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Effect of chitooligosaccharides on *cyclin D1*, *bcl-xl* and *bcl-2* mRNA expression in A549 cells using quantitative PCR

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Chitooligosaccharide (COS) is derived from the chemical and enzymatic hydrolysis of chitosan and has been reported to have potent antitumor activity. Our study investigated the effects of five chitooligomers ranging from the dimer form to the hexamer form (chitobiose, chitotriose, chitotetraose, chitopentaose, chitohexaose) on the expression of *cyclin D1*, *bcl-2* and *bcl-xl* mRNA in A549 cells using reverse transcription quantitative real-time PCR. We demonstrated that, of the five chitooligomers used, chitohexaose (COS6) had the most potent inhibitory effect on A549 cell proliferation. COS6 also significantly down regulated *cyclin D1* and *bcl-xl* mRNA expression levels. Our data suggested that COS6 exerts its antitumor activity by two different mechanisms: (1) COS6-mediated inhibition of Cyclin D1 levels leads to suppression of tumor cell proliferation; and (2) COS6-mediated down-regulation of the pro-survival protein, Bcl-xl, promotes the apoptosis of tumor cells.

chitooligosaccharides, *cyclin D1*, *bcl-xl*, *bcl-2***

Chitooligosaccharide (COS) is derived from the chemical or enzymatic decomposition of chitin or chitosan [1]. COS has been reported to have a lower molecular weight and a higher water solubility and activity when compared with chitosan. A number of studies have suggested that COS has antimicrobial [2], antibacterial [3] and antioxidant properties [4,5], and has also been shown to play an important role in antitumor responses [6,7].

The antitumor activity of COS is likely influenced by its chemical structure, molecular size, and strong electric charge [6–8]. Administration of N-acetylchitohexaose (NACOS-6) resulted in a significant increase in the candidacidal activity of macrophages and T lymphocytes in the early phase of tumor development [8] and COS was suggested to play a role in the induction of apoptosis of carcinoma cells via up-regulation of *Bax* [9]. The chitohexamer form was shown to down-regulate *VEGF* and *uPA* mRNA expression levels in ECV304 cells [10], and to inhibit tumor growth and metastasis by up-regulation of *p21* and *MMP-9*, and down-regulation of *PCNA*, *cyclin A* and *cdk-2* [11]. However, the effects of different forms of COS on the expression levels of cyclin D1 and the pro-survival proteins bcl-2 and bcl-xl are unknown. In this study, we investigated the role of five different fractions [chitobiose (COS2), chitotriose (COS3), chitotetraose (COS4), chitopentaose (COS5), chitohexaose (COS6)] on the regulation of *cyclin D1*, *bcl-2* and *bcl-xl* mRNA levels in A549 cells. We demonstrated that COS6 exhibited the most potent inhibitory activity on A549 cell proliferation by down-regulation of *cyclin D1* mRNA. Treatment of A549 cells with COS6 also resulted in suppression of *bcl-xl* mRNA expression levels.

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1 Materials and methods

(i) Materials. RPMI 1640 medium and fetal calf serum (FCS) was purchased from GIBCO, USA. Chitooligomers (chitobiose, chitotriose, chitotetraose, chitopentaose and chitohexaose) were from Dalian GlycoBio Co. Ltd. (China). Trizol reagent, M-MLV Reverse Transcriptase and the Platinum SYBR Green qPCR SuperMix-UDG kit were all from Invitrogen (Carlsbad, CA, USA). All primers were made by Sangon (Shanghai, China).

(ii) Cell culture. The A549 human lung carcinoma cell line was cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS, and maintained at 37°C/5% CO_2. Cells were treated with different chitooligomers (100 μg/mL) after serum starvation for 12 h.

(iii) Cell proliferation assay. The effect of the different COS fractions on proliferation of A549 cells was determined by the MTT assay. Cells cultured in 96-well plates were treated with different COS fractions (100 μg/mL) for 48 h at 37°C. A 20 μL volume of MTT (5 mg/mL; Sigma) was added to each well and cells were incubated for another 4 h before adding 120 μL DMSO to each well to dissolve the blue crystals. The absorbance was read at 490 nm using an MK3 microplate reader (Thermo Electron Corporation, Shanghai, China).

(iv) RNA extraction and reverse transcription. A549 cells were treated with chitooligomers (100 μg/mL) for 48 h, and total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using 20 U of M-MLV Reverse Transcriptase and Oligo(dT)$_{18}$ primers according to the manufacturer's instructions (M-MLV First Strand Kit; Invitrogen). The reaction was incubated at 37°C for 50 min and then 70°C for 15 min.

(v) Quantitative real-time PCR (qPCR). The amplification of cDNA amplification was performed by qPCR using the Platinum SYBR Green qPCR SuperMix-UDG kit according to the manufacturer's instructions. Briefly, a mix of the following reaction components was prepared: 1 μL cDNA; 12.5 μL 2× Platinum SYBR Green qPCR Super-Mix-UDG; 2.5 μL (0.2 μM) of each primer and 6.5 μL water. The forward and reverse primer sequences for each gene

product are listed in Table 1. Data were acquired with the Chromo4 Real-Time detector (MJ Research). Gene expression was normalized to the endogenous expression of β-actin, which was determined not to have significantly changed in the different experiments. The PCR cycling conditions involved an initial denaturation step at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 60 s, then completed with a melting curve from 55–95°C with a heating rate of 1°C/s and continuous fluorescence measurement. For analysis, a standard curve was constructed from β-actin amplification in two serial 10-fold dilutions of cDNA stock from untreated A549 cells. The target cDNA levels in each sample were quantified against a standard curve as sample molecule units and were normalized to the sample β-actin units. The concentration of the target gene was expressed as arbitrary units and calculated according to the following formula: relative target molecule production units = sample target molecule units/sample β-actin units.

(vi) Statistical analysis. Statistical results were obtained using the statistical software SPSS 13.0. The two sample *t*-test was used for statistical analysis. Values of $P < 0.05$ were considered significant.

2 Results

2.1 Effects of different chitooligomers on A549 cell proliferation

We used the MTT assay to analyze the effects of different chitooligomers on A549 cell proliferation. Cells were incubated with different chitooligomers [COS2–COS6 (100 μg/mL)] for 48 h. Control cells were incubated in the absence of COS. The results were presented as mean ± standard deviation (SD) of the data from six independent experiments. We demonstrated (Figure 1) a significant inhibition ($n = 6$, $p < 0.05$) of proliferation in A549 cells treated with COS5 (0.426 ± 0.037) or COS6 (0.376 ± 0.029) when compared with untreated control cells (0.574 ± 0.056). However, there was no significant change in the proliferation of A549 cells treated with COS3 (0.536 ± 0.052) or COS4 (0.557± 0.084).

Table 1 Sequences of primers used in qPCR

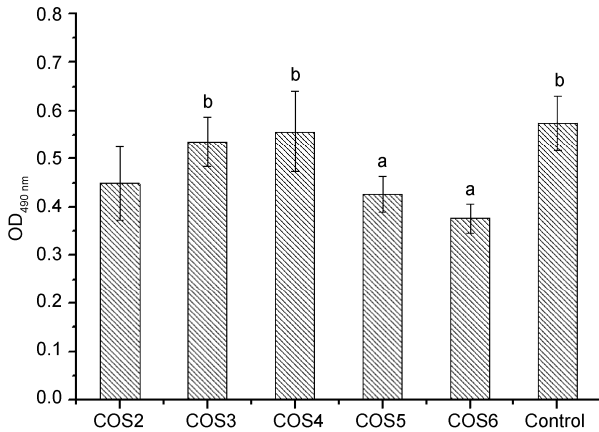| Gene	Primer sequences
cyclin D1 [12]	Forward: 5′-CCGTCCATGCGGAAGATC-3′ Reverse: 5′-GTCACACTTGATCACTCTGG-3′
bcl-2 [13]	Forward: 5′-CATGTGTGTGGAG AGCGTCAA-3′ Reverse: 5′-GCCGGTTCAGGTACTCAGTCA-3′
bcl-xl [13]	Forward: 5′-TCCTTGTCTACGCTTTCCACG-3′ Reverse: 5′-GGTCGCATT GTGGCCTTT-3′
β-actin [14]	Forward: 5′-GATGACCCAGATCATGTTTGA-3′ Reverse: 5′-ATGAGGTAGTCAGTCAGGTCC-3′

Figure 1 Effects of chitooligomers on the proliferation of A549 cells (a, $P < 0.05$ *vs* control, b, $P < 0.05$ vs. COS6 group).
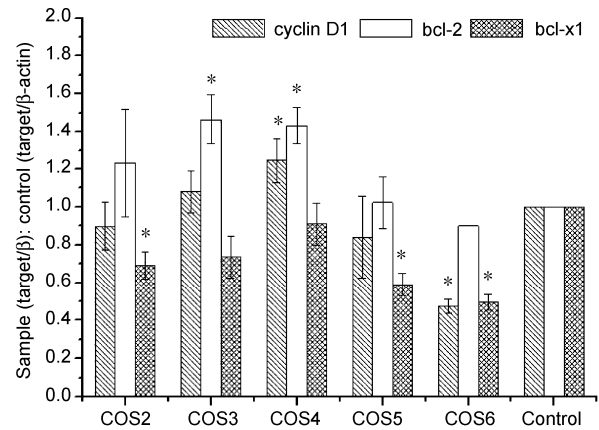

Figure 2 Effect of COS oligomers on the expression of *cyclin D1*, *bcl-xl* and *bcl-2* mRNA in A549 cells, (*, $P < 0.05$ vs. control).

2.2 Standard curve for RT-qPCR

The cDNA template was serially diluted for the standard curve to cover the expected concentrations of the target genes in all samples. The standard curves were analyzed using Real Quant Software.

2.3 COS6 down-regulated *cyclin D1*, *bcl-xl* and *bcl-2* gene expression

We explored the effects of different COS oligomers on *cyclin D1*, *bcl-xl* and *bcl-2* mRNA levels in A549 cells. Cells seeded in 6-well plates were treated with different COS oligomers (100 μg/mL) for 48 h and total RNA was prepared. After reverse transcription, cDNA samples were subjected to qPCR and results were expressed as the target:reference ratio of the samples divided by the target: reference ratio of the control ($n = 4$). β-actin cDNA was the reference gene used in all the experiments.

We demonstrated that different COS oligomers exerted different effects on the expression of *cyclin D1*, *bcl-xl* and *bcl-2* mRNA in A549 cells (Figure 2). We demonstrated suppression of *bcl-xl* mRNA levels in all COS-treated cells except the COS4 treated group (COS2: 0.691 ± 0.073; COS3: 0.734 ± 0.109; COS4: 0.910 ± 0.110; COS5: 0.589 ± 0.061; COS6: 0.493 ± 0.042), when compared with untreated cells. However, the different COS oligomers did not suppress *bcl-2* mRNA levels to the same extent as *bcl-xl*. Of all the COS oligomers, COS6 had the strongest suppressive activity, and most potently down-regulated *cyclin D1* (0.473 ± 0.037) mRNA levels in treated cells when compared with control cells ($p < 0.05$ *vs* control; Figure 2). We showed a significant upregulation in *cyclin D1* (1.245 ± 0.118) and *bcl-2* (1.431 ± 0.096) mRNA levels in COS4 treated cells while there was no significant difference in the expression levels of these tumor related genes in COS2 and COS5 treated cells when compared with untreated control cells.

3 Discussion

Malignant transformation of cells in most cancers is characterized by dysregulation of the cell cycle and inhibition of cell death. Overexpression of cyclin D1, Bcl-2 or Bcl-xl in a number of cancers, including lung, prostate, colorectal, breast, cervical, non-Hodgkin's lymphoma, and both acute and chronic leukemia, has been reported to contribute to increased resistance to chemotherapy. The *cyclin D1* gene, located on chromosome 11q13, is an important regulator of the cell cycle, which interacts with cyclin dependent kinases (CDKs) 4 and 6 to form a CDK 4/6-cyclin D complex. This complex phosphorylates the retinoblastoma tumor suppressor gene product (pRb), which forms an inhibitory complex with a group of transcription factors known as E2F-DP (E2F-1, -2 and -3), and functions to control the G_1/S transition point of the cell cycle [15–17]. Hyperphosphorylation of pRb by the Cyclin D1-CDK complex causes a dissociation of pRb from the E2F inhibitory complexes, permitting progression of the cell cycle. Many tumors evade apoptotic death signals by expressing anti-apoptotic proteins, such as the pro-survival Bcl-2 family members, Bcl-2 and Bcl-xl. These proteins are characterized by the presence of four Bcl-2 homology (BH) domains, which are required for their anti-apoptotic functions. The BH1–BH4 domains mediate interactions of these proteins with other protein partners, resulting in localization of these proteins at the cytoplasmic surfaces of intracellular membranes. The BH1–BH3 domains form a hydrophobic groove, while the N-terminal BH4 domain stabilizes this structure from behind the groove by burying additional hydrophobic residues, which would otherwise be exposed. The hydrophobic groove of Bcl-2 or Bcl-xl binds the BH3 α-helix of a pro-apoptotic Bcl-2 family member, such as Bax or Bak, inhibiting their binding to membranes, and uncovering the C-terminus to trigger the apoptotic cascade [18–20]. Overexpression of cyclin D1, Bcl-2 and Bcl-xl is strongly associated with tumorigenesis,

tumor proliferation, lymph node metastasis and prognosis of the cancer. They are valuable molecular markers in the early stages of cancer and are important molecular targets in tumor therapy [15,18].

In this study, we explored the effect of five COS fractions, ranging from the dimer form to the hexamer form, on *cyclin D1*, *bcl-2* and *bcl-xl* mRNA levels. Our results showed that COS6 was the most potent inhibitor of A549 cellular proliferation. Of all the chitooligomers, COS6 most potently down-regulated the mRNA levels of *cyclin D1* and *bcl-xl* mRNA. The data suggested that COS6 exerted its antitumor activity through inhibition of Cyclin D1 expression, which resulted in suppression of tumor cell proliferation, and inhibition of the expression of the pro-survival protein, Bcl-xl, which resulted in increased apoptosis of tumor cells.

4 Conclusions

We investigated the mechanisms underlying the anti-tumor effects of five COS fractions. Our results demonstrated that COS inhibited the proliferation of A549 cells. COS6 also suppressed *cyclin D1* and *bcl-xl* mRNA expression in these cells. We demonstrated that of the five fractions, COS6 was the most potent inhibitor of cell proliferation as well as *cyclin D1* and *bcl-xl* mRNA levels. COS6-mediated anti-tumor activity may be initiated *via* its binding with the cell surface receptor, or *via* endocytosis into the intracellular environment; however the exact mechanisms and signaling pathways underlying this function remain to be further elucidated.

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