of SW480 and SW620 cells and suppress their proliferation for long term treatment. GSPs arrests SW480 and SW620 cell cycle in G0/G1 phase and promote cell apoptosis significantly. The mechanism by which Akt signaling pathway is involved in this process.

Materials and Methods

Materials

Human colon cancer cells SW480 and SW620 were gifted by the Cancer Center Laboratory of the

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Received Date: *12 Feb 2019*
Accepted Date: *02 Apr 2019*
Published Date: *08 Apr 2019*

Citation:
Shi H, Chengcheng LV, Zhu Y. Grape Seed Proanthocyanidins Alters the Characteristics of Colon Cancer Cells Regarding an Akt Signaling. Clin Oncol. 2019; 4: 1602.

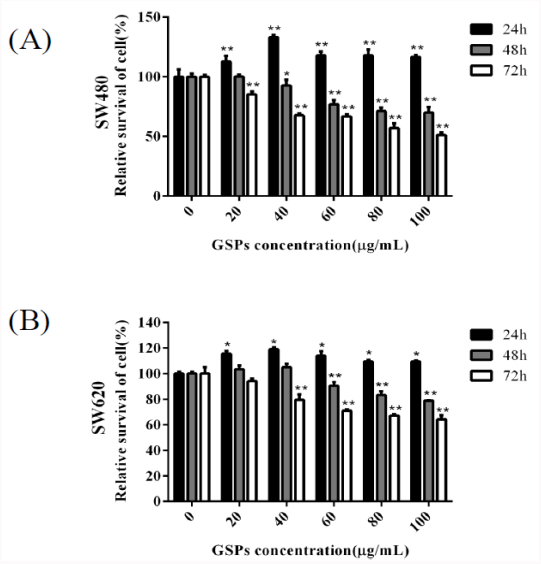

Figure 1: GSPs inhibited the proliferation of SW480 and SW620 cell. (A) The cell viability of SW480 cell treated with various concentrations of GSPs for 24 h, 48 h, and 72 h. (B) The cell viability of SW480 cell treated with various concentrations of GSPs for 24 h, 48 h, and 72 h.*p<0.05, **p<0.01

PLA General Hospital. GSPs (≥ 95%) were purchased from Jianfeng Natural Product Research and Development Co. Tianjin, China. Fetal bovine serum was purchased from Gibco, USA. RPMI1640 medium was purchased from Hyclone, USA. Dimethyl Sulfoxide (DMSO) and MTT were purchased from Sigma Company, USA. Annexin V-FITC cell apoptosis detection kit was purchased from KGI Biotechnology Development Co., Nanjing, China. Cell cycle detection kit, cell lysate were purchased from Haimen Biyuntian Institute of Biotechnology, Jiangsu and China. BCA protein concentration assay kit was purchased from Thermo Corporation, USA. Trizol was purchased from Invitrogen Corporation, USA. RNA reverse transcription kit was purchased from Promega USA. Antibody against β-actin was purchased from Easy Bio., USA. Antibodies against Cyclin D1, CDK4, Bax and Bcl-2 were purchased from Dr. De Bioengineering Co., Wuhan, China. Antibody against Bad was purchased from Abcam, USA.

Methods

Cell culture and treatment: Human colon cancer cells SW480 and SW620 were maintained in (1640) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37°C in a humidified incubator containing 5% CO_2 and 95% air. For experimental group, GSPs was added with the final concentration 100 μg/mL and the control group received no treatment. The cell morphological state was observed after culturing for 48 h.

Cell proliferation assay: Logarithmic growth cells SW480 and SW620 were seeded in 96-well plates at 2 × 10^4/well and different concentrations of GSPs were added to the experimental groups (20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL and 100 μg/mL), 6 replicates for each concentration, and no treatment for the control group. Thereafter, 20 μl of MTT solution was added to each well and incubated for 4 h. The number of viable cells was measured in a 96-well plate at an optical density of 490 nm on a micro plate reader. Cells viability were normalized by control and described as the relative percentage.

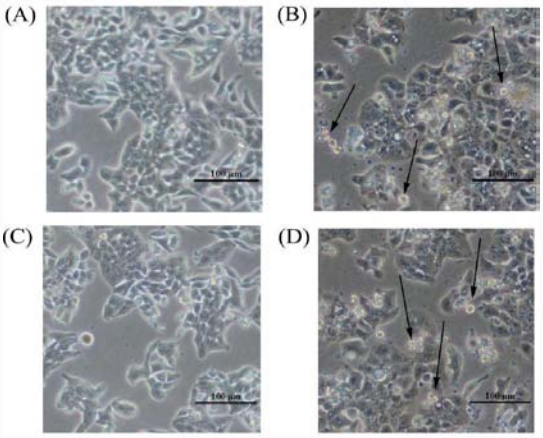

Figure 2: GSPs significantly changed the cell features. (A) SW480 cells control. (B) SW480 cells treated by GSPs with 100 μg/mL. (C) SW620 cells control. (D) SW620 cells treated by GSPs with 100 μg/mL. (Scale bar, 100 μm).

Annexin V/FITC staining. Cells were treated with GSPs (100 μg/ml) for 24 h, 48 h, 96 h respectively followed by harvesting and suspending with 1 × binding buffer (according to the supplier's instructions). Afterwards, the cells were stained with Annexin V and PI, followed by incubation on ice for 10 min in the dark. Results were analyzed using flow cytometry.

Measurement of cell-cycle distribution: Cells were treated with GSPs (100 μg/ml) for 48 h. The cells were then harvested, fixed with ice-chilled 70% ethanol, and stored at 4°C overnight. On the next day, they were centrifuged at 16,000 × g for 5 min with 1 ml of cold PBS. After discarding the supernatant, the cell pellets were stained with PI (500 μg/ml) in the presence of RNase A (10 μg/ml) and incubated at 37°C for 30 min. Cell cycle distribution was analyzed by using flow cytometry.

RNA isolation and reverse transcription: RNA from cell lines was isolated using TRIzol reagent; cDNA was prepared from 1 μg of RNA according to the manufacturer's instructions and stored at -20°C until used.

Real-time quantitative PCR analysis: Real-time PCR was carried out to measure the level of gene expression in both colon cancer cell lines. Real-time PCR was performed in 20 μl final volume by using 10ul of 2 × iQ™ SYBR Green Super mix, and 7 μl of nuclease-free water, 2 μl of cDNA, 0.5 μl forward primer and 0.5 μL reveres primer.

Cycling conditions: 95°C for 30 sec, followed by 40 cycles of 94°C for 5 sec, 60°C for 5 sec and 72°C for 25 sec. Triplicate qPCR reactions were performed for each cDNA sample for all experiments.

Western blotting analysis: Cells were harvested and lysed with RIPA buffer, and collected protein samples were quantified by using Pierce BCA Protein Assay Kit. Equal amounts of protein samples were loaded onto SDS/polyacrylamide gels. After running, the gels were transferred onto NC membranes followed by incubating with 5% skim milk (blocking solution) at room temperature for 1 h, and primary antibodies (Anti-β-Actin, -CDK, -cyclin D1,-Bcl-2, -Bad, -Bax,-Akt, -p-Akt) at 4°C overnight sequentially. On the next day, the membranes were incubated with Horseradish Peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h. Enhanced chemiluminescence was used to detect proteins immobilized on membranes.

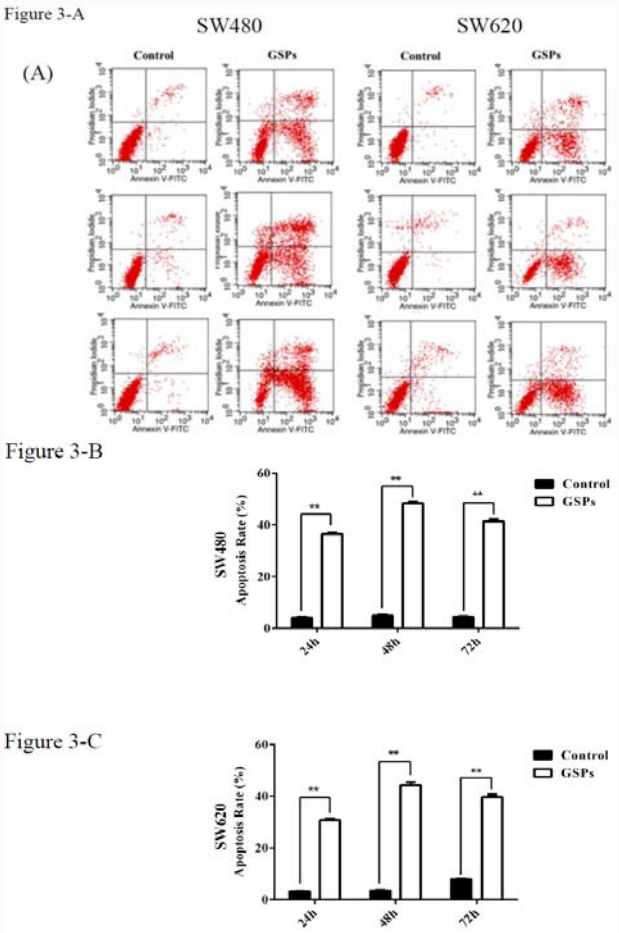

Figure 3: GSPs facilitated apoptosis of SW480 and SW620 cells. (A) The plot of SW480 and SW620 treated by GSPs (100 μg/mL) for 24 h, 48 h, and 72 h, respectively. (B) By normalizing with the control, the apoptotic rates in different GSPs groups were calculated in SW480 cells. (C) By normalizing with the control, the apoptotic rates in different GSPs groups were calculated in SW620 cells. *p<0.05, **p<0.01

Statistical analysis: All experiments were performed in triplicates; data are presented as mean ± Standard Deviation (SD). Statistical analysis was performed using SPSS19.0. Values were considered significant when p<0.05.

Results

GSPs inhibited the proliferation of SW480 and SW620 cell: SW480 and SW620 cells were treated by GSPs with different concentrations for 24 h, 48 h and 72 h, respectively. As shown in Figure 1, GSPs could suppress the proliferation of both SW480 and SW620 significantly for long term (48 h and 72 h) treatment, even that displayed a growth-promoting phenomenon for short term (24 h) treatment. The inhibiting rate was significant difference in low concentration comparing with high concentration (more than 60 μg/mL).

GSPs induced alteration of cells phenotype: SW480 and SW620 cells were treated by GSPs for 48 h, and the morphological changes of the two cells were observed. As shown in Figure 2, comparing with control, the cells phenotype was changed significantly in experiment groups, which were enlarged, mostly round and irregular.

GSPs facilitated apoptosis of SW480 and SW620 Cells: To investigate the effect of GSPs on cell apoptosis, the SW480 and SW620 cells treated by GSPs were subjected to measure by flow cytometry at different time points (24 h, 48 h, and 72 h) with Annexin V/PI staining. The results showed that GSPs can promote apoptosis of SW480 and SW620. As shown in Figure 3, the number of apoptotic cells of SW480 and SW620in GSPs group was increased significantly compared with the control at three time points, and most obvious effect is observed at 48 h.

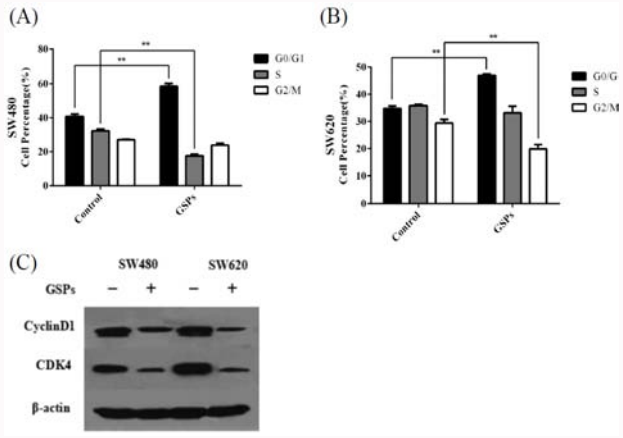

Figure 4: GSPs altered cell cycle of SW480 and SW620 cells. (A) The cells numbers in G0/G1 phase were increased in GSPs-treated SW480 cell. (B) The cells numbers in G0/G1 phase were increased in GSPs-treated SW620 cell. (C) GSPs suppressed the expression of CyclinD1 and CDK4 in SW480 and SW620.*p<0.05, **p<0.01

GSPs altered cell cycle of SW480 and SW620 cells: To investigate the effect of GSPs on cell cycle, SW480 and SW620 cells were treated by GSPs (100 μg/mL) for 48 h and subjected to detect by flow cytometry. As shown in Figure 4, G0/G1 arrest was observed in both GSPs treated cells. The expressions of CDK4 and cyclin D1 were identified and shown a significant decrease in GSPs groups.

Akt signaling was involved in GSPs regulation in SW480 and SW620 cells: Given Akt signaling was the key pathway in the regulation of cell growth and apoptosis; we identified some key components in them further. The expression and activation of Akt were investigated and showed that GSPs could down-regulate Akt as well as suppress its activation in both SW480 and SW620 cells, as shown in Figure 5A. The ratios of p-Akt/Akt were calculated and shown a significant decrease in GSPs treatment group as shown in Figure 5B. The expression of both Bad and Bax was up-regulated by GSPs in SW480 and SW620 cells, whereas Bcl2 was down-regulated, as shown in Figure 5C, 5D. Compared with control, GSPs elicited significant decrease of ratios of Bcl-2/Bax as shown in Figure 5E. The cleaved-caspase 9 and cleaved-caspase 3, the key components in apoptosis, were identified to be increased significantly, as shown in Figure 5F.

Discussion

GSPs as a dietary plant supplement, the effect on colon cancer cells of SW480 and SW620 was investigated and showed that GSPs could affect the biological characteristics of SW480 and SW620 significantly, e.g.: altering cell phenotype and cycle, inhibiting cell proliferation, promoting cell apoptosis. Consistently, this phenomenon is also observed in tongue squamous carcinoma cell Tca8113, which GSPs elicited a dose-dependent decrease of cell viability [11]. Still, other cancer cells of cervical cancer and pancreatic cancer also displayed the same results when they were treated by GSPs

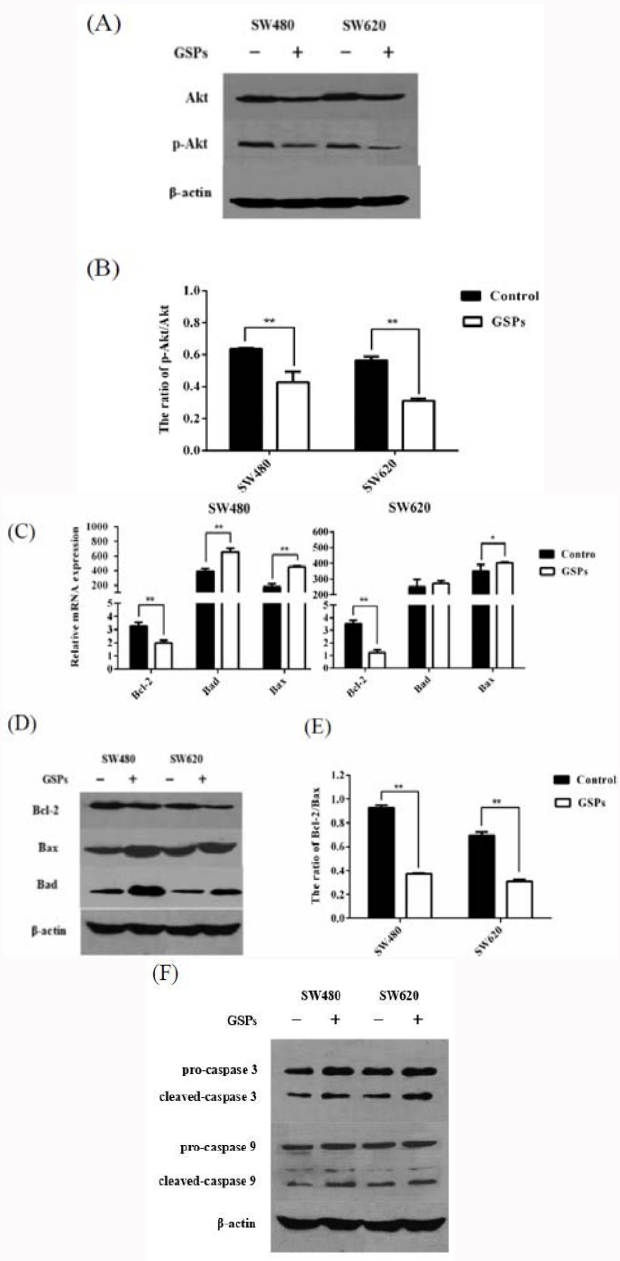

Figure 5: Akt signaling was involved in GSPs regulation in SW480 and SW620 cells.
(A) GSPs suppressed the expression and activation of Akt in both SW480 and SW620 cells. (B) The ratios of p-Akt/Akt in different groups were calculated and plotted. (C) GSPs down-regulated Bcl-2 and up-regulated Bad and Bax in SW480 and SW620 cells detected by qPCR. (D) GSPs down-regulated Bcl-2 and up-regulated Bad and Bax in SW480and SW620 cells detected by Western blotting. (E) The ratios of Bcl-2/Bax in different groups were calculated and plotted. (F) The cleaved-caspase-3 and cleaved-caspase-9 were detected in different groups.*p<0.05, **p<0.01.

[12,13]. This phenomenon was also observed in different tumors that GSPs induces apoptosis of nasopharyngeal carcinoma [14], breast carcinoma [15], human epidermoid carcinoma [16] and colorectal cancer [17]. Furthermore, tumor xenograft experiments have shown that GSPs also induce apoptosis in cervical cancer cells *in vivo* [12].

Annexin V/PI staining analysis by Flow cytometry showed that GSPs can cause G0/G1 arrest and induce apoptosis in both SW480 and SW620 cells. Consistently, it was reported that Grape Seed (GSE) and Grape Skin (GSK) combined treated ehrlich ascites carcinoma in vitro could result in a significant increase of G0/G1 population (diploid DNA content) [18]; Grape Seed Proanthocyanidin Extract (GSPE) caused a block in the G1 phase and inhibited human bladder cancer BIU87 cell growth [19]; GSPs induced G1 arrest in melanoma cells (A375, Hs294t), which elicited by miRNA-106b [20].

Given that Cyclin D1/CDK4 plays a crucial role in the G1 phase of the cell cycle and appeared an aberrant expression in many tumors [21,22], the expression of Cyclin D1 and CDK4 was also investigated and shown that GSPs suppressed the expression of Cyclin D1 and CDK4 significantly, which may account for the cause of G0/G1 arrest and inhibitory of cell growth.

The phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway is often activated as a cancer driver, leading to the development of various cancers, e.g.: renal cell carcinoma [23], breast cancer [24], and gastric cancer [25]. A variety of anticancer drugs identified for their activities were attributed to inhibit Akt signaling, such as Rg3 (ginsenoside) exerted anti-tumor activity in lung cancer cell lines A549 and H23 by inhibiting PI3K/Akt signaling pathway [26], Oridonin inhibited the OSCC (oral squamous cell carcinoma) tumors growth in xenograft nude mice by inhibiting phosphorylation of PI3K and Akt significantly [27]. Consistent with these reports, our results showed that GSPs suppressed the expression and activation of Akt in SW480 and SW620 cells.

As a downstream effector molecule of Akt, Bcl-2 and Bax are the most representative molecules of the Bcl-2 family concerned the regulation of tumor cells [28]. As same as some anticancer drugs identified above, GSPs not only suppressed activation of Akt signaling but also regulated expression of some downstream molecules, e.g.: down-regulating Bcl-2 and up-regulating Bad and Bax. Further, cleaved-caspase 9 and cleaved-caspase 3 as the key components of apoptosis were identified to be up-regulated significantly in GSPs treated cells of SW480 and SW620. Consistently, with HeLa and SiHa cells, Chen et al. [12] showed that GSPs can activate caspase-3 and facilitate the expression of proapoptotic protein Bak-1, and suppress the expression of anti-apoptotic protein Bcl-2.

Taken together, with colon cancer SW480 and SW620 cells, GSPs can significantly alter the morphology of the two cells, decrease cell proliferation, arrest G0/G1 cell cycle, and increase cell apoptosis. The mechanism by which the GSPs altered activation and expression of Akt signaling and its downstream molecules. Given the roles of GSPs in the regulation of SW480 and SW620 cells, this drug may shed a light on prevention and adjunctive therapy for colon cancer.

Acknowledgment

This project was supported by National High Technology Research and Development Program in China (2011AA02A110).

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