Oligosaccharide from apple induces apoptosis and cell cycle arrest in HT29 human colon cancer cells

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ABSTRACT

It is reported that apple polysaccharide can prevent colon cancer growth and impede colon cancer progression. Apple oligosaccharide was prepared by the combination of alkaline hydrolysis and enzymolysis of apple polysaccharides, and purified by anion column chromatography. The aim of this study is to explore the effect of apple oligosaccharide on the cellular viability of human colon canceroma cells (HT29 cells) and its mechanism. The results showed that apple oligosaccharide decreased the cellular viability of HT29 cells in dose-dependent manner. Meanwhile it enhanced the expression of Bax; and decreased the levels of Bcl-2 and Bcl-xl. Apple oligosaccharide induced cell cycle arrest in S phase, which correlated with the decreased expression of Cdk 2 and cyclin B1. These results indicated that apple oligosaccharide attenuated HT29 cell viability by inducing cell apoptosis and cell cycle arrest. Apple oligosaccharide is a potential chemoprevention agent or anti-tumor agent and is worthy of further study.

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1. Introduction

Colorectal carcinoma (CRC) is the third in males and the second in females leading causes of cancer-related death worldwide [1]. 5-Fluorouracil (5-FU) is widely used to treat colorectal carcinoma, but its side effects, such as coronary spasm [2], neurotoxic effects [3,4], and myelosuppression [5], hamper its clinical use.

During the past years, tremendous progresses have been made in our understanding of the carcinogenic process at the cellular and molecular level. This has led to the development of a promising new approach to cancer prevention, termed “chemoprevention” [6], which aims to block, inhibit or reverse the development and progression of precancerous cells through use of non-cytotoxic nutrients and/or pharmacological agents [7]. Accordingly, the validation and utilization of dietary components, natural products, or their synthetic analogs as potential cancer chemoprevention agents in the form of functional foods or nutraceuticals has become an important issue in current health- and cancer-related research.

Apples and apple juice are the most consumed fruit and fruit juice in the advanced country [8,9]. Several lines of evidence suggest that apples and apple products possess a wide range of biological activities which may contribute to health beneficial effects against cardiovascular disease, asthma and pulmonary dysfunction, diabetes, obesity and cancer [10].

It is reported that treatment of HT29 cells with low molecular weight apple polysaccharides (LMWAP, 1000–3000 Da) resulted in 333 genes expression over cutoff values (≥2-fold). When concentrations ranged from 0.001 to 0.1 mg/ml, LMWAP induced a G0/G1 phase block in HT29 cells in a dose-dependent manner. In vivo study revealed that oral administration of LMWAP effectively protected ICR mice against CRC. The results of Western blot suggested that LMWAP induced cell-cycle arrest in p53 independent manner. These data indicate that LMWAP inhibited the development of CRC through affecting cell cycle, and it has potential in clinical prevention of colon cancer [11]. Recently, it was found that when

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concentrations ranged from 0.1 to 1 mg/ml, apple polysaccharides (APs) showed apoptosis-inducing effect by activating NF-κB [12]. Oligosaccharides were found to have many pharmacological actions, including anti-infection properties [13,14], alteration of intestinal flora [15–17], anti-hypoglycemia [18], and anticancer effect [19–21]. Increasing evidence has indicated that oligosaccharides display anticancer effects toward different types of human tumors, including ovarian cancer, colon cancer, prostate cancer [22], and astrogloma [23]. It is found that oligosaccharides exert anti-cancer activity by blocking carcinogenesis, enhancing the efficacy of chemotherapeutic agents, or directly exhibiting cytotoxicity effect. Compared with polysaccharide, oligosaccharide is easier to prepare in large scale and control quality. Oligosaccharide is also better in drug like properties. Therefore, we prepared oligosaccharide from apple polysaccharide and investigate its antitumor activity and mechanism.

2. Materials and methods

2.1. Materials

RPMI 1640 and fetal calf serum (FCS) were purchased from HyClone (Thermo Scientific, USA). The antibodies against Bcl-2 and Bcl-xl were obtained from Anbo (California, USA), and antibodies against β-actin, bax, cyclin A1, cyclin B1, and Cdk 2 were purchased from Abcam (Cambridge, UK). All other chemicals were purchased from Sigma and Merck, and were of the highest grade available.

2.2. Preparation and structure analysis of the apple oligosaccharide

Apple pomace was purchased from Xi’an yi-le Technology Co., Ltd. (Shaanxi, China), and boiled with ethanol to remove alcohol-soluble components. Then residue was separated from ethanol solution, and boiled with water to extract polysaccharides. Proteins were removed using Sefag method. After being dialyzing and lyophilizing [12], apple polysaccharide was acquired (average molecular weight was 5000–10,000 Da). Polysaccharide was suspended in 1 mol/l NaOH–EtOH–water solution, stirred at room temperature for 2 h, and precipitated completely at 4 °C. The precipitation was separated from solutions by using vacuum filtration method, and washed with 0.5 mol/l HCl–EtOH–water. Then the precipitation was suspended in 50 mmol/l sodium acetate and the pectinase (2.5 ml, 1 mg/ml, from Aspergillus niger, Fluka, USA) was added. The mixed solutions was incubated at 37 °C for 4 h, and heated to 100 °C for 10 min to terminate the enzymatic hydrolysis. After being cooled, supernatant fluid was acquired by centrifugation (5000 rpm, 5 min). An oligosaccharide was separated from the supernatant fluid by an anion chromatography column with 0.57 mol/l ammonium formate solution as eluent, the resulting product was desalted by using a column of Dewex-50W × 8 resin (H+ form, 200 mesh). The oligosaccharide-containing eluent was concentrated, dried and one fraction was obtained. The structure of this fraction was determined by infrared spectroscopy (IR) and ESIMS.

2.3. Cell culture

HT29 cells were purchased from ATCC (USA). Cells were routinely cultured in RPMI 1640 medium containing 2.05 mM l-glutamine, 100 U/ml penicillin, and 100 units/ml streptomycin. The cells were cultured as a monolayer in a humidified atmosphere containing 5% CO2 at 37 °C. The medium was supplemented with 10% FCS and changed every 24 h.

2.4. Cell viability assay

The effect of apple oligosaccharide on cellular viability was assessed by using the MTT assay [24]. HT29 cells were seeded at a density of 2000 cells per well in 96-well microtiter plates (Costar, USA) and allowed to adhere. Cells were treated with different concentrations of 5-FU or apple oligosaccharide or apple polysaccharide for 12 h, 24 h, or 36 h. At each time point, the wells were washed three times with warm phosphate buffer solution (PBS) and incubated again for another 4 h with RPMI 1640 containing 5 mg/ml of MTT. After removing the culture medium, 150 μl of DMSO was added to dissolve the precipitates and the resulting solution was measured for absorbance at 570 nm using an ELISA reader (iMark, BioRad680, USA).

2.5. Annexin V–FITC/PI staining experiment

HT29 cells were plated at a modest density (approximately 2 × 10^5 cells/well) in 6-well plates and treated with various concentrations of 5-FU, apple oligosaccharide or apple polysaccharide for 36 h. After treatment, cells were digested and washed twice with PBS. The cells were re-suspended in 200 μl of binding buffer, stained with Annexin V–FITC (20 μg/ml) and 5 μl of propidium iodide (PI, 50 μg/ml) for 30 min in the dark room, and then diluted with an additional 300 μl of binding buffer. The apoptotic index was determined by flow cytometry (BD FACsCan™, USA), and the form scatter diagram was analyzed.

2.6. Cell cycle analysis

The ratio of HT29 cells in the G0/G1, S and G2/M phases of cell cycle was determined by their DNA content. HT29 cells were treated with various concentrations of 5-FU, apple oligosaccharide or apple polysaccharide for 36 h. Cells were then harvested, washed twice with cold PBS, and fixed with 75% ice-cold ethanol overnight. Fixed cells were washed twice with cold PBS and incubated with 5 μl of 100 μg/ml RNase A for 30 min at 37 °C. After incubation, the cells were stained with 50 μg/ml PI for 30 min in the dark room and analyzed by flow cytometry. Untreated cells were used as a control.

2.7. Western blotting analysis

After HT29 cells were treated with various concentrations of 5-FU or apple oligosaccharide for 36 h, the levels of Bax, Bcl-2, Bcl-xl, cyclin B1, cyclin A1, Cdk 2, and β-actin were measured by Western blotting. Total protein from the cells was extracted in RIPA lysis buffer (Beyotime, Nanjing, China) supplemented with protease inhibitor cocktail tablets (complete ULTRA Tablets, Roche) and quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 30 μg of protein was separated using 10% SDS–PAGE and electrophoretically transferred to a NC membrane (Millipore, MA, USA). The membrane was blocked with 5% nonfat milk and incubated at 4 °C overnight with the desired primary antibody. After washing three times with PBS containing 0.05% Tween-20 (PBST), the membrane was incubated for 70 min with horseradish peroxidase-conjugated secondary antibody diluted in PBST. Protein bands were visualized using enhanced chemiluminescence (Millipore, USA) and detected using a chemiluminescence detection system (Clixn Science Instrument, China).

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Fig. 1. IR spectra (A) and ESI-MS spectra (B) of apple oligosaccharide.
The relative protein levels were normalized to β-actin as a loading control.

2.8. Real time PCR array

HT29 cells were treated as previously described. Total cellular RNA was extracted by using TRIzol reagent (Invitrogen, Beijing, China) according to the manufacturer’s instructions. The total RNA (2 μg) was reverse transcribed using reverse transcriptase (TaKaRa Biotechnology Co., Ltd., Dalian, China). The first strand cDNA was used as the template for real-time quantitative PCR analysis. The following primer sequences were used: 5′-CCGCCCATGTTGTGTCAG-3′ (forward) and 5′-CTCAGGAGGTCTTTTTCCGAG-3′ (reverse) for bcl-2; 5′-GGTGGGCTCATGTCGTTGG-3′ (forward) and 5′-CCGCCATGTTGTGTCAG-3′ (reverse) for bcl-xL; 5′-GAGGCCATGTTGTGTCAG-3′ (forward) and 5′-GCAGGCCATGTTGTGTCAG-3′ (reverse) for cyclinA1; 5′-AATGAAGCAGAATACATGCC-3′ (forward) and 5′-TTGGTAGATAATTCC-3′ (reverse) for cyclinB1; 5′-CCAGGAGGTCTTTTTCCGAG-3′ (forward) and 5′-TTGAGGCATTGCAG-3′ (reverse) for cyclinD1; 5′-CTGGAGGAGGTCTTTTTCCGAG-3′ (forward) and 5′-CTGGAGGAGGTCTTTTTCCGAG-3′ (reverse) for cdk2; and 5′-CTGGAGGAGGTCTTTTTCCGAG-3′ (forward) and 5′-CTGGAGGAGGTCTTTTTCCGAG-3′ (reverse) for the internal quantitative control β-actin. The mRNAs were detected using SYBR Green PCR Master Mix (TaKaRa Biotechnology) and an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, UK) using the comparative threshold cycle method for relative quantification. The thermal cycling conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 34 s.

2.9. Statistical analysis

All data were processed and analyzed by Sigma-Plot 10.0 and GraphPad Prism 5.0 software. The statistical significances were evaluated by t-test of the software and p < 0.05 was considered significant.

3. Results

3.1. Structural analysis of apple oligosaccharide

In this work, IR and MS were used to characterize this compound, and the results are showed in Fig. 1. The characteristic infrared absorption peak of this compound was obtained through a conventional scan (Fig. 1A). The prominent band at 3421 cm⁻¹ was assigned to the OH stretching vibrations of the alcoholic hydroxyl. The signals at 3230 and 3145 cm⁻¹ represented C–H bending vibrations. The absorptions between 1740 and 1395 cm⁻¹ were typical of a carboxyl group, with 1616 and 1402 cm⁻¹ representing the C=O stretching vibration and C–O bending vibration, respectively. The bands from 1250 to 950 cm⁻¹ were assigned to the C–O–C stretching of the glycosidic linkages of the pyranose ring and OH bending vibration. The band at 844 cm⁻¹ was characteristic of an α-(1→4)-glycosidic bond in the compound. Molecular weight of oligosaccharide was detected by ESI-MS, molecular ion peak [M–H]⁻ was 897 (Fig. 1B). These data showed that this compound was an oligosaccharide with molecular weight 898.

3.2. Cytotoxicity of apple oligosaccharide

In this study, we investigated the effect of 5-FU, apple oligosaccharide and apple polysaccharide on viability of HT29 cells, the results are showed in Fig. 2. Apple oligosaccharide and 5-FU reduced the viability of HT29 cells in concentration- and time-dependent manner. Compared with 5-FU, apple oligosaccharide showed greater cytotoxicity on HT29 cells. Meanwhile, compared with apple oligosaccharide, apple polysaccharide showed much lower cytotoxicity on HT29 cells.

3.3. Apoptosis induced by apple oligosaccharide

The apoptosis induced by apple oligosaccharide was determined by using Annexin V–FITC/PI staining and flow cytometry. Representative pictures are showed in Fig. 3, and the statistic results of percentage of apoptosis cells (percentage in Q2 plus percentage in Q4) are showed in Fig. 4. When HT29 cells were incubated with apple oligosaccharide and 5-FU for 36 h, both of apple oligosaccharide and 5-FU induced cell apoptosis in concentration-dependent manner. Compared with 5-FU and apple polysaccharide, apple oligosaccharide induced more apoptosis on HT29 cells. The results also showed that 0.01 mg/ml and 0.1 mg/ml apple polysaccharide did not induce any apoptosis, when its concentration increased to 1 mg/ml, apple polysaccharide induced the apoptosis on HT29 cells. This result was consistent with the cytotoxicity of apple oligosaccharide, 5-FU and apple polysaccharide on HT29 cells.

3.4. The effect of apple oligosaccharide on cell cycle

Cell cycle progression is a complex and highly ordered process, which can be divided into mitosis (M phase) and inter phase [25]. The effect of apple oligosaccharide on cell cycle was investigated on HT29 cells. After the cells were treated with apple oligosaccharide, apple polysaccharide or 5-FU for 36 h, their distribution...
Fig. 3. The apoptosis induced by apple oligosaccharide, apple polysaccharide and 5-FU in HT29 cells. Cultures of HT29 cells were treated with different concentration of apple oligosaccharide (OS), apple polysaccharide (PS) and 5-FU for 36 h. Cells were harvested by trypsinization and centrifugation, then analyzed in a Becton Dickinson FACScan (excitation at 488 nm) equipped with Cell Quest software after staining with annexin V-FITC and propidium iodide. Results shown are of an experiment representative of apoptosis. Q1 showed that cells were undergoing necrosis, and Q2 showed that cells were at the end stage of apoptosis. Q3 showed that cells were viable, or there were no measurable apoptosis. Q4 showed that cells were undergoing apoptosis.
in different phases of the cell cycle is illustrated in Table 1. Representative pictures are shown in Fig. 5. The results showed that the percentage of cells in the G1 phase was reduced by incubating with apple oligosaccharide or 5-FU. This reduction was accompanied by an increase in the proportion of cells in the S phase in concentration-dependent manner. Apple oligosaccharide and 5-FU showed the same extent effects on cell cycle. Meanwhile, 0.01 mg/ml and 0.1 mg/ml apple polysaccharide did not induce cell cycle arrest in HT29 cells, when its concentration increased to 1 mg/ml, apple polysaccharide induced the cell cycle arrest in S phase.

It was found that that LMWAP induced a G0/G1-cell cycle block in HT29 cells in concentration-dependent manner and protected ICR mice against colitis-associated CRC induced by AOM/DSS. One of the possible mechanism of LMWAP inhibiting the growth of CRC involved that it induced a cell-cycle block in a p53 independent way. Another possible anti-CRC mechanism of LMWAP is that it competitively blocked the binding of the tumor-associated antigens to galectin-3 [26]. Meanwhile, LMWAP decreased the protein levels of COX-2, this led to the decrease of PGE2 level. It has been reported that when PGE2 couple with prostaglandin E receptors, the cell-cycle regulatory protein expression levels including Cdk-2 finally is increased [27,28]. Thus LMWAP inhibit the expression of cell-cycle regulatory protein through COX-2-PGE2 pathway.

3.5. Apoptosis related and cell cycle related protein expression after treatment with apple oligosaccharide

The Bcl-2 gene family plays an important role in anti-apoptosis. The pro-apoptosis protein Bax promotes cell death, Bcl-2 and Bcl-Xl control the activity of Bax [29]. In addition, the cell cycle of eukaryotic cells is regulated by two important components, cyclins and cyclin-dependent kinases (Cdk)s. Different cyclins reach their maximum activity during different phases of the cell cycle. The activity of these heterodimeric complexes of cyclins and the corresponding Cdk drive proper progression of the cell cycle.

In order to investigate the mechanism that apple oligosaccharide induce apoptosis and cell cycle arrest on HT29 cells, we determined the expression of the apoptosis and cell cycle associated proteins using Western blotting after HT29 cells were treated with apple oligosaccharide or 5-FU for 36 h. The results are shown in Figs. 6 and 7. Bax expression was enhanced after HT29 cells were treated with apple oligosaccharide or 5-FU. Meanwhile, the

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**Table 1**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Control</th>
<th>5-FU (μg/ml)</th>
<th>Oligosaccharide (μg/ml)</th>
<th>Polysaccharide (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1</td>
<td>0.013</td>
<td>0.014</td>
<td>0.68 ± 0.63</td>
<td>0.69 ± 0.63</td>
</tr>
<tr>
<td>G1</td>
<td>0.12</td>
<td>0.12</td>
<td>0.63 ± 0.63</td>
<td>0.64 ± 0.63</td>
</tr>
<tr>
<td>S</td>
<td>0.12</td>
<td>0.12</td>
<td>0.59 ± 0.59</td>
<td>0.60 ± 0.59</td>
</tr>
<tr>
<td>G2/M</td>
<td>0.32</td>
<td>0.32</td>
<td>0.28 ± 0.28</td>
<td>0.29 ± 0.28</td>
</tr>
</tbody>
</table>

* p < 0.01 vs control.
Fig. 5. The effect of apple oligosaccharide, apple polysaccharide or 5-FU on cell cycle in HT29 cells. HT29 cells were treated with different concentration of 5-FU, apple polysaccharide or apple oligosaccharide for 36h. At the end of treatment, cells were trypsinized, incubated with RNase, stained with propidium iodide (PI), and analyzed by flow cytometry. Results shown are of an experiment representative of cell cycle.
expression of the antiapoptosis proteins Bcl-2 and Bcl-xl was suppressed after HT29 cells were treated with apple oligosaccharide or 5-FU. These results indicated that apple oligosaccharide or 5-FU induced apoptosis in HT29 cells by modulating Bax, Bcl-2 and Bcl-xl protein expression.

After HT29 cells were treated with apple oligosaccharide or 5-FU for 36 h, the expression of cyclin B1 and Cdk 2 was significantly down-regulated. The expression of cyclin A1 was up-regulated but no significant difference when apple oligosaccharide concentration was less than 1 μmol/l. When apple oligosaccharide concentration increased to 10 μmol/l, the cyclin A1 expression was significantly up-regulated. 5-FU did not show this characteristic. In addition, apple oligosaccharide showed a greater effect on cell cycle than 5-FU did. These data indicated that the mechanism of cell cycle arrest in the S phase was related with the inhibition the formation of the Cdk 2–cyclin A1 complex by decrease of Cdk 2 expression.

### 3.6. Changes in mRNA expression after apple oligosaccharide treatment

To determine the possible mechanisms that apple oligosaccharide triggered apoptosis and blocked the cell cycle progression in HT29 cells, the mRNA expression of various genes was assessed using real time PCR. The results are shown in Fig. 8. The changes in mRNA level after HT29 cells were treated with apple oligosaccharide were consistent with the results that were observed by Western blotting method. mRNA levels of bcl-2, bcl-xl, Cdk 2 and cyclin B1 were decreased. HT29 cells did not undergo significantly changes in cyclin A1 expression after oligosaccharide or 5-FU treatment. The changes of bax in mRNA level are obviously observed when apple oligosaccharide concentration was 1 μmol/l and 10 μmol/l, 5-FU did not show this characteristic.
**Fig. 7.** Effects of apple oligosaccharide or 5-FU on the expression of cell cycle associated protein. HT29 cells were treated with oligosaccharide or 5-FU for 36 h, and the protein expression levels were estimated by Western blotting. Each bar represents the mean ± SD of three independent experiments. *p < 0.05 or **p < 0.01 vs control.

**Fig. 8.** mRNA level changes after HT29 cells were treated with apple oligosaccharide. The mRNA expression levels were estimated using real time PCR. Each bar represents the mean ± SD of three independent experiments. *p < 0.05 or **p < 0.01 vs control.
4. Conclusion

We conclude from the study that apple oligosaccharide inhibited the HT29 cells viability by inducing apoptosis and cell cycle arrest in S phase, this is different from what LMWAP showed. Apple oligosaccharide induced apoptosis in HT29 cells by modulating Bax, Bcl-2 and Bcl-xl protein expression. Apple oligosaccharide caused cell cycle arrest in the S phase in HT29 cells was related with the inhibition of the formation of the Cdk2-cyclin A1 complex by decreasing Cdk 2 expression. Apple oligosaccharide is a potential chemoprevention agent or anti-tumor agent. However, the antitumor mechanisms of apple oligosaccharide still need further clarification.

Conflict of interest statement

The authors declare that they have no competing interests.

Authors’ contributions

Qian Li, Qibing Mei, Siyuan Zhou, and Li Liu conceptualized and guided the research project. Qian Li, Juan Jing, Shuangyan Duan, Siyuan Zhou, Qibing Mei, and Li Liu performed the experiments. Qian Li, Siyuan Zhou, Tiehong Yang, Zhongfu Wang, Qibing Mei, and Li Liu analyzed the data. Qian Li, Siyuan Zhou, Qibing Mei, and Li Liu wrote the manuscript. All authors approved the final manuscript.

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