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RESEARCH ARTICLE

A Huaier polysaccharide inhibits hepatocellular carcinoma growth and metastasis

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Abstract This study was carried out to evaluate the effects of a Huaier polysaccharide (TP-1) on the tumor growth and immune function in hepatocellular carcinoma (HCC) H22-based mouse in vivo. Results showed that TP-1 was capable of repressing transplanted H22 solid hepatic tumor cell growth in vivo, prolonging the live time of mice bearing ascetic H22 tumors, and repressing the pulmonary metastasis of H22-bearing mice. Moreover, the relative weight of immune organ (spleen and thymus) and lymphocyte proliferation were improved after TP-1 treatment. Furthermore, the treatment with TP-1 could promote immune-stimulating serum cytokines, such as IL-2 and IFN-γ, but inhibit immune-suppressing serum cytokines IL-10 secretion in H22-bearing mice. Besides, the percentage of CD4+ T cells and NK cells was increased, whereas the number of CD8+ T cells decreased in tumor-bearing mice following TP-1 administration. In addition, this compound displayed little toxic effects to major organ of tumor-bearing mice at the therapeutic dose, such as the liver and kidney. This experimental finding suggested that TP-1 exhibited prominent antitumor activities in vivo via enhancement of host immune system function in H22 tumor-bearing mice. This product could be developed individually as a safe and potent biological response modifier for HCC therapy.

Keywords Huaier polysaccharide · Antitumor · Metastasis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent fatal malignancies and the third leading cause of cancer-related mortality [1, 2]. It is an important public health problem throughout the world, particularly in developing countries [3]. In China, HCC is one of the most common causes of malignancy-related death [4]. HCC at advanced stages usually carries a poor prognosis. The high recurrence and metastasis account for the poor prognosis in HCC patients. Despite endeavors to prevent postoperative metastasis in clinical practice, the overall survival rate is still rather dismal [5, 6]. Badly, there is no effective systemic chemotherapy for advanced HCC patients. The therapeutic success of chemotherapeutic agents is often limited by severe adverse effects [7]. Therefore, the development of potential agents, which exert greater efficacy to HCC with no or restricted toxicity to normal cells, has special priority for these patients.

Increasing evidence shows that traditional Chinese medicines can be considered as potential drugs to prevent invasion and metastasis of HCC. For example, brucine, an alkaloid from seeds of *Strychnos nux-vomica* Linn., represses HCC cell migration and metastasis via inhibition of the HIF-1 pathway [8]. Recently, reports showed that Huaier polysaccharide can inhibit the growth and metastatic potential of HCC cells through modulation of the AEG-1/EMT pathway [9]. AEG-1 shRNA-induced anti-metastatic potency was also enhanced by Huaier polysaccharide via inactivating downstream

P13K/Akt pathway as well as augmenting cell-mediated immune response [10]. In view of these meaningful results, we purified one homogenous polysaccharide, named TP-1, from this Huaier fungus water extract and examined its effect and relevant mechanism on HCC cell growth and metastasis. Data from authors' institution (2012–2014, unpublished) revealed that the inhibition of cell proliferation and metastasis by TP-1 was achieved not only by reducing the expression of AUF1, AGE 1, and N-cadherin but also by enhancing miR-122 and E-cadherin expression in HCC cells. We also provided novel in vivo evidence that TP-1 attenuated tumor growth and pulmonary metastasis by induction of apoptosis and inhibition of angiogenesis, which is consistent with the in vitro findings. To the best of our knowledge, many polysaccharides extracted from herbs exhibit antitumor and anti-metastatic activities through the immune response of the host organism [11]. However, whether TP-1 exerts anti-invasion and anti-metastasis activity through enhancing immure function remains unclear. Thus, we investigated the effect of TP-1 on tumor growth and pulmonary metastasis in a murine HCC H22 model in vivo, and its effects on host immune of animals were also examined.

Materials and methods

Materials and chemicals

Huaier extract was donated by Qidong Gaitianli Pharmaceutical Co., Ltd. (Jiangsu, China). Sepharose CL-6B and DEAE-cellulose-52 were purchased from Amersham Pharmacia Co. (Sweden). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), and propidium iodide (PI) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Fluorescein isothiocyanate (FITC)-labeled rat anti-mouse CD4+, phycoerythrin (PE)-CD8+, and PE-CD49b were purchased from Pharmingen (San Diego, CA). Cytokine (IL-2, IL-10, and IFN-γ) detecting ELISA kits were from US R&D Systems, Ltd. (Minneapolis, USA). All other chemicals were of the highest commercial grade available.

Isolation and purification of polysaccharide TP-1

After the filtered Huaier extract (8 %) was washed with Sevage reagent (isoamyl alcohol and chloroform in 1:4 ratio) to remove the protein [12], the supernatant was concentrated to 80 ml and precipitated by the addition of 95 % ethanol to a final concentration of 40 % (*v*/*v*) at 4 °C overnight. Next day, the precipitate was recovered by centrifugation and washed successively with absolute ethanol, acetone, and ether, followed by drying in vacuo at 45 °C, yielding the crude polysaccharide (TCP-40).

The filtrate of TCP-40 through a 0.45-μm millipore membrane was applied to DEAE-cellulose-52 gel filtration column chromatography (2.6 cm×30 cm) and eluted stepwise with distilled water, 0.1 and 0.5 M NaCl at a flow rate of 2 ml/min. Each eluted solution (5 ml) was collected and monitored by carbohydrate content based on phenol-sulfuric acid method at 490 nm absorbance. The 0.1 M NaCl elution was concentrated, dialyzed, and lyophilized to yield one fraction (TCP-40-1). The resulting TCP-40-1 was purified on a Sepharose CL-6B column (2.6×100 cm) and eluted with 0.15 M NaCl at a flow rate of 1 ml/min to yield one purified polysaccharide (TP-1).

Cell line and animals

Murine H22 ascitic hepatoma cells were maintained by weekly transplanting them into the peritoneal cavities of Kunming mice. Kunming mice weighing 18–22 g were purchased from the Laboratory Animal Center of Capital Medical University (Beijing, China). The animals were housed for 1 week in a room maintained at 25±1 °C with 60 % relative humidity under a standard specific pathogen free (SPF) condition and had free access to food and water. All animal procedures were performed following the protocol approved by the Institutional Animal Care and Use Committee at Capital Medical University.

Evaluation of antitumor effect in vivo

To determine the effect of TP-1 on tumor growth, the mice were injected subcutaneously into the left axillary region of each mouse with 0.2 ml cell suspensions (5×10^6 cells per mouse). When the tumor size grow to an average diameter of 100 mm^3, the H22-bearing mice were randomly divided into three groups with 10 mice for each group receiving normal saline (NS), 25, or 50 mg/kg of TP-1 via intraperitoneal injection once daily for 14 days, respectively. The doses were chosen according to their acute toxicity (1/2 LD10). The volume of the solid tumor (TV) was measured with a digital caliper every other day after treatment and was calculated according to the following formula: TV (mm^3)=$[a\times b^2]/2$, where a and b represent the largest diameter and the smallest diameter, respectively. At the end of experiment, all the mice were sacrificed and the transplanted tumors were dissected out and weighted immediately. The tumor inhibition rate (IR) was calculated by the following formula: IR (%)=(1−average tumor weight of treated group/control group)×100 [13].

Evaluation of life prolonging effect in vivo

For the ascites tumor model, the mice were injected with 0.2 ml of H22 cells (5×10^7) by intraperitoneal injection. The next day, the tumor-bearing mice were randomly assigned to one of the following three treatment groups (n=10 mice per

group): NS, TP-1 (25 mg/kg), and TP-1 (50 mg/kg). Treatments were administered via intraperitoneal injection once daily for seven consecutive days. Then, mice in each group were observed daily to monitor their survival time beginning the first drug administration. The life prolongation survival rate (SR) was calculated using the following formula: SR (%)=(1−numbers of mice died in each group/10)×100. The test was continued for 60 days and those survived for more than 60 days were defaulted as 60 days.

Evaluation of anti-metastatic effect in vivo

To establish an in vivo pulmonary metastasis model, H22 cells (5×10^6 cells per mouse) suspended in saline were injected into male Kunming mice via the tail vein. Each mouse was weighed immediately after inoculation. Drug administration began after pulmonary metastasis was allowed to develop for 10 days. On day 11, the tumor being mice were randomly divided into three groups (n=10 mice per group) and treated in the same way of the former experiment for 1 week. Another 10 mice as normal control group received 0.9 % normal saline for the same conditions. Twenty-four hours after the final administration of tested drug on the 7th day of the experiment, blood samples were drawn from the eyes of those experimental mice and collected in heparinized tubes and EDTA tubes for assessment of blood physiochemical parameters. All the mice were killed by pulling and breaking of the cervical vertebra, and the whole body and relevant organs, including the lung, spleen, and thymus, were dissected out and weighted immediately. After the lung was fixed in Bouin's solution (saturated picric acid/formalin/acetic acid = 15:5:1), the metastatic nodules in the lungs of mice from all groups were counted under a dissecting microscope to evaluate the therapeutic effect of TP-1. The metastasis inhibitory rate was calculated as follows: MIR=(1−average number of lung metastatic nodes in treated group/average number of lung metastatic nodes in control group)×100 %. The organ indexes of the spleen and thymus were calculated as follows: Organ index=average organ weight (mg)/average body weight (g)×10.

Lymphocyte proliferation assay

Spleen lymphocytes were prepared under aseptic conditions as described by Li et al. [14]. Lymphocytes were plated into 96-well plates (1×10^7 cells per well) with concanavalin A (ConA, 2 mg/l) or lipopolysaccharide (LPS, 5 mg/l) and incubated for 68 h at 37 °C in a humidified 5 % CO_2 incubator. After incubation, 50 μl of MTT solution (2.0 mg/ml in PBS) was added to each well and incubated for another 4 h at 37 °C. The precipitated formazan was dissolved in 150 μl DMSO and the plates were read at 570 nm with a 630-nm reference using an ELISA reader (Bio-Rad, USA). The stimulation index (SI) was calculated based on the following formula: SI=the absorbance value for experimental/the absorbance value for control.

Flow cytometry analysis

At the end of the study, the spleens were dissected out and their single-cell suspensions were stained with the following rat anti-mouse antibodies: fluorescein isothiocyanate (FITC)-labeled anti-CD4, phycoerythrin (PE)-CD8, and PE-CD49b. The percentages of CD4+ T, CD8+ T, and natural killer (NK) cells (indicated by CD49b positive) were determined by a flow cytometry (FACScalibur, Becton Dickinson, USA) and analyzed using CellQuest software as previously described [15].

Blood physiochemical assays

The blood samples of mice in each group were centrifuged at 3,000×g for 10 min, and the serum was collected for the detection of a series of physiochemical indexes, including aspartate aminotransferace (AST), alanine aminotransferace (ALT), blood urea nitrogen (BUN), uric acid (UA), and creatinine (CRE) using an automatic Burker hemocytometer according to the manufacturer's instructions.

Serum cytokine concentration determination

The serum levels of cytokines, such as IL-2, IL-10, and IFN-γ, were determined by commercial ELISA kit according to the manufacturer's instructions.

Statistical analysis

All experimental data were expressed as mean±SD, and statistical analysis was performed using Student's t test to compare the results from the untreated group.

Results and discussion

Effect of TP-1 on tumor growth, life extension, and pulmonary metastasis of H22-bearing mice

To further evaluate the antitumor activity of TP-1 in vivo, three H22 tumor transplant models were performed as following: solid tumor for tumor growth inhibition evaluation; ascites tumor for life survival evaluation; and pulmonary metastasis tumor for tumor metastasis evaluation.

The antitumor effects of TP-1 were checked on H22-bearing mouse solid model in vivo. As shown in the curves of tumor growth volumes (Fig. 1a), on day 14, the average volume of the tumors in control group grew rapidly and

reached 761 mm^3, while that in TP-1-treated group had increased relatively slowly and attained only 452 and 389 mm^3 at 25 and 50 mg/kg, respectively. Also, the tumor weight in H22 tumor-bearing mice treated with TP-1 was less than that of control mice (Fig. 1b), and the difference showed a statistical significance ($P<0.01$). TP-1 at doses of 25 and 50 mg/kg significantly inhibited the growth of H22 hepatoma by 49.60 and 57.26 %, respectively.

The effects of TP-1 on live time of H22-bearing mice were evaluated by measuring the increase of lifespan (Fig. 1c). The average survival time of H22-bearing mice treated with TP-1 at doses of 25 and 50 mg/kg was extended at least three times compared to that in NS-treated control. Survival rate in TP-1-treated groups was much high than those in NS-treated control group, suggesting that the survival time of tumor-bearing mice treated with the TP-1 were significantly prolonged.

In the lung metastasis model, mice treated with TP-1 (50 and 100 mg/kg) displayed a statistically significantly lower incidence of lung metastases ($P<0.01$ or $P<0.001$), whereas extensive metastatic nodules in the lungs were observed in mice treated with NS (Fig. 1d). The MIR in two doses of 50 and 100 mg/kg of TP-1 was 38.67 and 61.05 %, respectively, with relative to control as 100 %. In addition, there was no obvious change in the lung mass index or body weights of the mice treated with NS or TP-1 (data not shown).

Effect of TP-1 on the weight of immune organ and lymphocyte proliferation of H22-bearing mice

Many evidences had documented that the anticancer activities of polysaccharide–protein complexes could be associated with their immunostimulating properties [16]. To evaluate whether TP-1 administration had an influence on the immune system, we first determined the immune organ indexes in tumor-bearing mice following TP-1 or NS treatment. The results in Fig. 2a showed that two indexes in all TP-1 groups increased, which was near to or exceed the normal levels, but was not statistically different with those in model control ($P>0.05$)

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses [17]. Therefore, we investigated the effect of TP-1 on lymphocyte proliferation in tumor-bearing mice. As shown in Fig. 2b, spleen lymphocyte proliferation induced by ConA and LPS

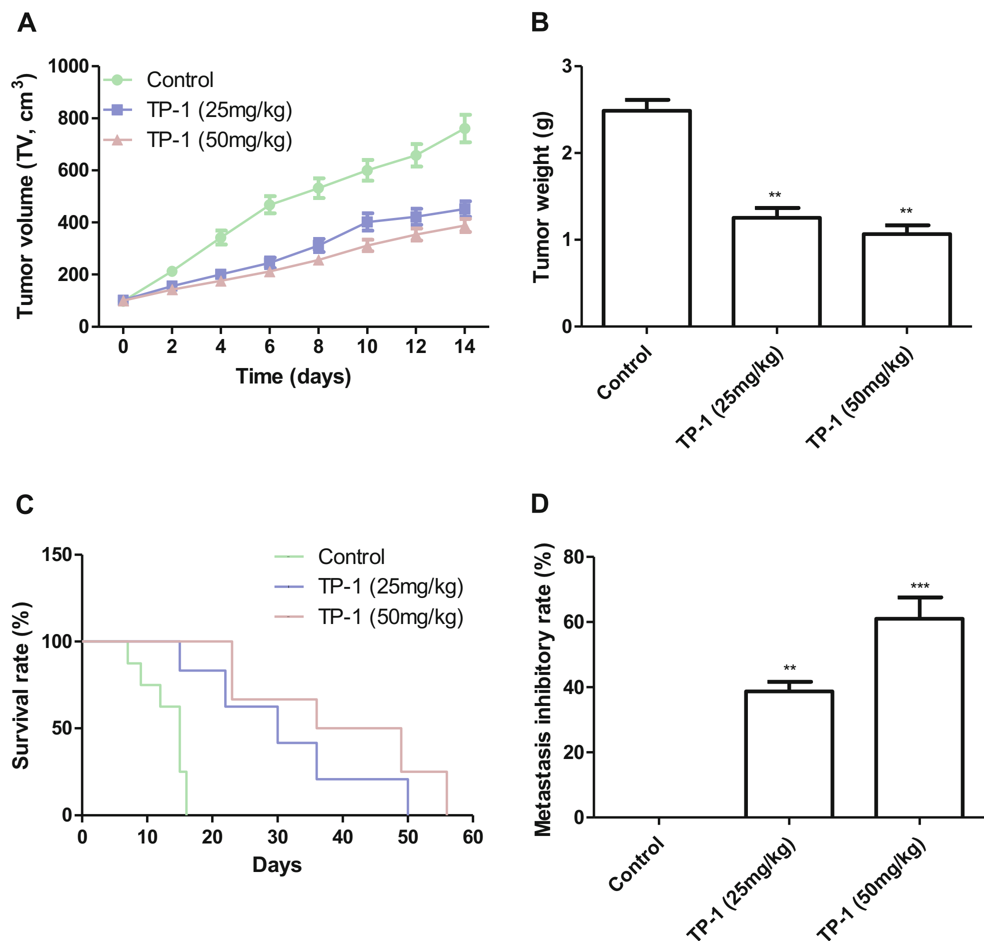

Fig. 1 Antitumor activity of TP-1 was evaluated in vivo. **a** The mean tumor weight in subcutaneous model; **b** tumor volume of mice bearing solid H22 in three groups; **c** survival rate (SR) of mice bearing ascites H22 in three groups; **d** metastasis inhibitory rate (MIR) of mice bearing pulmonary metastasis H22 in three groups. Data are presented as mean±SD (n=10). $^{**}P<0.01$, $^{***}P<0.001$ significantly different from control group

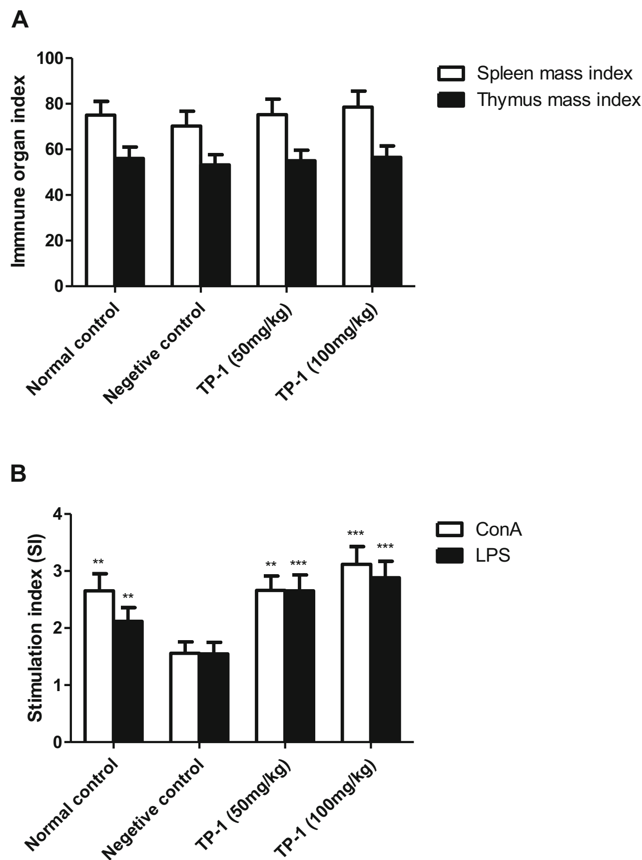

Fig. 2 **a** Effects of TP-1 on spleen and thymus index in pulmonary metastasis H22-bearing mice; **b** effects of TP-1 on spleen lymphocyte proliferation in pulmonary metastasis 22-bearing mice. Data are presented as mean±SD (n=10). $^{**}P$<0.01, $^{***}P$<0.001 significantly different from negative control group

declined significantly in H22 tumor-bearing mice compared with normal mice (P<0.01), suggesting the repressing effect of tumor on host immune functions. However, TP-1 administration at two doses markedly prompted the T- or B lymphocyte proliferation induced by ConA or LPS, respectively (P<0.01 or P<0.001), compared to those in H22-bearing mice. These data suggested that CSP had a beneficial effect to strengthen immunological response in H22 tumor-bearing mice.

Effect of TP-1 on hepatic and renal function parameters of H22-bearing mice

A perfect chemotherapeutics include not only the virtue of repressing tumorigenesis and metastasis but also the minimum toxicity to their normal organs. As we know, blood ALT, AST, and ALP are usually believed to be hepatic function markers, and blood BUN, UA, and CRE are considered as renal function markers. Their accumulation in blood usually result from hepatic disorders or the malfunctions of the kidney [18, 19]. In view this, we examine the potential toxicological effects of TP-1 administration on the kidney and liver of pulmonary metastasis 22-bearing mice by evaluating their serum hepatic function parameters including ALT and AST, and serum renal function parameters such as BUN, UA, and CRE. The data in Fig. 3a demonstrated ALT and AST level in model control had a marginal elevation than those in normal control, but is not significant (P>0.05). This slight increase in ALT and AST was attenuated and restored to the normal level in H22-bearing mice after TP-1 administration, although they were not significant (P>0.05). Simultaneously, tumor transplantation gave rise to a substantial increase in the level of BUN, UA, and CRE as compared to the normal control (P<0.05). All these indexes were ameliorated, at least in part, after TP-1 administration (Fig. 3b). Collectively, these data indicated that TP-1 exhibited very few toxicological effects to major organ of tumor-bearing mice at the dosages of 25 and 50 mg/kg.

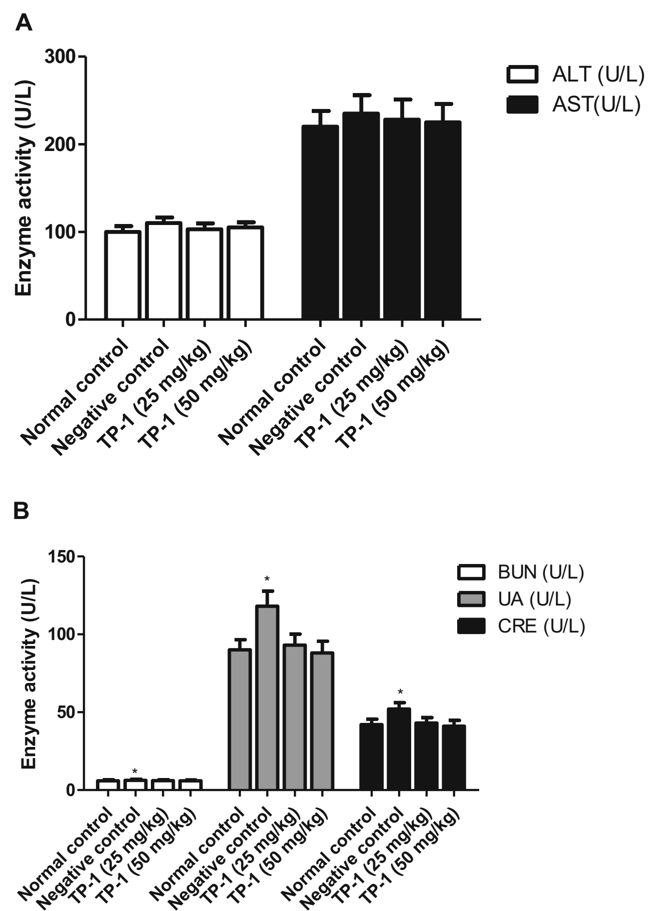

Fig. 3 **a** Effects of TP-1 on serum hepatic function parameters ALT and AST activities in pulmonary metastasis H22-bearing mice; **b** effects of TP-1 on serum renal function parameters BUN, UA, and CRE activities in pulmonary metastasis H22-bearing mice. Data are presented as mean± SD (n=10). $^{*}P$<0.05 significantly different from negative control group

Effect of TP-1 on the level of serum cytokines of H22-bearing mice

Cytokines have been shown to play a critical role in the modulation of innate and adaptive immune responses for fighting against the tumor growth [14, 20]. IFN-γ and IL-2 are immune-stimulating factors, but IL-10 is an immune-suppressing factor [21]. As shown in Fig. 4, inoculation with ascitic H22 tumor cells to mice resulted in a decrease of IFN-γ and IL-2, but induced an increase in IL-10 level in serum ($P<0.05$ or $P<0.01$, compared to normal mice), implying a status of cancer-induced immunosuppression. However, the concentration of IL-2 and IFN-γ in serum of H22-bearing mice was remarkably restored, and IL-10 was decreased after TP-1 treatment in H22 tumor-bearing mice, compared with those in model control mice administrated with NS. All the data imply that TP-1 might have the potential to alleviate tumor transplantation-induced immunosuppression in H22 tumor-bearing mice by modulating the cytokines release.

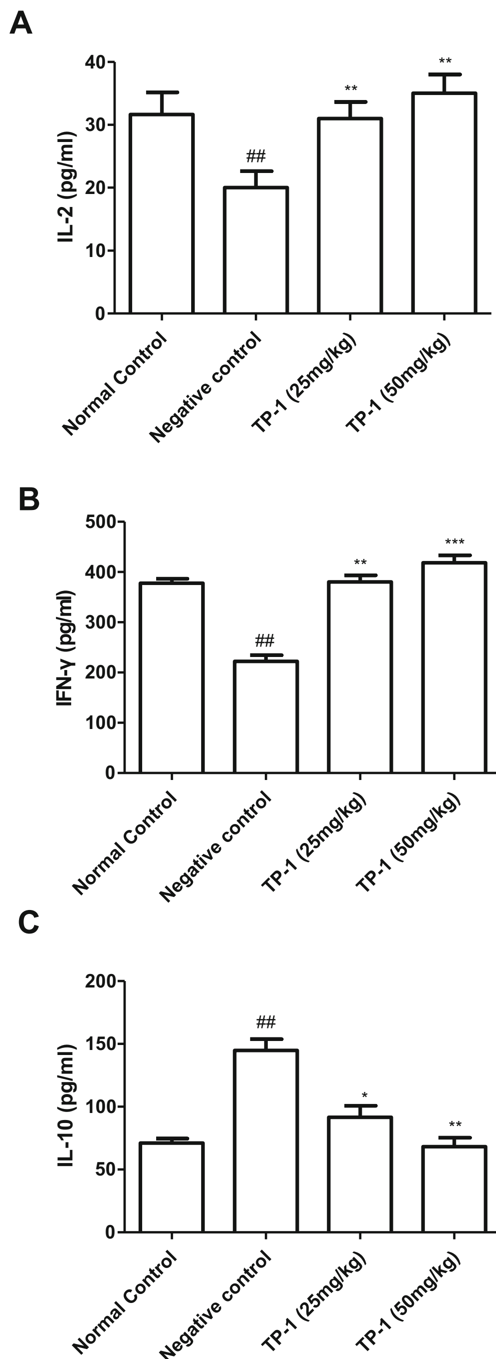

Fig. 4 Effects of TP-1 on the concentration of serum cytokine **a** IL-2; **b** IFN-γ; and **c** IL-10 in pulmonary metastasis H22-bearing mice. Data are presented as mean±SD (n=10). $^{\#\#}P<0.01$ significantly different from normal control group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ significantly different from negative control group

Effect of TP-1 on the percentage of splenic CD4+, CD8+ T lymphocytes, and NK cells of H22-bearing mice

CD4+ T, CD8+ T, and NK cells are capable of inducing the death of tumor cells and played pivotal roles in host antitumor immune response [22, 23]. To further study the antitumor immunity trigged by TP-1, we counted the numbers of CD4+ T, CD8+ T, and NK cells in the splenocytes of the tumor-bearing mice using flow cytometry analysis (FACS). Figure 5 demonstrated that the percentages of CD8+ T cells and NK cells remained almost unchanged in normal control and negative control mice. However, the percentage of splenic CD4+ T cells decreased significantly in H22-bearing mice treated with NS as compared to their non-tumor counterparts ($P<0.01$). Treatment with TP-1 resulted in significantly increased quantities of CD4+ T cells and NK cells compared with the model control group ($P<0.05$ or $P<0.01$), while that of CD8+ T cell was significantly decreased by TP-1, especially at the dose of 50 mg/kg ($P<0.05$). As the results, the frequency of CD4+ T and NK cells and the ratio of CD4+/

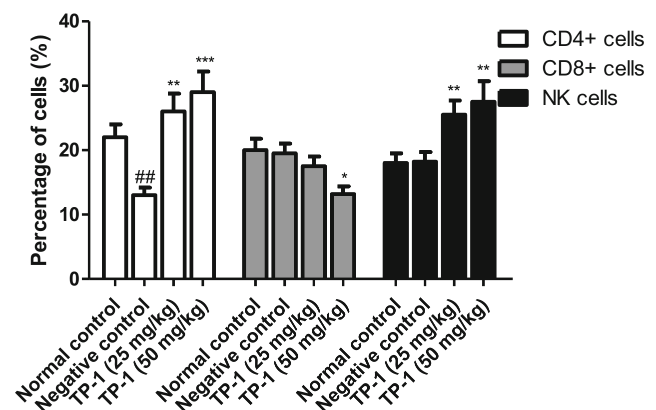

Fig. 5 Effects of TP-1 on the percentages of (a) CD4+ T; (b) CD8+ T; and (c) NK cells in pulmonary metastasis H22-bearing mice. Data are presented as mean±SD (n=10). $^{\#\#}P<0.01$ significantly different from normal control group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ significantly different from negative control group

CD8+ in the spleen of tumor-bearing mice were elevated following treatment with TP-1. To the best of our knowledge, helper T cells are called as CD4+ T cells, which can excrete cytokines and express CD4 molecules on their surface, and cytotoxic T cells, also known as CD8+ T cells, which express CD8 on their surface [24]. These results suggested that TP-1 administration could activate helper T cells to exert their immunomodulatory effects in tumor-bearing mice.

Conclusions

The present study demonstrated that TP-1, a purified polysaccharide from Huaier, exerted more potent antitumor activity in inhibiting subcutaneous xenograft tumor growth, prolonging survival time, and repressing lung metastasis in mice. The antitumor and immunostimulatory activities of TP-1 have been demonstrated in pulmonary metastasis H22-bearing mice models, which may be related to its ability to prompt the lymphocyte proliferation, enhance the secretion of IFN-γ and IL-2, decrease IL-10 level and CD8+ T cells proportion, and upregulate the percentage of CD4+ T cells and NK cells. Encouragingly, preliminary toxicological evaluation demonstrated that TP-1 had no obvious systemic toxicity to major organ of tumor-bearing mice at the therapeutic dose, evidenced by the decreased concentrations of hepatic function markers, ALT and AST, and renal function parameters, BUN, UA, and CRE. These results suggest that TP-1 deserves further study as a potential adjuvant reagent to stimulate anticancer immune response in clinical HCC treatment.

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Conflicts of interest None

References

1. Yang JD, Roberts LR. Hepatocellular carcinoma: a global view. Nat Rev Gastroenterol Hepatol. 2010;7:448–58.
2. Linnemann U, Schimanski CC, Gebhardt C, Berger MR. Prognostic value of disseminated colorectal tumor cells in the liver: results of follow-up examinations. Int J Colorectal Dis. 2004;19:380–6.
3. Parkin DM, Pisani P, Ferlay J. Estimates of worldwide incidence of eighteen major cancers in 1985. Int J Cancer. 1993;54:594–606.
4. Yuan SL, Wei YQ, Wang XJ, Xiao F, Li SF, Zhang J. Growth inhibition and apoptosis induction of tanshinone II-A on human hepatocellular carcinoma cells. World J Gastroenterol. 2004;10: 2024–8.
5. El-Serag HB. Hepatocellular carcinoma. N Engl J Med. 2011;365: 1118–27.
6. Parkin DM, Bray F, Ferlay J. Global cancer statistics CA. Cancer J Clin. 1999;55:74–108.
7. Coradini D, Zorzet S, Rossin R, Scarlata I, Pellizzaro C, Turrin C, et al. Inhibition of hepatocellular carcinomas in vitro and hepatic metastases in vivo in mice by the histone deacetylase inhibitor HA-But. Clin Cancer Res. 2004;10:4822–30.
8. Shu G, Mi X, Cai J, Zhang X, Yin W, Yang X, et al. Brucine, an alkaloid from seeds of *Strychnos nux-vomica* Linn., represses hepatocellular carcinoma cell migration and metastasis: the role of hypoxia inducible factor 1 pathway. Toxicol Lett. 2013;222:91–101.
9. Zheng J, Li C, Wu X, Liu M, Sun X, Yang Y, et al. Huaier polysaccharides suppresses hepatocarcinoma MHCC97-H cell metastasis via inactivation of EMT and AEG-1 pathway. Int J Biol Macromol. 2014;64:106–10.
10. Zheng J, Li C, Wu X, Liu M, Sun X, Yang Y, et al. Astrocyte elevated gene-1 (AEG-1) shRNA sensitizes Huaier polysaccharide (HP)-induced anti-metastatic potency via inactivating downstream P13K/Akt pathway as well as augmenting cell-mediated immune response. Tumour Biol. 2014;35:4219–24.
11. Wasser S. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Appl Microbiol Biotechnol. 2003;60:258–74.
12. Staub AM. Removal of protein-Sevag method. Methods in Carbohydr Chem. 1965;5:5–6.
13. Zhu BD, Yuan SJ, Zhao QC, Li X, Li Y, Lu QY. Antitumor effect of Gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, combined with cytotoxic agent on murine hepatocellular carcinoma. World J Gastroenterol. 2005;11:1382–6.
14. Li H, Sun M, Xu J, Li H, Zang M, Cui Y. Immunological response in H22 transplanted mice undergoing *Aconitum coreanum* polysaccharide treatment. Int J Biol Macromol. 2013;55:295–300.
15. Zhang Y, Yu G, Wang D, Hu Y, Lei W. ERK1/2 activation plays important roles in the opposite effects of Trichostatin A in non-cancer and cancer cells. Toxicon. 2011;57:932–7.
16. Ooi VEC, Liu F. Immunomodulation and anticancer activity of polysaccharide protein complexes. Curr Med Chem. 2000;7:715–29.
17. Singh SS, Haldar C, Rai S. Melatonin and differential effect of L-thyroxine on immune system of Indian tropical bird, *Perdicula asiatica*. Gen Comp Endocrinol. 2006;145:215–221.
18. Fu J, Fu J, Yuan J, Zhang N, Gao B, Fu G, et al. Anti-diabetic activities of *Acanthopanax senticosus* polysaccharide (ASP) in combination with metformin. Int J Biol Macromol. 2012;5:619–23.
19. Zeng G, Ju Y, Shen H, Zhou N, Huang L. Immunopontentiating activities of the purified polysaccharide from evening primrose in H22 tumor-bearing mice. Int J Biol Macromol. 2013;52:280–5.
20. O'Garra A, Arai N. The molecular basis of T helper 1 and T helper 2 cell differentiation. Trends Cell Biol. 2000;10:542–50.
21. Wang N, Yang J, Lu J, Qiao Q, Wu T, Du X, et al. A polysaccharide from *Salvia miltiorrhiza* Bunge improves immune function in gastric cancer rats. Carbohyd Polym. 2014;111:47–55.
22. Gerloni M, Zanetti M. CD4 T cells in tumor immunity. Springer Semin Immunopathol. 2005;27:37–48.
23. Brunda MJ, Luistro L, Warrier RR, Wright RB, Hubbard BR, Murphy M, et al. Antitumor and antimetastatic activity of interleukin-12 against murine tumors. J Exp Med. 1993;178:1223–30.
24. Shen H, Tang G, Zeng G, Yang Y, Cai X, Li D, et al. Purification and characterization of an antitumor polysaccharide from *Portulaca oleracea* L. Carbohydr Polym. 2013;93:395–400.