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Effects of tumor-specific antigen induced by lentinan on murine H22 hepatocellular carcinoma immunoprophylaxis

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Abstract. – OBJECTIVE: Hepatocellular carcinoma (HCC) is one of the most prevalent tumor types and the third most common form of morbidity in cancer-related deaths worldwide. Lentinan isolated from *Lentinus edodes*, is known to be a biologically active macromolecule with extremely strong activation of the human immune system such as host-mediated anti-cancer activity. The aim of this study is to investigate the immunoprophylaxis effect of the antigens induced by lentinan on murine hepatocellular carcinoma.

MATERIALS AND METHODS: The antigens were prepared by a co-culture method (HCL) and purified by ammonium sulfate fractionation precipitation (Z1, Z2, Z3). The effects of antigens on murine hepatocellular carcinoma immunoprophylaxis were determined *in vivo*. The cellular immunity of the immunized mice was tested by spleen lymphocyte proliferation tests and peritoneal macrophage phagocytosis assays. The tumor-specific antigen was confirmed by Western blot analysis.

RESULTS: Results *in vivo* revealed that the antigens (HCL/Z1) activated immunoprophylaxis against hepatocellular carcinoma with a better survival status. The survival rates (60%, 100%) of the HCL/Z1 group were better than the model group ($p < 0.01$). The quantity of lymphocytes in the spleen in the HCL or Z1 groups treated with ConA or LPS were higher than that of the model group ($p < 0.01$). The phagocytosis ability of macrophages in the HCL or Z1 groups was better than that of the control group or model group ($p < 0.01$). The characterization of Western blot analysis showed that about 59.6kDa tumor specific antigen combined with antiserum of immunized mice specifically appeared in antigens.

CONCLUSIONS: The newly generated tumor-specific antigen played a key role in the anti-tumor immune response and in activating the immune system. Our results suggest that this protein could serve as a tumor vaccine, and it could generate new ideas for tumor immunoprophylaxis.

Key Words:
Hepatocellular carcinoma (HCC), Lentinan, Immunoprophylaxis, Tumor-specific antigen.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent tumor types and the third most common form of morbidity in cancer-related deaths worldwide. The major risk factor for HCC is chronic infection with hepatitis B virus (HBV)[1,2], which accounts for 52% of all HCC, followed by chronic infection with hepatitis C virus (HCV)[3], human immunodeficiency virus (HIV)[4,5] and alcohol consumption[1]. Although surveillance with ultrasonography allows diagnosis at an early stage, when the tumor might be curable by resection, liver transplantation, or ablation, the optimal profile is that the HCC smaller than 2 cm[6,7]. US-guided biopsy is the gold standard, but a biopsy of such small nodules in a cirrhotic liver (cirrhosis) is not entirely reliable; sampling error may occur and it is very difficult to distinguish well-differentiated HCC from dysplastic nodules[8-11]. Therefore, a more effective

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path for HCC prevention or therapy, such as HCC immunoprophylaxis, should be developed to reduce the incidence of liver cancer.

Lentinan, a (1-3)-beta-d-glucan extracted from the mushroom *Lentinus edodes*, is a potent anti-cancer drug licensed in China for anti-tumor therapy since 1995[12]. The biological functions of lentinan include anti-inflammatory activity[13], cellular immunity promotion, immunostimulation, and anti-cancer effects[14]; the mushroom polysaccharides cause no harm and place no additional stress on the human body[15]. The proposed mechanism by which mushroom polysaccharides exert anti-tumor effects include direct anti-tumor activity by inducing the apoptosis of tumor cells (direct tumor inhibition activity)[16,17].

The immune system is a built-in host defense mechanism against infectious agents, including cancer[18]. In cancer immunology, the immune system is either passively or actively exploited to target and kill cancer cells[19,20]. Successful examples of immunoprophylaxis include vaccination for preventing cancers with a clear viral etiology such as cervical cancer[21]. A cancer antigen is the safety fuse for activation of cancer immunity. These antigens are produced in response to one or several mutated proteins that are typical of cancer, and they are the products of non-mutated genes that are expressed preferentially by cancer cells (for example, cancer-testis antigens), or differentiation antigens associated with the cancer's tissue of origin[22,23].

Preventative vaccines have been used successfully for the prevention of cancers of viral origin, such as hepatitis B virus and human papilloma virus (HPV), where the etiological agent is known[24,25]. Another prophylactic vaccine (CHCP, H22 cells and cartilage polysaccharide), which induces humoral immunity and cellular immunity in mice against hepatocarcinoma, has been reported by us[26,27]. Cartilage polysaccharide has not been used clinically and background information on this approach is also deficient. Therefore, we chose lentinan, which has been approved for its curative effect in the clinic as a surrogate for cartilage polysaccharide as an effective immunogen for murine H22 hepatocarcinoma immunoprophylaxis.

Materials and Methods

Ethics Statement

All experiments were conducted following the Guide for the Care and Use of Laboratory Animals of National Institutes of Health. This study was approved by the Animal Ethics Committee of Hebei University of Science and Technology (Permission number: 2014-02-01). The animals were allowed free access to food and water at all times and were maintained on a 12 h light/dark cycle in a controlled temperature (20 to 25°C) and humidity (50% ± 5%) environment for 1 week before use. The health of the mice was monitored by every day.

Animals and Cell Lines

Female Kunming mice that weighed 18-22 g were purchased from the animal center of Hebei Medical University, Shijiazhuang, China (Certificate Number: 1410014). Murine H22 hepatocarcinoma cell lines (China PLA General Hospital, Beijing, China) were passaged in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS).

Preparation of Antigens

1. Lentinan from *Lentinula edodes*, a β-(1→6) branched β-(1→3)-glucan used in this study was gifted from Kang Yuan (state medical permit No.H20067183, Jiangsu, China), and the compounding ingredient was sorbitol. H22 hepatocarcinoma cells (5×10^7cells/mL) were cultured (37°C, 5% CO_2) in RPMI 1640 containing 10% heat-inactivated FBS and Lentinan (165 μg/mL) for 48 h. The co-culture was frozen, thawed 5 times to inactivate living H22 hepatocarcinoma cells, and prepared as H22 cells with lentinan (HCL).
2. The supernatant of HCL, which was prepared by centrifugation at 8000 rpm/min for 15 min, was precipitated by adding ammonium-sulphate ($(NH_4)_2SO_4$) to create 30%, 60%, or 80% saturation at 25°C, to be labelled as groups Z1, Z2, or Z3, respectively. The solution was allowed to stand overnight at 4 □ to permit precipitation of proteins. After that, samples were centrifuged at 10,000 rpm/min for 15 min. The supernatant was discarded; the precipitate was dissolved in phosphate buffered saline (PBS) and dialyzed over night with PBS.

Animal Immunization Protocol

A sample of 120 mice were randomly and equally divided into 6 groups, including divided equally among the control group, model group, H22 cells plus lentinan (HCL) group, Z1 group, Z2 group, and Z3 group (n = 20). The HCL, Z1, Z2, and Z3 groups were immunized (intraperito-

nially (i.p.)) with HCL, Z1, Z2, and Z3 samples (0.2 mL/mouse), respectively, once per week for 3 weeks. The mice in control and model groups were treated with 0.9% normal saline. One week after the third immunization, all of the mice except the control group were injected i.p. with H22 hepatocarcinoma cells (1.5×10^6 cells/mouse) to establish the H22 hepatocarcinoma mouse model. Twelve days after establishment of the H22 mouse model, the body weights of the mice in each group were recorded. Ten mice in each group were selected randomly to calculate the immune organ (thymus and spleen) index or cellular immunity (pleen lymphocyte proliferation tests and peritoneal macrophages phagocytosis assays). The spleen or thymus was collected from each rat, and the growth index for each thymus and spleen was recorded.

Growth index of spleen = spleen weights (mg)/BW(g)

Growth index of thymus = thymus weights (mg)/BW(g)

Four weeks after establishment of the H22 mouse model, the successfully immunized mice and control mice of another 10 mice in each group were selected for blood collection by enucleation of the eyeball. After centrifugation, blood serum was stored at -80□ for the following studies. The average survival time of each group was recorded and the increase in life span (ILS) was calculated, according to this formula: ILS (%) = (average life span in an experimental group-average life span in the model group)/(average life span in model group) × 100.

Spleen lymphocyte Proliferation Tests

For the lymphocyte proliferation assay, spleen lymphocyte cells from each group were suspended at concentration of 2×10^6 viable cells/mL in RPMI 1640 (containing 10% FBS) medium. Triplicate cultures of spleen cells were set up in 96-well flat-bottom microtitre plates (Corning, NY, USA) in 100 μL volumes of the cell suspension with the Concanavalin A (ConA) or Lipopolysaccharide (LPS) (10 μg/mL). The cell cultures were incubated at 37°C in a humidified 5% CO_2 for 72 h. Four hours before the cells were harvested the methyl thiazolyl tetrazolium (MTT, Solarbio, Beijing, China) solution (0.5 mg/mL in PBS) was added. The formazan precipitate was dissolved in 150 μL dimethyl sulphoxide (DMSO, Solarbio, Beijing, China) and the absorbance of each well was read at 570 nm and 630 nm using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA).

Stimulus index (SI) = experimental $OD_{570-630}$/control $OD_{570-630}$

Peritoneal Macrophage Phagocytosis Assays

In order to test the phagocytosis activity of peritoneal macrophages, bouillon culture medium (Amidulin, 6%) was injected (i.p.) into the mice of each group once a day. Three days later, chick erythrocytes (1% suspension) was injected (i.p.) into mice and then the specimens of peritoneal fluid were harvested. Duplicate peritoneal fluid samples of each group were smeared on glass slides and stained with Wright Giemsa stain. One-hundred macrophages of each sample were observed. When chick erythrocytes appeared to be inside the cytoplasm, the macrophages cells were considered to be actively phagocytic. The numbers of activated macrophages and the chick erythrocytes in the macrophages were recorded. The phagocytosis rate and phagocytosis index were calculated, according to the reference[26].

Phagocytosis index = number of phagocytosed chick erythrocyte/100

Phagocytosis rate (%) = (number of activated macrophages/100) × 100%

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein concentration levels of the HCL supernatant were determined using the Bradford assay. The samples were then dissolved in SDS sample buffer solution containing 1% SDS, 1% b-mercaptoethanol, 1 mM EDTA, and 20% glycerin, and heated for 5 min at 100°C. Bio-Rad Precast Gel TGX 4%-15% was prepared and the samples were subjected to gel electrophoresis at a constant current of 20 mA per gel. The running buffer solution contained 0.1% SDS and 25 mM Tris-glycine buffer, at a pH of 6.8. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R250.

Western Blot Analysis

After finishing the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

as we described above, the proteins were transferred onto a wet nitrocellulose membranes. The membranes were incubated in blocking non-fat milk solution for 1h at 4°C to prevent non-specific background binding of the primary and/or secondary antibodies to the membranes. The primary antibodies (antiserum) as we described above (1:200) were incubated on the membrane at 4°C overnight. HRP-conjugated goat anti-mouse IgG (Solarbio, Beijing, China) diluted at 1:1500 as the secondary antibodies, was incubated on the membrane for 1h at 24°C. A DAB Color Developing Reagent Kit (Solarbio, Beijing, China) was used for strap coloration and analyzed with Quantity One software (Image Lab 5.0, BIO-RAD, Hercules, CA, USA).

Statistical Analysis

The data were analyzed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) and the difference was considered significant if $p < 0.05$. The data were presented as mean ± SD.

Results

Immunoprophylaxis Effects of Antigens on Mice in vivo

In order to establish a direct evidence for the immunoprophylaxis effects of antigens we have prepared, the body weight gain, survival rate and increases in life span of the mice were tested. The variation in average body weight gain of the mice in the Z1 or HCL groups was significantly different from that of model group ($p < 0.01$). These results suggest that less ascitic tumors developed in mice in the Z1 or HCL groups than in other groups (Figure 1a). The survival rate in the Z1 group (100%) was the best among all the experimental groups (Figure 1b).

Protection of Antigens on Immune Organ-spleen

The appearance of the spleen in HCL or Z1 mice, which had a carmine color and a fresh appearance, was similar to the control mice (Figure 2a). The growth index of spleens decreased in model mice. The growth index of spleens in the HCL ($p < 0.01$) and Z1 ($p < 0.01$) groups were significantly higher than those in the models (Figure2b).

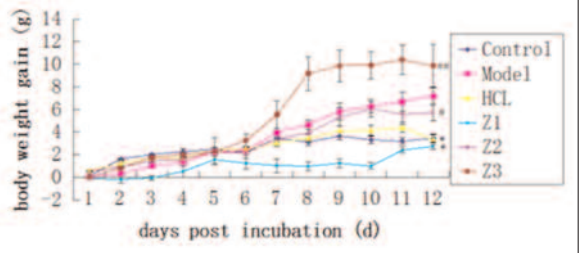
The Dynamic Changes in the Proliferation of Lymphocytes

The cell-mediated immune response to the antigens was measured after 72h of cultureing. The number of lymphocytes in the spleen in the HCL or Z1 groups treated with ConA or LPS was higher than that of the model group (Table I). The results showed that HCL or Z1 can arouse a T-or B-cell-mediated response in mice, which was greater than that observed in model mice.

Effect of Antigens on Macrophage Phagocysis in Immunized Mice

Peritoneal macrophages are important for cancer immunity, because they can kill cancer cells directly or indirectly. The phagocytosis ability of peritoneal macrophages were observation directly. Chick erythrocytes were ingested more avidly by macrophages from the mice that survived in the HCL or Z1 groups (Figure 3a, 3b). The

A



B

Group	Increases in life span (%)	Survival rate (%)
Model	0	0
HLC	98.42*	60*
Z1	109.07*	100*
Z2	37.31	40
Z3	0.63	0

Figure 1. Effects of antigens on body weight of mice and survival. HCL, H22 cell and lentinan; Z1, the precipitate of HCL supernatant to which was added ammonium-sulphate ($(NH_4)_2SO_4$) to 30% saturation; Z2, the precipitate of HCL supernatant to which was added $(NH_4)_2SO_4$ to 60% saturation; or Z3, the precipitate of HCL supernatant to which was added $(NH_4)_2SO_4$ to 80% saturation. ***A,*** Average body weight gain of mice in each group after modeling. ***B,*** The increase in life span (ILS) or survival rate of each group was calculated. *$p < 0.01$ compared to model.

B

Group	Thymus Index (ti)	Spleen Index (si)
Control	3.61±1.18	4.74±1.90
Model	0.53±0.26	1.29±0.35
HCL	2.68±0.51	4.56±0.41*
Z1	3.72±0.29*	4.49±0.63*
Z2	1.49±0.41	2.34±0.16
Z3	0.98±0.71#	1.17±0.64#

Figure 2. Effects of antigens on anti-cancer immunity. HCL, H22 cell and lentinan; Z1, the precipitate of HCL supernatant to which was added ammonium-sulphate ($(NH_4)_2SO_4$) to 30% saturation; Z2, the precipitate of HCL supernatant to which was added $(NH_4)_2SO_4$ to 60% saturation; or Z3, the precipitate of HCL supernatant to which was added $(NH_4)_2SO_4$ to 80% saturation. ***A,*** The spleen appearance of each group of mice was recorded. ***a,*** Control group, ***b,*** Model group, ***c,*** HCL group, ***d,*** Z1 group, ***e,*** Z2 group, ***f,*** Z3 group. ***B,*** ti (thymus index) and si (spleen index) of each group were tested. *$p < 0.01$ vs. model group, #$p < 0.01$ *vs.* control group.

phagocytosis rate and the phagocytosis index of immunized mice were significantly different ($p < 0.01$) compared with control mice (29%, 1.59) or model mice (28%, 1.31).

Table I. Spleen lymphocyte proliferation test results.

Group	SI (ConA)	SI (LPS)
Control	12.23 ± 0.25	9.10 ± 0.48
Model	1.87 ± 0.10	1.63 ± 0.03
HCL	6.27 ±0.21*	5.74 ± 0.34*
Z1	9.34 ± 0.16*	6.28 ± 0.36*
Z2	3.74 ± 0.09#	2.98 ± 0.11#
Z3	1.82 ± 0.14#	1.61 ± 0.08#

SI-stimulus index induced by ConA or LPS. HCL, H22 cell and lentinan; Z1, the precipitate of HCL supernatant to which was added ammonium-sulphate ($(NH_4)_2SO_4$ to 30% saturation; Z2, the precipitate of HCL supernatant to which was added $(NH_4)_2SO_4$ to 60% saturation; or Z3, the precipitate of HCL supernatant to which was added (NH4)2SO4 to 80% saturation. *$p < 0.01$ vs. model group; #$p < 0.01$ *vs.* control group.

Tumor-Specific Antigen Tested by Western Blotting

To investigate the tumor specific antigen, the serum of normal mice or the antiserum of immunized mice were used as the primary antibodies in western blot tests (Figure 4a). We observed the specific binding between the antiserum and HCL as shown in Figure 4a. According to the binding analysis, the molecular weight for specific binding was 59.6 kDa (Figure 4b,c).

Discussion

Hepatocellular carcinoma is a serious threat to humans. This type of carcinoma has a 90% incidence rate in primary liver cancer patients, because it can induced immune evasion against the immune reactions of the hosts to enhance cancer cell proliferation[28,29]. Cancer immunoprophylaxis has become an important subject for scientists globally in their attempt to prevent people from developing this type of cancer. Our previous studies suggested that H22 cells and cartilage polysaccharide (CHCP) can offer a new approach to induce immunomediated tumor reductions in murine H22 hepatocarcinoma[26,27]. Because cartilage polysaccharide has not been used clinically and background information on this approach is deficient, we chose lentinan, which has been approved for its anti-cancer effect on patients in a clinical setting[30-32]; lentinan may be a useful surrogate for cartilage polysaccharide in the search for an effective vaccine for murine H22 hepatocarcinoma immunoprophylaxis.

In the present study, HCL was used to immunize mice to verify the immunoprophylaxis effect on murine liver cancer. Compared with the model group, the results from the HCL group suggests a new approach to induce immune-mediated tumor suppress proliferation in murine H22 hepatocarcinoma. Our *in vivo* experiments showed that the survival rate and increase life span of the HCL group were better than that of the model group. The protective effect of lentinan in HCL (survival rate 60%) against H22 cells was better than that of cartilage polysacchride in CHCP (survival rate 50%)[26].

Thymus and spleen are important immune organs for vertebrates[33,34]. The growth index of the spleen (spleen weight normalized to body weight, SI) is the ratio of spleen weight and body weight, and it indicates the change in the immune function of the spleen[35-37]. The immune or-

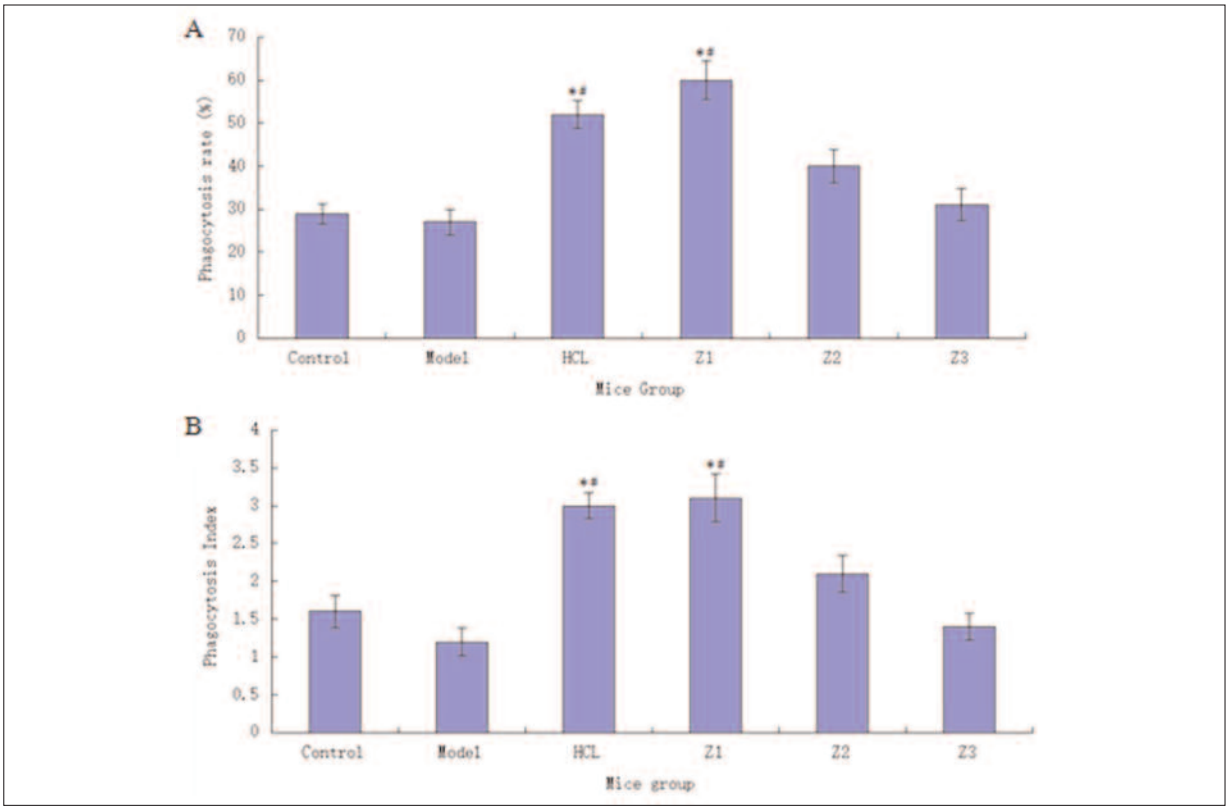
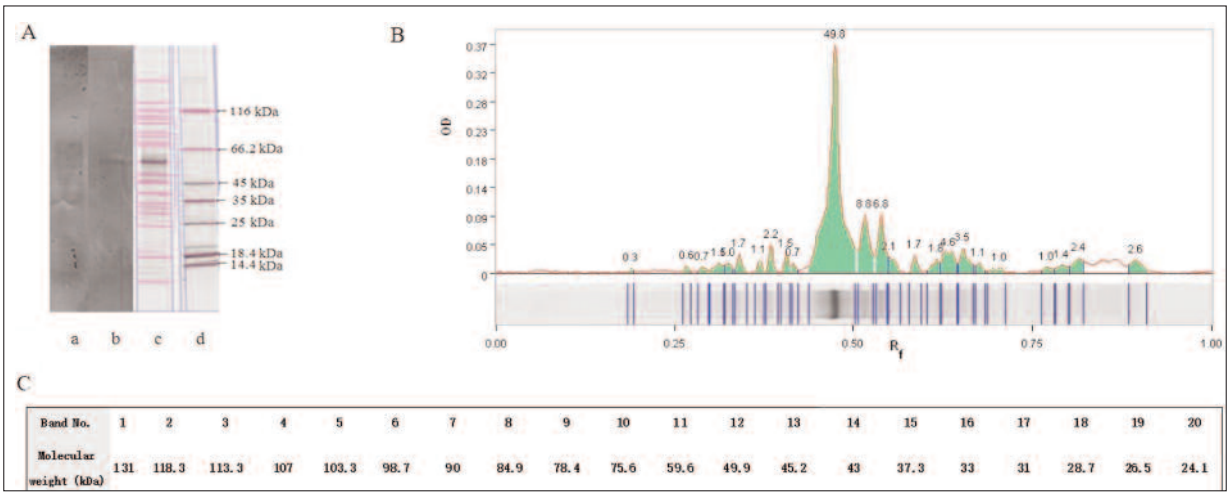


Figure 3. Effects of antigens on mice peritoneal macrophages phagocytosis. HCL, H22 cell and lentinan; Z1, the precipitate of HCL supernatant to which was added ammonium-sulphate ($(NH_4)_2SO_4$) to 30% saturation; Z2, the precipitate of HCL supernatant to which was added $(NH_4)_2SO_4$ to 60% saturation; or Z3, the precipitate of HCL supernatant to which was added (NH4)2SO4 to 80% saturation. A, Macrophages phagocytosis rate of each group (control, model, HCL, Z1, Z2 and Z3) was calculated. ***B,*** Macrophages phagocytosis index of each group (control, model, HCL, Z1, Z2 and Z3) was tested. *$p < 0.01$ vs. model group, $^{\#}p < 0.01$ *vs*. control group.



Band No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Molecular weight (kDa)	131	118.3	113.3	107	103.3	98.7	90	84.9	78.4	75.6	59.6	49.9	45.2	43	37.3	33	31	28.7	26.5	24.1

Figure 4. Tumor-specific antigen tested by Western blotting. Two blots were prepared using 10 μg total protein per blot from the supernatant of HCL. The blots were incubated with primary antibodies such as the serum of normal mice ***(a)*** or serum of immunized mice ***(b)*** respectively. ***A,*** Specific binding in Western blotting. a, the primary antibody was serum of normal mice; b, the primary antibody was serum of immunized mice; ***c,*** SDS-Page of proteins from HCL supernatant; ***d,*** Marker. ***B,*** Strap magnitude analysis of SDS-Page. ***C,*** Molecular weight analysis of SDS-Page.

gan (thymus and spleen) weight index and physical appearance organs of mice in Z1 group were more similar to that of control group than model group. This phenomenon may be explained by that the thymus and spleen of the immunized mice could inhibit proliferation of cancer cells.

Tumor-specific antigen(s) is vital in activating the immune system to protect mice from HCC[38]. The preliminary purification of a given protein which always be considered as an antigen is salting-out. The commonly used salt is ammonium sulfate, as it was soluble in water, and this salt has no adverse effects on enzyme or antibody activity[39,40]. In the present study, the Ammonium Sulfate Precipitation Method was chosen to purify antigens. We chose three ammonium sulfate concentrations of 30%, 60%, and 80% to isolate the protein in HCL in Z1, Z2, and Z3, respectively. These proteins were then used as immunogens to protect mice from HCC. The most effective treatment was shown by the Z1 group, which miraculously had a survival rate of 100%, and an increase in life span of 109.07%.

Perhaps, Z1 was the solution for which we were searching. In addition, the antigen-specific cellular immune responses against the H22 hepatocarcinoma antigen can be induced by Z1 immunization in a mouse model. According to the results *in vivo*, the immunity variance between Z1 group and model group was obvious. When the immune response was activated, it exhibited characteristics of cellular immunity, such as immuno-cell activation. In this study, the proliferation of splenic lymphocytes in Z1 group was higher than that of model mice. Peritoneal macrophages phagocytosis was also improved in Z1 group compared to that in the model group.

Western blot analysis is widely used to detect and compare the relative levels of particular proteins in cells, serum, and other biological materials[41,42]. To reveal the cancer-specific antigens, western blot tests helped us initially. Treated with the anti-serum of Z1 mice as the primary antibody, the specific binding protein of the HCL supernatant was located at about 59.6kDa. The protein with molecular weight of about 59.6kD may be the unknown protein we are looking for. On the other hand, the solubility of proteins varies according to the ionic strength of the solution, and salt concentration[43-45]. The protein purified by 30% ammonium sulfate always had higher hydrophobicity than hydrophilicity. Thus, the 59.6kDa protein that had the higher hydrophobicity will be our focus of attention in the future.

Conclusions

The present study provided evidence for the effects of HCL/Z1 on murine H22 hepatocellular carcinoma immunoprophylaxis. The cellular immunity resisted the hepatocarcinoma cell generation have been enhanced by the antigens (HCL/Z1). The 59.6kDa protein appeard to be reactive with the antiserum of immunized mice specifivally, to prove this protein maybe the tumor-specific antigen we were looking for. Therefore, the expression of potential tumor-specific antigen may play an important role in future liver cancer immunoprophylaxis.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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