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Anti-tumor effects of proteoglycan from *Phellinus linteus* by immunomodulating and inhibiting Reg IV/EGFR/Akt signaling pathway in colorectal carcinoma

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ABSTRACT

Proteoglycan (P1) purified from *Phellinus linteus* has been reported to have anti-disease activities. The objectives of our research were to determine the anti-tumor effect and possible mechanisms of P1 on human cancer cells. Cell inhibition assay showed that P1 has an antiproliferative effect on HepG2, HT-29, NCI-H 460 and MCF-7 human colon cancer cells, especially it was very effective in inhibiting HT-29 cells. When HT-29-bearing mice were treated with P1(100 mg/kg), there was relative increase in spleen and thymus weights, the plasmatic pIgR and IgA levels were significantly increased, also there was a notable decrease in plasmatic PGE2, Reg IV, EGFR and Akt concentrations measured by ELISA. RT-PCR analysis suggested that P1-induced HT-29 apoptosis appeared to be associated with a decrease in the levels of expression of Reg IV and EGFR. These results suggest that P1 might have two potential roles in treating cancer; it acts as an immunopotentiator partly through protecting T cells from escaping PGE2 attack and enhancing the mucosal IgA response, and as a direct inhibitor by disrupting the Reg IV/EGFR/Akt signaling pathway.

1. Introduction

Phellinus linteus (PL), is a kind of mushroom that grows mainly on wild mulberry tree trunks, has been used as a traditional medicine in China, Korea, Japan and other Asian countries for treatment of various diseases, including oral ulcer, gastroenteric disorder, lymphatic disease and various cancers. Today, PL draws greater attention by more and more people because of its anti-tumor properties. Polysaccharides and proteoglycans extracted from PL are thought to be the active ingredients involved in anti-tumor effect [1]. To investigate the potential anticancer mechanisms of these polysaccharides, a large number of studies have focused on the functional activations of immune cells. It has been reported that polysaccharides and proteoglycans extracted from PL could stimulate the proliferation of T lymphocytes and the humoral immune function, including acting as a polyclonal activator on B cells, thereby inducing the secretory and cellular macrophage response [2,3]. In recent years, polysaccharides isolated from PL have also been shown direct antitumor effects through apoptosis and blocking cell cycle progression, adhesion and invasion of human colon cancer cells [3,4]. These results suggested that the effects of these polysaccharides not only are the immunomodulatory activity, but also could directly act on the tumor cells.

Although the antitumor effect of PL extract is apparent, the underlying mechanism(s) is still not clear. In this study, to explore the underlying mechanism(s) of the anticancer effects for this famous traditional medicine, a novel heteropolysaccharide named 'P1' was isolated from PL by HPLC isolation method and its effect was studied. Further experiments demonstrated that P1 could inhibit cellular proliferation of HT-29 cells by down regulating the expression of Reg IV and EGFR genes, and up-regulating pIgR mRNA level. In HT-29-bearing model mice, the significant changes of plasma biochemical parameters show that P1 inhibited colorectal carcinoma by not only by enhancing the immune response of T cells and IgA, but disrupting the Reg IV/EGFR/Akt signaling pathway as well.

2. Materials and methods

2.1. Preparation of the polysaccharide (P1)

Fresh-fruiting bodies of *P. linteus* (PL) were collected from the mulberry trees on the Tong-lu Mountain in Zhejiang province of China, authenticated by Lin Hong, Department of Botany, Zhejiang Academy of Agricultural Science. The dried mushroom powder was defatted with ethanol and ethyl acetate three times at room temperature. Then it was extracted three times with boiling water for 2 h, concentrated and treated with three volumes of ethanol. The

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deposit collected was redissolved in distilled water and further purified using HPLC (Waters, USA). Its purity was more than 95% based on HPLC analysis (column: Ultrahydrogel™ Linear, mobile phase: water; flow rate: 0.6 ml/min; detection: 2414, and temperature: 50 °C). The purified solution of polysaccharide (P1) was collected, concentrated and lyophilized to dryness by low pressure evaporation at −50 °C.

2.2. Analysis of polysaccharide and protein contents

The protein content was measured by Lowry's method using bovine serum albumin as the standard protein [5]. The average molecular weight was determined by HPLC using a Waters 600 HPLC system, a Model 600 pump, a Waters 2410 RI detector, a Waters 2487 dual wavelength absorbance detector and an on-line de-gasser. In order to analyse the monosaccharide compositions, P1 was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100 °C for 2 h, and the monosaccharides were identified by high-performance anion-exchange chromatography (HPAEC) using a Dionex LC30 equipped with a CarboPac™PA20 column (3 mm × 150 mm) [6,7].

2.3. Cell culture media and supplements

HepG2 (hepatocellular carcinoma), HT-29 (colon carcinoma cell), NCI-H460 (lung cancer cell), MCF7 (breast cancer cell) and L-929 (mouse fibroblast cell) cells were obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences and grown in RPMI 1640 containing 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. P1 was dissolved with 0.1% DMSO and adjusted to final concentrations with culture medium before use. RPMI 1640 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). RevertAid™ First-Strand cDNA Synthesis Kits were purchased from Fermentas (Lithuania). Cisplatin (CDDP) was purchased from Kunming Gui-Yan Pharmaceutical Co. Ltd. All other chemicals were of the highest purity available.

2.4. Cell inhibition assay

The inhibitory effects of P1 on the cells (HepG2, HT-29, NCI-H 460, MCF-7 and L-929) were evaluated by the analysis of viable cells number determined with a MTT-based colorimetric assay [8]. Briefly, the cells were cultured in RPMI 1640 complete medium. Sterilized test samples were added to a 96-well plate, containing all tested cells (1×10^5 cells/well) to give a final concentration of P1 (15.625, 31.25, 62.5, 125, and 250 μg/ml), DMSO was used as negative control, and CDDP was used as a positive control. After cultivation for 48 h at 37 °C in a humidified 5% CO_2 incubator, the percentage of viable cells was determined by MTT assay, reading absorbance at 570 nm with a Benchmark microplate reader (Bio-Rad, California). The inhibitory rates of cells were calculated by the following formula: %Inhibitory rate = 1 − (mean absorbency in test wells)/(mean absorbency in control wells) × 100%.

2.5. HT-29 cell proliferation assay

Cells were plated at a density of 1×10^5 cells/well on 24-well plates with various final concentrations of P1 (16.125 and 32.25 μg/ml) and cultured up to 96 h. At the indicated time points after the treatment with P1, the number of viable cells was determined by trypan blue exclusion at 24, 48, 72, and 96 h.

Table 1
Primers used in quantitative real-time reverse transcription-PCR.

Primer	Sequence 5′–3′	PCR product size (bp)
DDX32-F	AGCAAACACAAGGTGCAGCA	106
DDX32-R	AGACCGTCCTGTGGATGTGAA	
MCM2-F	TGTGATCGAAGACGACGTCAA	101
MCM2-R	CAAAAGTCTTGCGCATGCTG	
PIgR-F	AGCCGTCTATGTGGCAGTTGA	110
PIgR-R	CCCGAAAACCAGAGTCTAGCA	
RegIV-F	GCCAACACTTCCTGTGCAAGT	105
RegIV-R	GCAGATTTAGCCAGGCTAGCA	
EGFR-F	ATAGACGCAGATAGTCGCCCA	106
EGFR-R	GCATTCTTTCATCCCCCTGA	
β-Actin – F	CCATCATGAAGTGTGACGTGG	102
β-Actin – R	TCTGCATCCTGTCGGCAAT	

Key-DDX32: DEAH (Asp-Glu-Ala-His) box polypeptide 32, MCM2: minichromosome maintenance protein 2, pIgR: polymeric immunoglobulin receptor, RegIV: regenerating gene IV, EGFR: epidermal growth factor receptor.
F: forward primer, R: reverse primer, bp: base pairs (length of nucleic acid sequence).

2.6. Quantitative real-time reverse transcription-PCR analysis

Cells were plated at a density of 1×10^6 cells/well on 24-well plates with final concentrations of P1 (16.125 and 32.25 μg/ml) and cultured up to 48 h. At the indicated time points after the treatment, total RNA from HT-29 cells was extracted using TRIZOL reagent according to the supplier's instruction. RNA was quantitated by optical density measurement at 260 and 280 nm using a spectrophotometer, and integrity was confirmed by running 4 μl RNA on a 1.2% agarose gel.

The PCR primers of MCM2 (minichromosome maintenance protein 2), DDX32 (DEAH (Asp-Glu-Ala-His) box polypeptide 32), pIgR (polymeric immunoglobulin receptor), Reg IV (regenerating gene IV), EGFR (epidermal growth factor receptor) and β-actin were synthesized by Shanghai Shenergy Biocolor BioScience and Technology. The sequences of the primers used in this study are shown in Table 1. Reverse-transcription (RT) was performed from total cellular RNA using RevertAid™ First-Strand cDNA Synthesis Kit for RT-PCR, and carried out according to the method of Li et al. [9].

2.7. In vivo anti-tumor activity test and PGE2, pIgR, IgA, Reg IV, EGFR and Akt levels in plasma

Female BALB/c-nu/nu mice (6 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. All mice were housed under controlled conditions (a 12 h light/12 h dark cycle, 60% humidity and 25 ± 1 °C) with free access to standard pellet diet and water. 50 mice were divided into 5 groups. Except the normal control group, 4 groups were inoculated 0.2 ml of HT-29 cells suspension (2.5×10^7 cells/ml) at the nape subcutaneously to establish the colorectal carcinoma model mice at day 0. Test sample P1 (50 and 100 mg/kg), and 0.2 ml saline (as negative control) were administered intragastrically everyday, CDDP (2 mg/kg, as positive control) was injected into peritoneal cavity every other day. After the tumor inoculation, the maximal (a) and minimum (b) diameters of solid tumors were measured with vernier caliper every week, and the tumors volume calculated as $V = a \times b^2/2$. Compared with the control group, the anti-tumor activity of the tested samples was expressed as an inhibition ratio (percent) calculated as $((A - B)/A) \times 100\%$, where A and B are the average tumor volume of the control and treated groups. To determine the potential toxicity of P1, the body weights of mice were determined every week. At the end of experiment, plasma were collected from the tumor-bearing mice and measured using murine enzyme-linked immunosorbent assay (ELISA) kits (Purchased from R&D Systems (USA)) for PGE2, pIgR, IgA, Reg IV, EGFR and Akt according to the manufacturer's instruction. Spleen and thymus were excised from the animal and

weighed immediately. The spleen/thymus index was expressed as the spleen (thymus) weight (mg) relative to body weight (g). All animals used in the current study were handled and treated in accordance with guidelines of National Institutes of Health.

2.8. Statistical analysis

Results are reported as means ± S.D. ANOVA was used to evaluate the difference between multiple groups. If significance was observed between groups, a Duncan's multiple range test (DMRT) was used to test the means of two specific groups by a commercially available statistics software package (SPSS for Windows, V. 12.0, Chicago, USA), with $P < 0.05$ considered as significant.

3. Results

3.1. Investigation of the proteoglycan

The P1 was isolated from precipitated polysaccharide (crude proteoglycan fraction) of the *P. linteus*. P1 appeared as a symmetrical peak on HPLC and the average molecular weight was about 18.8 kDa. The monosaccharide analysis showed that it was composed of L-fucose, D-rhamnose, D-galactose, D-glucose, D-xylose and D-mannose, and a novel monosaccharide 3-O-Me-D-galactose was detected, which is similar to the report by Yang [6]. The corresponding molar ratios for the monosaccharides above were approximately 1:3.12:33.51:2.03:4.03:1.09:2.87 and the protein content was 8.45% in the extraction.

3.2. Inhibitory effects of P1on cancer cells and its cytotoxicity to normal cells

The inhibitory effects of P1 were tested on HepG2, HT-29, NCI-H 460 and MCF-7 human colon cancer cells under different dosages of 15.625, 31.25, 62.5, 125, and 250 μg/ml in vitro. As shown in Fig. 1A, a significant inhibition of P1 in all the cancer cells were observed at high dose of 250 μg/ml and the highest inhibition ratios were 88.91%, 92.79%, 79.71% and 73.99%, respectively. Meantime, P1 also exhibited a significant cytotoxicity against normal cells (L-929), the inhibition ratio was as high as 83.72%. However, when the dose was under 62.5 μg/ml, cytotoxicity to the normal cells (L-929) was not significant (inhibition ratio fewer than 10.0%). But no significant inhibitory activity was also observed to cancer cells at the low doses (under 62.5 μg/ml) except for HT-29 cells. The standard reference drug (CDDP) exhibited a significant dose-dependent inhibition of cell proliferation in all cell lines including the normal cells (L-929) (Fig. 1B). So the P1 may have a specific concentration range to treat colorectal carcinoma, and the proliferation of cells HT-29 was further investigated.

3.3. Effect of P1 on HT-29 cell proliferation

To explore the inhibitive effect and its mechanism(s) of P1 on HT-29 cell proliferation at non-toxic dose levels, P1 was added to the HT-29 cell culture medium maintained for 24, 48, 72, and 96 h, respectively. P1 treatments showed significant inhibition of HT-29 cell proliferation in the presence of 15.625 and 31.25 μg/ml (Fig. 2). The growth morphology of cells HT-29 treated with P1 for 48 h is shown in Fig. 3 (×100) after P1-treatment for 48 h. The control cells showed normal karyokinesis and proliferation as exponential growth (Fig. 3 A), the proliferations were inhibited and the number of the cells was lower evidently than control ($P < 0.01$) after treatment with P1 (15.625 and 31.25 μg/ml) and CDDP (0.625 μg/ml) (Fig. 3B–D). These results suggest that P1 may have a direct antitumor activity on HT-29 cells.

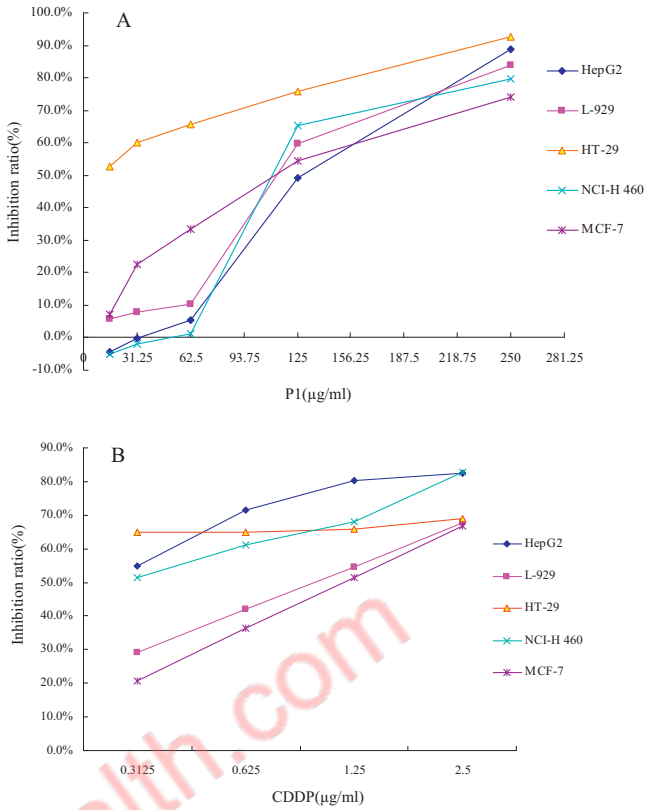

Fig. 1. Effect of P1 and CDDP on the proliferation of HepG2, HT-29, NCI-H 460, MCF-7 and L-929 cells. Cells were plated at a density of 1×10^5 cells/well on 96-well plates with various concentrations of P1 (15.625, 31.25, 62.5, 125, and 250 μg/ml) (A) and CDDP (0.3125, 0.625, 1.25, and 2.5 μg/ml) (B), and then cultured up to 48 h. After indicated time of culture, cell numbers were determined by MTT colorimetric assay. Data are mean ± S.D. of three repeated experiments.

3.4. Expression of MCM2, DDX32, pIgR, Reg IV and EGFR mRNA in HT-29 cells

Recent findings have demonstrated that many functional gene expression are frequently abnormal in various gastrointestinal tumors, in which the expression of MCM2 [10], DDX32 [11], Reg IV [12] and EGFR [13] are up-regulated, while pIgR is down-regulated in colorectal cancer cells, adenoma and carcinoma tissues [14]. In this study, to validate the inhibitory mechanism(s), the mRNA gene levels of MCM2, DDX32, pIgR, Reg IV and EGFR in the HT-29 cells were detected by quantitative real-time reverse transcription-PCR.

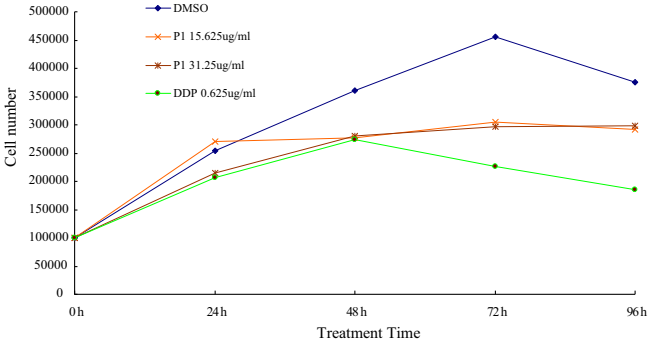

Fig. 2. The antiproliferative effect of P1 in HT29 cells. Cells were plated at a density of 1×10^5 cells/well on 24-well plates and treated with various concentrations of P1 (15.625 and 31.5 μg/ml) and CDDP (0.625 μg/ml) for 24, 48, 72 and 96 h. After treatment, cell viability was estimated by trypan blue dye exclusion method. Data are mean ± S.D. of three repeated experiments.

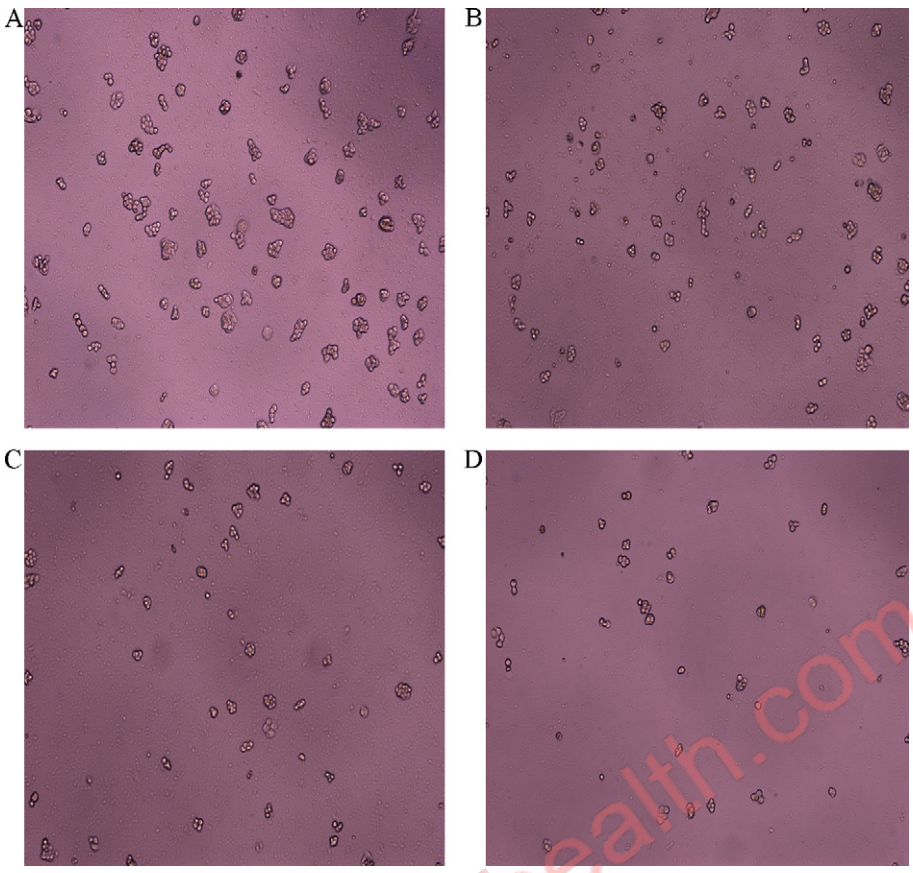

Fig. 3. The growth morphology of cells. HT-29 cells observed with a light microscope (×100) after P1 and CDDP treatment for 48 h. (A) DMSO; (B) P1 (15.625 μg/ml); (C) P1 (31.25 μg/ml); (D) CDDP (0.625 μg/ml).

As shown in Fig. 4A and B, the expression of Reg IV and EGFR were remarkably decreased, however, pIgR was evidently up-regulated with P1-treated cells at dose of 32.5 μg/ml. Although the down-regulated tendency of MCM2 and DDX32 were observed, there was no statistically significant change. These results suggest that P1 possessed prominent inhibitory properties against HT-29 cells through regulation of the functional gene expression of Reg IV, EGFR and pIgR.

3.5. Anti-tumor activity and plasma levels of PGE2, pIgR, IgA, Reg IV, EGFR and Akt

The anti-tumor effect of P1 was tested on HT-29-bearing mouse model at the dose of 50 and 100 mg/kg in vivo. As shown in Table 2 and Fig. 5, a significant tumor inhibition was observed at dose 100 mg/kg of P1 compared these in the tumor-bearing (TB) control mice. Anti-tumor activity of P1 obtained result indicated gradual increase and generated the highest inhibition ratio at 51.4% after 42 days of treatment. There was no significant difference in body weight of mice between TB control and P1treatment groups, suggesting that P1 has no toxicity to mice at the dose of 100 mg/kg (Fig. 5B). The standard reference drug (CDDP, 2 mg/kg) also exhibited a high inhibitory rate (63.42%), however, considerably decreased the body weight in HT-29-bearing mice ($P < 0.01$), and the mental conditions and coat color indicated that CDDP was harmful to TB control mice. It was noteworthy that the weight of the spleen and thymus indices also significantly increased in P1 group mice after treatment with P1 (50 and 100 mg/kg) for 42d, as compared with the TB control mice (Table 2). Therefore, it would be interesting to analyze the substantial adaptive immuno response and immunological responses. The pIgR and IgA levels in plasma were also significantly increased, but the PGE2 level was evidently

Table 2
In vivo anti-tumor activity of P1 from *Phellinus linteus*.

Sample	Inhibition degree (%)						Relative spleen weight (mg/g)	Relative thymus weight (mg/g)
	7d	14d	21d	28d	35d	42d		
TB control							4.84 ± 0.47	2.14 ± 0.16
P1 (50 mg/kg)	7.72	7.29	7.43	18.85	21.98	26.05	6.42 ± 0.38**	2.53 ± 0.19*
P1 (100 mg/kg)	22.66	21.16	27.22	27.83	40.67	51.41	6.84 ± 0.43**	2.65 ± 0.24**
CDDP (2 mg/kg)	22.91	35.01	38.50	58.98	63.42	58.54	4.07 ± 0.50	2.07 ± 0.18

TB control: tumor-bearing (TB) control; HT-29 cells were injected subcutaneously in female BALB/-nu/nu mice on day 0. The proteoglycans P1 (50 and 100 mg/kg) were dissolved in saline and TB control received 0.2 ml saline by intragastric administration everyday, CDDP (2 mg/kg, as positive control) was injected into peritoneal cavity every other day. The inhibition degree (%) is calculated according to the following formula: (average tumor volume of NaCl group − average tumor volume of sample group/average tumor volume of NaCl group) × 100%, relative spleen (thymus) weight was measured in the ratio of the spleen (thymus) weight (mg) to body weight (g). Values are mean ± S.D. ($n = 10$ each group).

* $P < 0.05$ vs. TB control.

** $P < 0.01$ vs. TB control.

Table 3
Effect of P1 from *Phellinus linteus* on levels of PGE2, pIgR, IgA, Reg IV, EGFR and Akt in plasma.

Sample	PGE2 (pg/ml)	pIgR (μg/ml)	IgA (μg/ml)	Reg IV (ng/ml)	EGFR (pg/ml)	Akt (ng/ml)
Control	118.77 ± 12.53	2.72 ± 0.39	3.14 ± 0.37	4.49 ± 0.37	281.40 ± 90.56	5.63 ± 0.58
TB control	400.77 ± 22.20**	2.11 ± 0.31*	2.09 ± 0.21**	5.29 ± 0.36*	715.51 ± 65.19**	6.62 ± 0.48**
P1 (50 mg/kg)	379.01 ± 25.26**	2.43 ± 0.49	2.36 ± 0.29**	4.93 ± 0.57	677.23 ± 70.08**	6.08 ± 0.49
P1 (100 mg/kg)	350.62 ± 20.30**,++	2.57 ± 0.26+	2.55 ± 0.23**,++	4.75 ± 0.35+	619.92 ± 40.64**,+	5.69 ± 0.80+
CDDP (2 mg/kg)	362.87 ± 17.39**,++	2.27 ± 0.43	2.21 ± 0.19**	4.96 ± 0.37	686.1 ± 95.25**	6.15 ± 0.41

TB and positive control and the proteoglycan (P1)-groups were inoculated HT-29. The mice were supplied orally daily with NaCl, P1 (50 and 100 mg/kg) or injected CDDP (2 mg/kg) into peritoneal cavity every other day. Plasma was collected from the tumor-bearing mice after 42 days. The biochemical parameters were determined using ELISA. Results are presented as mean ± S.D. (n = 10 each group).

* $P < 0.05$ vs. normal.
** $P < 0.01$ vs. normal.
\+ $P < 0.05$ vs. TB groups.
++ $P < 0.01$ vs. TB groups.

decreased in colorectal carcinoma model mice compared with the HT-bearing model (Table 3). The thymus is a specialized organ in which the T lymphocytes develop, differentiate, and mature, while spleen contains T-cells and B-cells [15,16]. The present results indicated that P1 is a potent immunomodulating and immunoenhancing agent, which is in accordance with previous reports of some of the functions of various polysaccharides [15,16]. Moreover, statistically significant decreases of the Reg IV, EGFR and Akt concentrations in plasma measured by ELLISA were observed (Table 3). These results were consistent with our foregoing RT-PCR results measured in HT-29 cells in vitro, suggesting that P1 also could directly inhibit colorectal carcinoma via disrupting the Reg IV/EGFR/Akt signaling pathway.

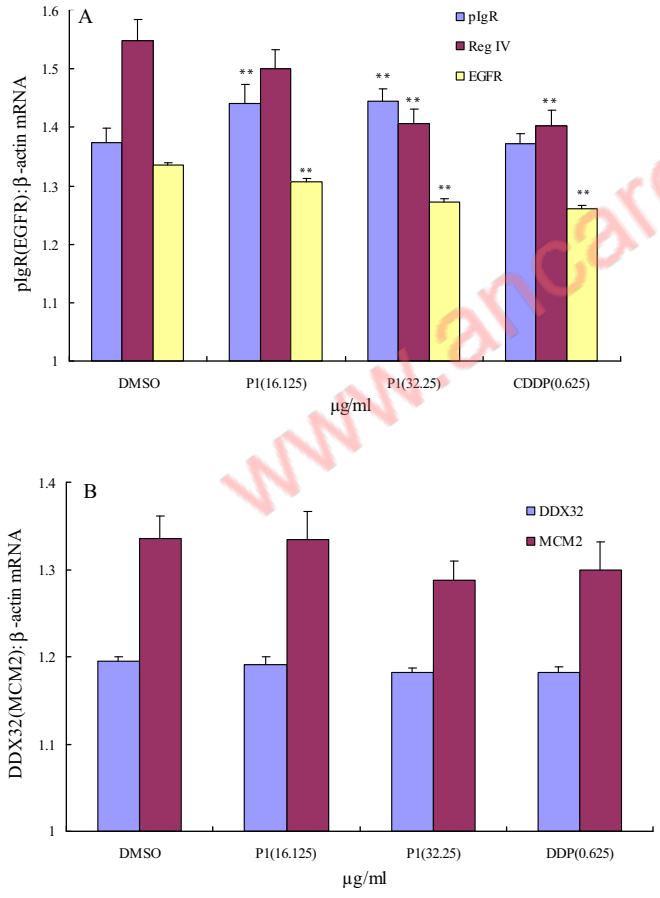

Fig. 4. Analysis of relative changes of pIgR, Reg IV, EGFR(A), DDX32 and MCM2(B) mRNA levels in HT-29 cells following treatment with P1 (15.625 and 31.5 μg/ml) and CDDP (0.625 μg/ml) for 48 h as detected by real-time RT-PCR. All expression data were corrected by the control gene β-actin. Standard error bars were calculated based on the mean of three replicate treatments. Statistically significant differences compared to the DMSO control were determined by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) and are indicated by $^{**}P < 0.01$.

4. Discussion

This study demonstrated that the proteoglycan (P1) from *P. linteus* (PL) has an antiproliferative effect on HepG2, HT-29, NCI-H 460 and MCF-7 human colon cancer cells, especially good effect on HT-29 cells. Growth inhibition of HT-29 cells by P1 treatment is mediated through the induction of immunoreaction and apoptosis. Further studies found that the immune function of P1 may be associated with increasing spleen and thymus relative weights, enhancing the mucosal IgA response by up-regulating the expression of pIgR and allowing T cells escape PGE2 attack through decreasing plasmatic PGE2 level. We also demonstrated for the first time that P1 could inhibit HT-29 cells proliferation, suggesting that P1 plays an important role in mediating the proliferative action stimulated by down-regulating the Reg IV and EGFR, and may lead to a disruption of the Reg IV/EGFR/Ak signaling pathway [12]. Further research is needed to better understand the Reg IV, EGFR and Akt levels in plasma in vivo. So we expect that P1 can be used as an adjuvant chemotherapeutic and chemopreventive agent as well as immunostimulant against the potential threat of colonic carcinoma.

Mushroom polysaccharides are increasingly being used to treat a wide variety of diseases. Previous reports showed that the polysaccharides and proteoglycans from PL had inhibitory effects on tumor growth and metastasis in a murine model [1], and also could inhibit the proliferation and colony formation of SW480 human colon cancer cells [3]. But whether all the polysaccharides from PL have the anti-tumor activities and which cancers are suitable to treatment draw our attention. In this study, a significant tumor inhibitory effect of P1 from PL on HepG2, HT-29, NCI-H 460 and MCF-7 human colon cancer cells was observed, but the cytotoxicity to normal cells (L-929) was also evident at high doses. Although previous researches deemed that protein-bound polysaccharides are thought to have no cytotoxicity on cancer cells, recently increasing studies reported that the proteoglycan could kill cancer cells directly when the doses were high [3]. Our results showed that the P1 has an effective inhibition on HT-29 cells under 62.5 μg/ml, which was a dose of non-toxic to normal cells.

To data, there are a large number of studies on anticancer activities of polysaccharides and their molecular mechanisms have been documented. Previous studies investigated the anticancer mechanisms of polysaccharides have focused upon the large part on the functional activations of immune cells, such as T cells, B cells, nat-

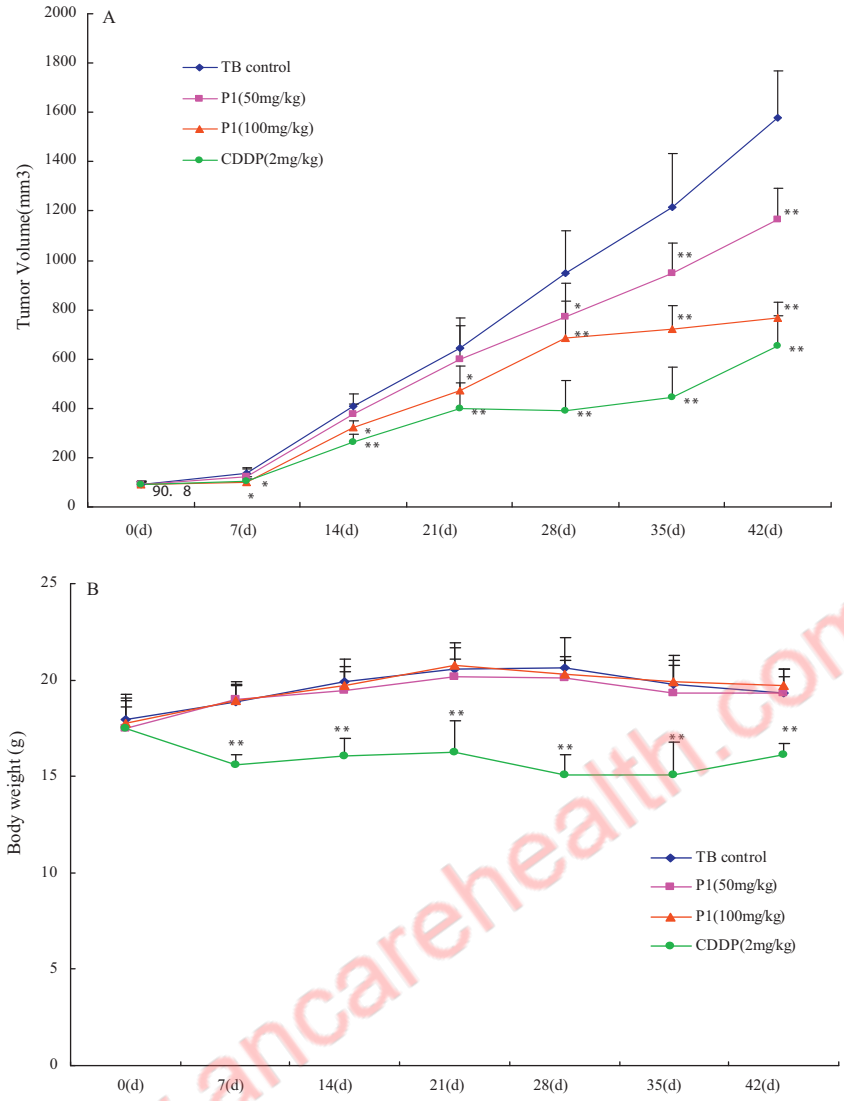

Fig. 5. The anti-tumor activity of P1 from *Phellinus linteus* in vivo. TB control: tumor-bearing (TB) control. HT-29 cells were injected subcutaneously in female BALB/c-nu/nu mice on day 0. The proteoglycans P1 (50 and 100 mg/kg) were dissolved in saline and TB control received 0.2 ml saline by intragastric administration everyday, CDDP (2 mg/kg, as positive control) was injected into peritoneal cavity every other day. The maximal (a) and minimum (b) diameters of solid tumors (A) and the body weights (B) of mice were measured every week. The tumors volume was calculated as $V = a \times b^2/2$. Compared with the TB group, Values are mean ± S.D. ($n = 10$ each group). $^*P < 0.05$ and $^{**}P < 0.01$ vs. TB control.

ural killer cells, dendritic cells, and macrophages, which all have the capability to eliminate transformed cancer cells [2,17–19]. In our study, we found that P1 has a direct antitumor effect through inducing apoptosis and inhibiting the karyokinesis of HT-29 cells, the results are consistent with the previous reports by Li et al. [3]. Meanwhile, the highest inhibition ratio of 51.4% after 42 days treatment with P1 also observed in HT-29-bearing model mice. In an attempt to clarify the potential mechanism(s), the effects of P1 on the relative spleen and thymus weights were measured, and the expression patterns (up/down-regulation) of many functional genes (MCM2, DDX32, pIgR, Reg IV and EGFR) in the plasma of colorectal carcinoma were also detected. The relative thymus and spleen weight were important indices for nonspecific immunity. The increase in thymus and spleen indices of mice treated with P1 indicated that P1 is a potent immunomodulating and immunoenhancing agent. It is well known that the overproduction of PGE2 has been associated with numerous pathological conditions including chronic inflammation and colon carcinogenesis. PGE2 from colon cancer cells can inhibit T cell response [20], impair differentiation and function of dendritic cells by suppressing poly (I:C)/LPS mediated IFN-α and IL-12 production [21]. In addition, PGE2 from colon cancer cells can also induce generation of regulatory T cells [20] and myeloid-derived suppressor cells, which allow the tumor cell to escape of immune surveillance [22]. IgA refer to as the first line immune defense, protecting mucosal surfaces against environmental and sexually transmitted pathogens [23]. Several investigators have observed an increased mucosal IgA response by long-term treatment with multiple polysaccharides [24,25]. The pIgR is a transmembrane glycoprotein expressed on secretory epithelial cells, which mediates the transport of polymeric immunoglobulins, primarily polymeric IgA (pIgA), into external secretions. Up-regulation of pIgR expression increases the capacity of mucosal epithelial cells to transport pIgA, thus enhancing the functions of IgA [26]. So the expression of pIgR is regulated with respect to the effects of specific immune modulators [17]. Our findings show that P1 could up-regulate the pIgR mRNA level in HT-29 cells (Fig. 4A). Correspondingly, the pIgR level change in colorectal carcinoma model mice was in well accordance with the RT-PCR results in vitro (Table 3), especially, the increased IgA concentration suggested that P1 exert their effects, at least part through protect-

ing T cells from escaping PGE2 attack and enhancing the mucosal IgA response.

Reg IV, a dominant member of the Reg multigene family, is of considerable interest because of its up-regulated expression in the colorectal carcinoma (CRC) and colon adenocarcinoma cell lines [27,28]. Recently, Bishnupuri et al. reported that Reg IV protein is a potent activator of the EGFR/phosphoinositide 3-kinase/Akt/AP-1 signaling pathway in human colon cancer cell lines [12]. Activation of EGFR signaling pathway results in mitogenic signaling in colon cancer cells, increase cell proliferation, angiogenesis, metastasis and decreased apoptosis [13,29,30]. Interestingly, Nanakin et al. found that EGF and TGF-α enhanced Reg IV gene expression by ERK signaling pathway in SW403 cells line [31]. Taken together, these studies suggest that a positive expression feedback loop between EGF and Reg IV exists in the signaling pathways. So disruption of Reg IV/EGFR signaling may be used as a therapeutic intervention for human gastrointestinal adenocarcinomas. Many researchers reported that fucoidans from *Laminaria gurjanovae* mainly consisting of glucose, fucose, galactose and mannose could bind to vascular endothelial growth factor 165 (VEFF165) and its receptors [32,33]. Recently, Lee et al. (2008) found that fucoidan could bind with EGF, blocked EGF-induced phosphorylation of EGFR, resulting to inhibit EGF-induced cell transformation [34]. Our studies showed that treatment of colonic adenocarcinoma cells with P1 resulted in a significant dose-dependent inhibition in cell numbers (Fig. 2) and cells' mitosis (Fig. 3), so we identified the expression of Reg IV and EGFR in P1-treatment HT-29 cells. RT-PCR results showed that P1 had the capacity to down regulate the expression of Reg IV and EGFR (Fig. 4A), these effects may be important for its growth inhibitory capacity to HT-29 cells. Therefore, we hypothesized that the proteoglycan P1 composed of L-fuc, D-gal, D-glu, D-man and other monosaccharides may bind with EGF as fucoidan, blocked EGF-induced phosphorylation of EGFR and feedback loop induced Reg IV down-regulation, thereby inhibiting intracellular signaling and decreasing the expression of EGFR without activation. In order to testify this hypothesis, HT-29 cells were inoculated in mice to establish the colorectal carcinoma model. The differences in the P1 dose intake for 42 days significantly lower the tumors volume, indicating that P1 has an anti-tumor effect in vivo (Table 2 and Fig. 5), decrease of Reg IV, EGFR and Akt levels in plasma (Table 3) which strongly supports that the P1 inhibited colorectal carcinoma by disrupting the Reg IV/EGFR/Akt signaling pathway. Although MCM2 and DDX32 play pivotal roles in tumor growth, unfortunately, no significant change in their levels were noted in HT-29 cells after P1-treatement (Fig. 5).

In summary, the polysaccharide (P1) from *P. linteus* is an immunostimulator that has therapeutic activity against cancers. Here, we show that P1 markedly inhibits the growth of HT-29 cells and directly effect on cancer cells' mitosis and proliferation through down-regulate the Reg IV and EGFR, and upregulating the expression of pIgR. In HT-29-bearing model mice, the significant changes of plasma biochemical parameters approved P1 inhibitory colorectal carcinoma effect. These results suggest that P1 has two antimetastatic functions, it could act not only as an immunopotentiator, but also as an anti-Reg IV/EGFR/Akt signaling pathway agent inhibiting HT-29 tumor directly.

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