

RESEARCH ARTICLE

Different Immunology Mechanisms of *Phellinus igniarius* in Inhibiting Growth of Liver Cancer and Melanoma Cells

Cui Zhou¹, Song-Song Jiang¹, Cui-Yan Wang¹, Rong Li², Hui-Lian Che^{1,3*}

Abstract

To assess inhibition mechanisms of a *Phellinus igniarius* (PI) extract on cancer, C57BL/6 mice were orally treated with PI extractive after or before implanting H22 (hepatocellular carcinoma) or B16 (melanoma) cells. Mice were orally gavaged with different doses of PI for 36 days 24h after introduction of H22 or B16 cells. Mice in another group were orally treated as above daily for 42 days and implanted with H22 cells on day 7. Then the T lymphocyte, antibody, cytokine, LAK, NK cell activity in spleen, tumor cell apoptosis status and tumor inhibition in related organs, as well as the expression of iNOS and PCNA in tumor tissue were examined. The PI extract could improve animal immunity as well as inhibit cancer cell growth and metastasis with a dose-response relationship. Notably, PI's regulation with the two kinds of tumor appeared to occur in different ways, since the antibody profile and tumor metastasis demonstrated variation between animals implanted with hepatocellular carcinoma and melanoma cells.

Keywords: *Phellinus igniarius* - hepatoma - melanoma - cell lines - immunological mechanism

Asian Pac J Cancer Prev, 15 (8), 3659-3665

Introduction

Phellinus igniarius (PI) is known as one of the best anti-cancer fungi, especially its inhibition on hepatoma (Zhang et al., 2006; Guo et al., 2010). The polysaccharide contained in PI is more complex than other fungal polysaccharide (Kim et al., 1994). The main medicinal ingredient in PI is glucan (1.18%) which is β (1 \rightarrow 3) polysaccharide (Figure 1) primarily. Besides, it also contains β (1 \rightarrow 6) polysaccharide, galactose, mannose, arabinose, fucose and so on, resulting extremely complex structure of PI (Ge, 2009). Many hydroxyl groups connecting to the skeleton of (1 \rightarrow 3)-D-glucan play important role in its antitumor activity, which improve body's immune function (Zhang and Huang, 2007), anti-tumor activity (Ladanyi, 1993), and the body's resistance to bacteria, parasites and virus. Researches revealed that PI can affect the body's immune function through influencing producing of complement, interferon, interleukin, etc. (Li et al., 1997). It can also inhibit the growth and metastasis of tumor (Han et al., 1999) through activating macrophages, reticuloendothelial system, T lymphocytes, B lymphocytes, NK, LAK and TIL

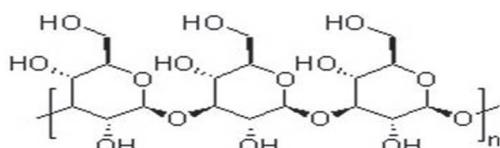


Figure 1. The Structural Formula of β (1 \rightarrow 3) Polysaccharide

cells, stimulating the production of non-specific antibodies and improving the lymphocyte conversion rate (Wang, 2008).

Liver cancer and melanoma are two kinds of tumor with higher malignant degree than the other tumors. Liver cancer develops rapidly with poor prognosis and the melanoma is one of the tumors with strongest immunogenicity. At present, main measures used in treatment including surgery and intervention chemotherapy, that often cause a variety of adverse reactions as these methods do not have selectivity between tumor and normal cells (Zhang and Ning, 2003). In the recent two years, some functional components have been reported to be inhibitor of many cancers. The *Rhizophara apiculata* extract can inhibit the lung metastasis and tumor masses of B16F10 melanoma cells in C57BL/6 mice (Prabhu and Guruvayoorappan, 2013). Besides, the Penfluridol (C28H27CIF5NO) also can inhibit growth of B16F10 melanoma cells through dysregulation of cholesterol homeostasis *in vitro* and *in vivo* (Wu et al., 2014). But the inhibition effect and mechanism of polysaccharide substance on cancer has not been investigated clearly and there are two reports about the inhibition mechanism of Barbigerone (Yang et al., 2014) and Hepatocellular Carcinoma Vaccine (Su et al., 2013).

Japan scholars Lkekawa (Ikekawa et al., 1968) and his co-workers found that the inhibition rate of *Lentinus edodes* in mice sarcoma S180 was 80.7%. Kim et al. found that PI had inhibitory effect on tumor growth and

¹College of Food Science and Nutritional Engineering, China Agricultural University, ²Management Center for 12320 National Health Hotline10/F, ³The Supervision, Inspection and Testing Center of Genetically Modified Organisms, Ministry of Agriculture, Beijing, China *For correspondence: chehuilian@cau.edu.cn

metastasis without toxic side effects (Kim et al., 1996). Nowadays, although it has been reported widely about PI's inhibition effect on tumor, its action mechanism is still known rarely. To investigate PI's immunological mechanism of inhibiting tumor, we analyzed and compared its inhibitory effect on the growth and metastasis of liver cancer and melanoma. Besides, PI's influence on the immunological function of tumor-burdened mice was also researched in this study. The results revealed that PI inhibited liver cancer and melanoma by modulating the immune function of experimental animals, but there are differences between the two kinds of inhibition in immunological mechanisms.

Materials and Methods

Materials

150 C57BL/6 mice with male and female in half and weighting of 20 ± 2 g were obtained from Vital River Laboratories (VRL, Beijing, Animal qualified number: 2002-0003). After one week of adapting feed in SPF animal house, the mice were divided into three groups according to their weight randomly for treatment and prevention experiments of liver cancer, the treatment experiment of melanoma respectively. There were 50 mice in each group with male and female half and half.

Hepatoma carcinoma H22 cell line was purchased from Heilongjiang Institute of Tumor Prevention and Control and the melanoma B16 cell line was from Vital River Laboratories. The phellinus igniarius was kindly presented by Risheng Kabushiki Kaisha (Japanese) and it was tan powder with a moisture of 5.4%, a total bacterial count of $5.8 \times 10^3 \text{ g}^{-1}$, negative in fecal coliform and a mass fraction of 85.3%.

Solid tumor-bearing mice models

Animals in each large group were randomly divided into five small groups by weight, three treatment groups with different doses of PI, tumor negative control group and normal group, each group contained 5 male and 5 female mice and their specific treatment was described in Table 1. Animals in treatment experiments were oral administrated daily for 36 days with different doses of PI 24h after carcinoma cells' inoculation. In prevention trial, inoculation of carcinoma cells was administrated 7d after giving PI daily and followed by given PI daily for 36d.

Determination of serum antibody and cytokines levels

To evaluate the humoral immunity in tumor-bearing

mice with or without administration of PI, sera of animals in different groups were collected at the end of experiments and detected levels of several specific antibodies (IgG1, IgG2a, IgG2b, IgG3, IgM and IgA) and cytokines (IL-2, IL-12, TNF- α) using double sandwich ELISA kits (eBioscience, USA) method followed manufacture's instruction referred the previous research (Zhang et al., 2006).

T cell subpopulation analysis by flow cytometry

To detect the PI's influence on animals' immune cells, the lymphocyte subsets in peripheral blood were analyzed using flow cytometry as described previously (Zhang, 1998). Briefly, 200 μ l anti-coagulated peripheral blood was added in tube followed by adding FITC labeled anti-mouse CD4 mAb (NO. 11-0043-81, eBioscience, Beijing, China) and PE labeled anti-mouse CD8a mAb (NO. 12-0081-81, eBioscience, Beijing, China). Then the tube was kept in dark for 20min at room temperature, and 2 ml hemolysis was added and incubated for 10min in dark after reversing. After washing twice with PBS for 5 minute, 0.6 ml of the PBS liquid containing 1% formaldehyde was added in tube and then stabilize 10-30min. At last, FASCalibur flow cytometry instrument (American Becion Dickinson) was used to analyze the cell population.

In vitro immunocompetence of splenocyte

The effect of administration of PI on the *in vitro* immunocompetence of splenocyte was determined by MTT assay method as described in previous publication (Qiu et al., 2010). Briefly, the splenocyte was isolated and cultured in complete RPMI 1640 medium in 96 well plate (6×10^5 /well) for 5 days with adding IL-2 (1000 u/ml) in the suspension and then lymphokine-activated killer cell (LAK cell) was obtained. 5×10^3 LAK cells were seeded in 96-well plate with or without the existence of K562 cell (5×10^3) as target cell. Put the 96 well plates in 37 °C, 5% CO₂ and cultured for 20h followed by adding 10 μ l MTT in each well and continue to culture 4h. After that, 100 μ l acidification isopropyl alcohol was added in each well. The absorbance was measured at 570 nm (OD570) by a 96-well microplate reader (N06355, Thermo Scientific Varioskan Flash, American), and then the activity was shown as cytotoxicity percentage value that was calculated according to calculation formula as follows:

$$\text{Cytotoxicity percentage (\%)} = [1 - (AE + T - AE) / AT] \times 100 \%$$

$AE + T = OD$ of the mixed well of effector cells and target cells

$$AE = OD \text{ of the well of effector cells}$$

$$\text{The } AT = OD \text{ of the well of target cells}$$

In addition, the immunocompetence of NK cells in splenocytes was also determined by method of lactate dehydrogenase (LDH) referred as described previously (Zhang, 1998). Splenocytes were isolated as described above and cultured 4h in complete RPMI 1640 medium in 96 well plate (2×10^6 /well) with or without YAC-1 cells (4×10^4 /well). Then the suspension was obtained and added in a new 96 well plate (100ul/well) with 100ul LDH together. 30 μ l 1 mol/L HCl was added in the wells after 3min and then the absorbance was measured at 490 nm (OD490) and then the immunocompetence of NK cell was

Table 1. Treatment Factors of Mice

Groups	Carcinoma cell	PI extractive	Volume (μ l / d kg bw)	Concentration (g/ d kg bw)
Normal	-	-	25	0
Untreated	+	-	25	0
Low-dose	+	+	25	40
Mid-dose	+	+	25	200
High-dose	+	+	25	1000

PI = Phellinus Igniarius. All of mice were orally gavaged with same volume of saline with or without PI. And the mice in normal group were not inoculated carcinoma cells

calculated according to below formula: **NK cell activity (%) = (OD effector cells well - OD negative well / ODmax - ODnegative well)**

Histopathology, tumor cell apoptosis and related protein. To investigate the inhibition of PI on the survival and progression of tumor in H22- and B16-bearing mice, animals' liver, kidney, lung, thymus, spleen and tumor tissue were weighed after execution. Then indexes of the thymus, spleen, tumors and inhibition rate of tumors were calculated. In addition, the morphology, malignant degree, lymphocytic infiltration, cancer cells metastasis and necrosis status in organs and tumor tissues were observed after HE staining. The related calculated formulas are as below:

Tumor Index = [mice tumor weight(g) / body weight (g)] × (T/B)

Inhibition (%) = [average weight of untreated(g) - average weight of experimental group (g) / average tumor weight of the untreated groups (g)] × 100

Organ index (%) = [organ's weight(g) / body weight(g)] × 100

Besides, to determine the apoptosis condition of tumor cell DNA rupture of cell in h22- and B16-bearing mice was detected by TUNEL (terminal-deoxynucleotidyl transferase mediated d-UTP Nick end ling) method. The expression of induced nitric oxide synthase (iNOS) and proliferating cell nuclear antigen (PCNA) that involving in cell proliferation in tumor tissues were examined using immunohistochemical method to speculate the inhibition mechanism of PI to tumor survival and progression.

Statistical analysis

The data were analyzed using SAS 6.12 software package and were expressed as the mean ± SD. The statistical significance of the values was evaluated by analysis of variance. $p < 0.05$ was considered as significant difference.

Result of TUNEL was analyzed by IPP pathological image analysis system. The images was 40× and counted 1000 tumor cells randomly for evaluating apoptosis degree of tumor cell and calculate the percentage of apoptosis positive cell (apoptosis index, AI).

Result of Immunohistochemical staining was also analyzed by IPP pathological image analysis system. The sections were investigated and evaluated in double-blind conditions by two experienced experts of department of pathology respectively and the results expressed as positive and negative. Three fields of view from each tissue section were selected randomly and collected at 40×. The area of chosen typical positive expression and total area of the entire field of vision were calculated respectively. The ratio of positive expression area and area of the entire field of vision was considered as evaluation index.

Results

Regulation effects of PI on serum antibodies and cytokines

To investigate the PI's effect on humoral immunity in tumor-bearing mice, different doses of PI were orally administrated to mice that planned hepatocellular

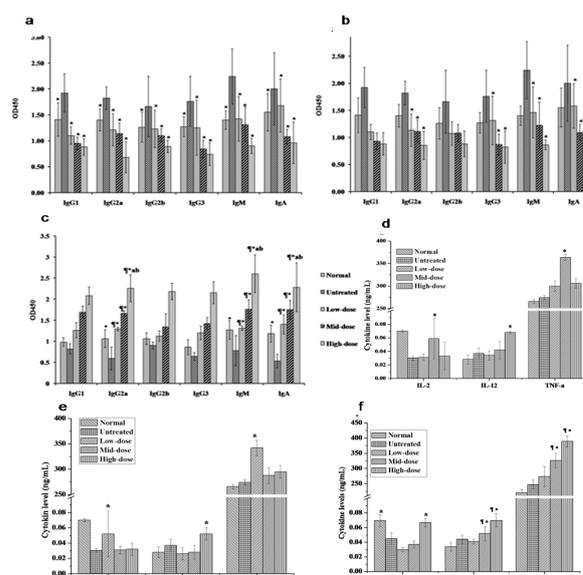


Figure 2. Levels of Sera Antibodies and Cytokines in Different Experiments. a) The treatment experiment of liver cancer lasting 36 days. b) The prevention experiment of liver cancer lasting 42 days. c) The treatment experiment of melanoma lasting 36 days. d) The treatment experiment of liver cancer lasting 36 days. e) The prevention experiment of liver cancer lasting 42 days. f) The treatment experiment of melanoma lasting 36 days. * $p < 0.05$ vs untreated group; † $p < 0.05$ vs normal group; †† $p < 0.05$ vs low dose group; ††† $p < 0.05$ vs middle dose group

carcinoma cell (H22) or mouse melanoma cell (B16). In the treatment experiment of mouse hepatocellular carcinoma cell (H22), as shown in Figure 2a, levels of IgG1, IgG2a, IgG2b, IgG3, IgM and IgA in sera in untreated group were significantly higher than that the other control or treated groups ($p < 0.05$). But notably, the antibody levels in each dose treated group showed a trend of decrease along with the increase of dose. In H22 prevention experiment, levels of antibodies appeared similar trend to that in treatment experiment besides IgG1 and IgG2b (Figure 2b). On the other hand, in prevention experiments of melanoma cell (B16), the influence of PI on serum primary antibody in mice was converse with that in the other two H22 experiments and its oral administration dramatically increased levels of IgG2a, IgM and IgA compared to untreated group ($p < 0.05$) with a positive correlation relationship to the gavaged dose of PI (Figure 2c).

Additionally, level of cytokines in every experiment was assayed to determine PI's *in vivo* regulation effect. In the H22 treatment experiment, IL-12 in high dose treated group and both of IL-2 and TNF- α in middle dose group were significantly increased when comparing with untreated group ($p < 0.05$) (Figure 2d). And all of three cytokines in the high dose group were significantly higher than that in untreated group in H22 prevention experiment ($p < 0.05$) (Figure 2e). Similarly, in B16 prevention experiment, levels of the three cytokines in high dose treatment group, IL-12 and TNF- α in middle dose group were significantly higher than that in untreated group ($p < 0.05$). Besides, levels of all these three cytokines were presented an increasing trend along with increase of PI dose in H22 prevention experiment (Figure 2f).

Table 2. The Effect of PI Extractive on CD4+/CD8+

Groups	36 days (H22)	42 days (H22)	36 days (B16)
Normal	2.63±0.23	2.63±0.23	2.49±0.54
Untreated	0.75±0.06	0.75±0.06	0.67±0.08
Low-dose	1.73±0.10 ^{¶*}	2.06±0.11 ^{¶*}	1.63±0.09 ^{¶*}
Mid-dose	2.12±0.07 ^{¶*}	1.72±0.17 ^{¶*}	2.03±0.11 ^{¶*}
High-dose	1.93±0.23 ^{¶*}	1.87±0.30 ^{¶*}	2.16±0.23 ^{¶*}

Each value represents the mean±SD (n=5). CD4+/CD8+= percentage of CD4+ T cell/percentage of CD8+ T cell; [¶] $p<0.05$ vs normal group; ^{*} $p<0.05$ vs untreated group

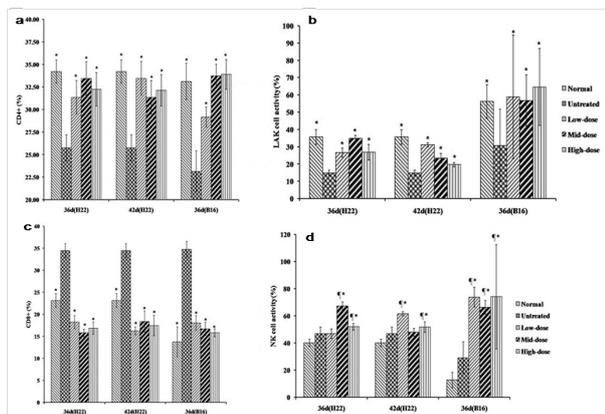


Figure 3. Effect of PI Extractive on T Lymphocyte Population and Cell Activity * $p<0.05$ vs Untreated Group; [¶] $p<0.05$ vs Normal Group

Regulation effect of PI on T lymphocyte population

The effect to immune cell of oral administration of PI on tumor bearing mice, population of CD4+ T lymphocyte and CD8+ T lymphocyte were detected using flow cytometry. T lymphocyte subsets in three experiments appeared similar trend that the frequencies of CD4+ T cell in every dose treatment group were marked higher than that in untreated group ($p<0.05$) and the CD8+ T cell shown an opposite results (Figure 3a and b). As shown in Table 2, CD4+/CD8+ T cells in PI treated groups were higher than that in untreated group ($p<0.05$) and this is consistently with the performance that in CD4+ T population. However, no statistical significance was appeared among different dose groups in the two H22 experiments. While the levels of CD4+ T cell and CD4+/CD8+ appeared an positive correlation relationship with the dose of PI in the B16 prevention experiment (Figure 3a and Table 2).

PI can increase anti-tumor activity of splenocyte

In addition to detection of PI's effect on tumor-bearing mice's humoral immunity and T lymphocyte population, its improvement influence on immune cells' anti-tumor activity also should be evaluated. At the end of three experiments, spleens were isolated and the splenocytes were collected for assay of their *in vitro* anti-tumor activity. In all of the three experiments, it was appeared that activity of LAK cells in all dose treatment groups were significantly higher than that in untreated group in ($p<0.05$) (Figure 3c). In H22 prevention experiment, LAK cell activity appeared a negative correlation relationship to treatment dose of PI and this was inverse to that in prevention of B16, however, the LAK cell activity did not

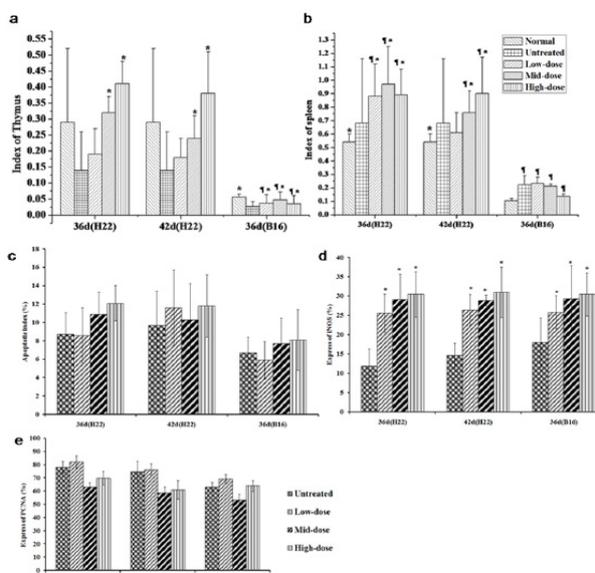


Figure 4. Effect of PI Extractive on Relative Weight of Thymus. a) Spleen **b)** Tumor cell apoptotic index **c)** Express of iNOS **d)** PCNA, **e)** * $p<0.05$ vs untreated group; [¶] $p<0.05$ vs normal group

show clearly difference among doses groups ($p\geq 0.05$).

Effect of PI on NK cell activity of splenocyte in mice was also administrated. Anti-tumor activity of NK cell from animals in high and middle dose treatment groups of H22 treatment experiment, as well as high and low dose group in H22 prevention experiment, and all the three doses groups in B16 treatment experiment appeared significantly higher than that in untreated groups ($p<0.05$) (Figure 3d). And notably, activity of NK cell in high dose groups was stronger than that in other doses treatment groups.

The effect of PI on the relative weight of immune organs.

Most kinds of cancers often affect body's immune system, especially the immune organs thymus and spleen. So animals' body weight and relative weight of spleen and thymus in every group were examined. Both of H22 and B16 cell decreased relative weight of tumor-bearing animals' thymus, while increased index of spleen ($p<0.05$) (Figure 4). Different doses oral administration of PI made a marked increase in indexes of the two organs in both of H22 treated experiments with a dose dependent trend (Figure 4a, b). On the other hand, relative weights of thymus in three treated groups were significantly higher than that in untreated group ($p<0.05$) in the B16 treatment experiment (Figure 4a). Conversely, PI appeared a reduction effect on spleen index without a significance compared to untreated group ($p>0.05$) in B16 treatment experiment.

Effect of PI on tumor survival and progression

To investigate the effect of oral administration of PI on survival and progression of H22 and B16 in tumor-bearing mice, the ratio of tumors (T/B), inhibition rate and metastasis of two tumor cells were detected. The T/B in each dose group was extremely significantly lower than that of untreated group ($p<0.01$) in both of H22 treatment

Table 3. The Effect of PI Extractive on T/B and Inhibition rate of Tumor

	Groups	T/B (%)	Inhibition rate of tumor (%)
36 days (H22)	Untreated	27.74±12.39	
	Low-dose	17.40±6.95*	27.33
	Mid-dose	12.87±7.89*	57.4
	High-dose	8.71±5.45*	65.65
42 days (H22)	Untreated	27.74±12.39	
	Low-dose	12.73±1.06*	50.99
	Mid-dose	10.93±4.06*	76.18
	High-dose	14.30±6.92*	38.78
36 days (B16)	Untreated	30.55±10.94	
	Low-dose	25.99±12.91	15.13
	Mid-dose	28.34±10.42	1.65
	High-dose	13.81±14.33*	29.71

T/B=tumour weight (g)/body weight (g). * $p<0.01$ vs untreated group

Table 4. The Effect of PI Extractive on Cancer's Metastasis Rate (%)

		36 days (H22)	42 days (H22)	36 days (B16)
Liver	Untreated	0	0	66.7
	Low-dose	0	0	66.7
	Mid-dose	0	0	33.3
	High-dose	0	0	25
Kidney	Untreated	0	0	66.7
	Low-dose	0	0	33.3
	Mid-dose	0	0	0
	High-dose	0	0	0
pulmonary	Untreated	33.3	33.3	100
	Low-dose	0	20	66.7
	Mid-dose	0	12.5	33.3
	High-dose	0	0	25

*Metastasis rate=number of occurrence metastasis /total number×100%

and prevention experiments and appeared a decrease trend along with the increase of PI dose (Table 3). While in the B16 treatment experiment T/B performed lower comparing to that in untreated group was only in high dose group ($p<0.01$).

On the other hand, to the aspect of tumor inhibition rate, a dose dependent trend was just shown in the H22 treatment experiment. When the PI was used in H22 prevention, its inhibition effect was strongest in the middle dose group (76.18%) and weakest in the high dose group (38.78%). Besides, PI was also shown a notably inhibitory effect in the B16 treatment experiment. The highest (29.71%) and lowest (1.65%) inhibition rates were appeared in the high dose and middle dose treatment groups respectively.

The tumor metastasis status was also determined in this research. Tumor metastasis was appeared only in pulmonary, but not in liver and kidney, in untreated group of H22 treatment experiment. However, in H22 prevention experiment, PI performed a dose dependent inhibition effect in pulmonary (Table 4). Unlike H22 cell line, the grade malignancy of melanoma B16 cell line was higher and shown metastasis in all the three organs of liver, kidney and pulmonary. And PI also inhibited B16 metastasis with a dose dependent trend that the tumor metastasis was decline along with PI's increased dose

(Table 4). These results indicate that the PI can not only inhibit tumors' development but also their metastasis.

PI's effect on tumor cells apoptotic and express of related protein

The above results and many other researches indicated that PI performed an inhibition influence to both of H22 and B16. But the possible mechanism remains to be understood, so we hypothesised that PI maybe cause the tumor cell apoptotic to play its inhibition role. To verify this hypothesis we investigated the tumor cell apoptosis and expression of related proteins, iNOS and PCNA. It was appeared that PI showed dose independent increased influence to cell apoptosis index in all of the three experiments without appearance of statistical differences (Figure 4c).

Result obtained from the IPP pathological image analysis system showed the effect of PI on expression of iNOS and PCNA (Figure 4d, e). The positive areas of iNOS expression in all the dose treated groups were significant higher than that in untreated groups ($p<0.05$). Furthermore, the positive area of iNOS expression in tumor tissue performed a increasing trend with the increase of PI treated dose. However, the expression of PCNA did not appear any difference among all the treatment groups ($p>0.05$). These results demonstrate that the synthesis of NO induced by iNOS may be play an important role in the inhibition of tumor progress.

Discussion

Mortality is one of the aggregative indicators reflecting physical condition in tumor-bearing mice. There was no death appeared in the untreated H22-bearing with the exception of a series of weak appearance, such as the lost of appetite, the reduced activity and body weight with dim hairs. Whereas these conditions were meliorated in the mice orally treated with PI extractive. On the other hand, in B16 treatment experiment the appearance of mice burdened B16 was worse than that in H22 experiments regardless of PI administered. And at later stages, due to the fast growth of tumor, ulceration was observed in some mice as a result of the tumor perforation out of the skin, meanwhile, mice death occurred in every B16 planned group. However, the mortality appeared a decline trend along with the increasing dose of PI treatment. This indicated that PI plays an important role in the inhibition of tumor cells development and progression *in vivo*, extension of tumor-bearing animals' lifetime and reduce of the mortality, all of which were similar to the reported effect of radix aconiti coreani polysaccharide (Liang et al., 2012). Besides, previous researches reported a promoting role of male hormone in the proliferation of cancer cell (Liu et al., 2001), and hereby we show a more obvious inhibition of PI in male mice. So it is speculated that PI can bond to the hormone receptors on cancer cell and stop the hormone playing promotion effect on development of cancer, which make the PI perform anti-cancer activity indirectly.

As the reported lentinan's anti-cancer effect on mice S180 of lentina (Li et al., 1997), weight of cancer tissue

was inhibited in all the PI treated groups in both of the H22 experiments. Besides, the PI's inhibition ratio to H22 was performed as a dose dependent trend in the H22 treatment rather than that in H22 prevention. The difference between two experiments was nothing but the PI's treatment time. So we suggest that the high dose of PI (1g/g. body weight) can initial body's immune response more quickly in cancer planed mice in a short time in treatment experiment. On the other hand, body's immune function may has been stimulated by PI in advance of H22 injection regardless treated dose and this may explain the lack of dose dependent relationship of PI in H22 prevention experiment. Therefore the PI's inhibition effects on H22 may relate to the initial point of stimulation rather than treated dose. Whereas, the PI's high dose treatment performed strongest inhibition to B16 cell without dose dependent trend and this indicated a high contact dose is needed for inhibiting a cancer with high malignancy. So we suggest that the PI's inhibition efficiency maybe different on cancers with different malignancy.

Both of H22 and B16 are more prone to be metastatic tumors than the others and the reported PI's effect on tumor metastasis are few currently. In this research, it appeared that PI performed obvious inhibition with dose-effect relationship on B16 in lung, liver and kidney. However, this effect only shown in lung in H22 experiments. These results are similar to that reported by Lee, Kang and their partners (Lee et al., 2005; Kang et al., 2003), so it is speculated that the PI's inhibition effect will be stronger on more serious cancer.

Many anticancer drugs play role of anti-tumor by inducing tumor cells' apoptosis directly. By contrast, in this study, the PI appeared neither induction of tumor cells' apoptosis directly nor inhibition of their proliferation, although performed its inhibition to cancer's progression. This is consistent with other reported results that revealed PI's weak effects on apoptosis of tumor cell (Zhao and Zhang, 2008), but conflicted with another study showing that it can inhibit S180 tumor cell directly (Wen et al., 2002; Wu et al., 2007). So it is still in controversy over PI's direct inhibition to tumor and this remains to be further in-depth study.

The survival and development of cancer often interrelate with disfunction of immune system. The PI raised frequency of CD4+ T cell along with declining percentage of CD8+ T cell and thus improved ratio of CD4+/CD8+T cells, which as other fungi polysaccharides reported in literatures (Liu et al., 1995; Xiong and Lu, 2000).

When respecting to cytokine, hereby we show PI increased levels of IL-2, IL-12 and TNF- α in animals inoculated tumor cells. As one of the most important regulating factors of immune system, IL-2 can promote the proliferation and differentiation of T cells and B cells, activate the tumor killer cells (LAK, TIL) and improve production of TNF (Li et al., 1998). IL-12 can stimulate macrophages producing high levels of NO to suppression tumor's development through promoting secretion of IFN- γ by T cell. Furthermore, TNF plays its role of suppression on tumor growth by inducing cell apoptosis

through multiple ways (Yang, 2002; Fan et al., 2004). Therefore, PI may suppress tumor's progression by many mechanisms that contain regulation of T lymphocyte population, affection of cytokines secretion and promotion of LAK and NK cells, which is consistent with other researches (Xiong and Lu, 2000; Li et al., 2012; Liu et al., 2013). Besides, the PI can also stimulate synthesis of NO by macrophages through improving expression of iNOS in tumor tissues. The high level of NO could destroy the replication, transcription and translation of nuclear acid directly, which may prevent tumor cells proliferation.

Cellular immune response often causes humoral immunity reaction. In present research we found that the influence of H22 and B16 to body's antibody production were opposite as well as the effect of PI on animals transplanted the two kinds of tumor cells. This indicated that PI can regulate and maintain balance of antibody. This different regulating effect on humoral immune of PI may be related to the different stimulation mechanisms of different cancers on immune system.

Results obtained from present study maybe relevant to the various structures of polysaccharides contained in PI. Both of primary and advanced structure of polysaccharide play important role in the regulation of body immunity, induction of tumor cell apoptosis and so on. The mechanism of PI's above immune regulation function may upon its recognition and combine with β -1, 3 GR expressing on the membrane of mononuclear cell, macrophages and grain cell (Thomton et al., 1996). Furthermore, the anti-tumor activity maybe different between β -glucans regardless they have similar structures, which indicates that its anti-tumor activity not only attribute to the structure of fungi polysaccharide, but also the molecular, water solubility and conformation and so on.

In present study, we established solid tumor-bearing mice model of mouse hepatocellular carcinoma cell (H22) and mouse melanoma cell (B16), investigated and compared the PI's immune mechanisms of suppression effect on different carcinoma cells. The obtained results indicated that anti-cancer ability of PI is exerted through regulating and promoting immune function rather than direct induce tumor cell apoptosis. In conclusion, PI's different inhibition effects on the two cancers are performed as follows: (1) PI plays a more obvious inhibition on the cancer relating to hormone. (2) In the H22-bearing animals, PI performed a clear inhibition to survival and progression of tumor cell regardless the dose, while this effect just appeared in the high dose treated B16-burdened animals. (3) PI's inhibitory effect on melanoma B16 metastasis performed in liver, kidney and lung, but only shown in liver when aspect to H22. (4) PI can regulate antibody levels in opposite ways between animals transplanting H22 and B16 cells, and make the humoral immunity tend to be normal eventually.

Acknowledgements

The authors gratefully acknowledge all the colleagues who contributed to this project. This work was supported by a grant from the Key Program of National Natural Science Funds of China (No.81072305).

References

- Fan J, Wang Q, Xue M, et al (2004). Primary research on culture medium of generating extracellular polysaccharide of *Phellinus ignarius*. *Food Science and technology*, **2**, 93-5.
- Ge Q (2009). Isolation, purification, Structural elucidation and chemical modification of polysaccharides from the fruiting bodies of *Phellinus baumii pilat*. Dissertation, Zhejiang University.
- Guo X, Zou X, Sun M (2010). Optimization of extraction process by response surface methodology and preliminary characterization of polysaccharides from *PI*. *Carbohydr Polym*, **80**, 344-9.
- Han SB, Lee CW, Jeon YJ, et al (1999). The inhibitory effect of polysaccharide isolated from *Phellinus linteus* on tumor growth and metastasis. *Immunopharmacology*, **41**, 157-64.
- Ikekawa T, Nakanishi M, Uehara N, et al (1968). Antitumor action of some basidiomycetes, especially *Phellinus linteus*. *Gann*, **59**, 155-7.
- Kang IC, Kim SA, Song GY, et al (2003). Effects of the ethyl acetate fraction of *Spatholobi caulis* on tumor cell aggregation and migration. *Phytother Res*, **17**, 163-7.
- Kim HM, Han SB, Oh GT, et al (1996). Stimulation of humoral and cell mediated immunity by polysaccharide from mushroom *Phellinus linteus*. *Intern J Immunopharmacol*, **18**, 295-303.
- Kim YS, Park KS, Park HK, et al (1994). Compositional sugar analysis of antitumor polysaccharides by high performance liquid chromatography and gas chromatography. *Arch Pharm Res*, **17**, 337-42.
- Ladanyi H (1993). Effect of lentinan on macrophage cytotoxicity against metastatic tumor necrosis factor. *Immunol Immunother*, **36**, 123-6.
- Lee HJ, Lee HJ, Lim ES, et al (2005). Cambodian *Phellinus linteus* inhibits experimental metastasis of melanoma cells in mice via regulation of urokinase type plasminogen activator. *Biol Pharm Bull*, **28**, 27-31.
- Li DF, Ge HQ, Tai JH, et al (1997). Effects of astragalus and ginsenoside on the *in vitro* anti-tumor activities of infiltrating lymphocyte in pulmonary carcinoma. *Chinese J Immunol*, **13**, 296-8.
- Li GJ, Wu GY, Cui JC, et al (1998). Culture and application of mycelia of *Phellinus linteus*. *Edible fungi of China*, **17**, 27-8.
- Li MJ, Wu DL, Wu DZ, et al (1997). Antitumor effect of Lentinan on murine sarcoma S180 model. *J Immunological*, **13**, 243-5.
- Li XL, Wang ZH, Zhao YX, et al (2012). Isolation and antitumor activities of acidic polysaccharide from *Gynostemma pentaphyllum* Makino. *Carbohydrate Polymers*, **89**, 942-7.
- Liang M, Li S, Shen B, et al (2012). Anti-hepatocarcinoma effects of *Aconitum coreanum* polysaccharides. *Carbohydrate Polymers*, **88**, 973-6.
- Liu L, Jia J, Zeng G, et al (2013). Studies on immunoregulatory and anti-tumor activities of a polysaccharide from *Salvia miltiorrhiza* Bunge. *Carbohydrate Polymers*, **92**, 479-83.
- Liu MG, Tao LX, Liang XQ, et al (2001). Analysis of effects of aweto against planted tumor of liver carcinoma ascetic strain on juvenile mice of different sexes. *J Guangxi Med University*, **18**, 21-3.
- Liu SX, Li NC and Fei HB (1995). Effects of on Lentinan infusion immune function in children with recurrent respiratory tract infections. *Shanghai J Immun*, **15**, 27-8.
- Prabhu VV, Guruvayoorappan C (2013). Inhibition of metastatic lung cancer in C57BL/6 mice by marine mangrove *Rhizophora apiculata*. *Asian Pac J Cancer Prev*, **14**, 1833-40.
- Qiu S, Huang S, Huang J, et al (2010). Antitumor activity of the water-soluble polysaccharide from *Hyriopsis cumingii* *in vitro*. *Toxicol Industrial Hlth*, **26**, 151-61.
- Su S, Zhou H, Xue M, et al (2013). Anti-tumor efficacy of a hepatocellular carcinoma vaccine based on dendritic cells combined with tumor-derived autophagosomes in murine models. *Asian Pac J Cancer Prev*, **14**, 3109-16.
- Thornton BP, Vetvicka V, Pitman M, et al (1996). Analysis of the sugar specificity and molecular location of the β -glucan-binding lectin site of complement receptor type 3 (CD11b/CD18). *J Immunol*, **156**, 1235-46.
- Wang YH (2008). Effects of dry matter of culture broth and its fractions from *PI* in submerged culture on transplanted murine Hepatoma 22 in mice. Dissertation, Jiang Nan University, 2008.
- Wen K, Chen J, Li H, et al (2002). Comparison of antitumor activity among four antitumor drugs including *Phellinus linteus*. *J Jilin University*, **28**, 247.
- Wu L, Liu YY, Li ZX, et al (2014). Anti-tumor effects of penfluridol through dysregulation of cholesterol homeostasis. *Asian Pac J Cancer Prev*, **15**, 489-94.
- Wu WW, Zhang P, Li SH, et al (2007). The direct repress sive effect of polysaccharide from *Phellinus baumii pilat* on tumor cell line S180. *J Jinzhou Medical College* **28** 18-20.
- Xiong JA, Lu HD (2000). Effect of Lentinan on immune functions of patients with recurrent respiratory tract infection. *Chinese J Pharmacoepidemiology*, **9**, 126-7.
- Yang JH, Hu J, Wan L, et al (2014). Barbigeron inhibits tumor angiogenesis, growth and metastasis in melanoma. *Asian Pac J Cancer Prev*, **15**, 167-74.
- Yang Q (2002). Liquid fermentation of *PI* and antitumor activity of proteoglycan from its mycelia. *China excellent master thesis database*, **9**, 126-7.
- Zhang BH and Ning CQ (2003). The research progress of liver cancer chemotherapy. *Foreign Med Sci Oncol Sect*, **30**, 384-7.
- Zhang CF and Huang RH (2007). The pharmacological effects of R are Medicinal Bacterium-*PI*. *Special wild economic anima land plant research*, **1**, 68-70.
- Zhang JT (1998). Modern Experimental Methods in Pharmacology. Beijing Medical University Peking Union Medical College Press, Beijing, 739-43.
- Zhang M, Ji XG, Bei ZC (2006). Studies on the anti tumor activity of *Phellinus linteus*. *Pharmacol Clinics Chinese*, **22**, 56-8.
- Zhao L, Zhang HF (2008). Inhibitory effects of polysaccharides extracted from *Phellinus linteus* on the proliferation and metastasis of Ho-8910 PM and Bcap-37. *J East China Normal University*, **2**, 78-84.