Student number：　2014321023

Hunan University of Chinese Medicine

Ph.D. Dissertation

The Study to Evaluate the Synergistic Effect of Ginsenosides and Curcumin in Hepatocellular Carcinoma (HCC)

Specialized subject：　Integrative Chinese and Western Medicine

Research direction : HCC treatment based on Integrative Chinese and Western Medicine research

Student's name　　:

Supervisor's name, title　：

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Abstract

Objective : This research is aim to investigate the synergistic effect of Ginsenoside and Curcumin as PD-1/PD-L1 pathway blocker and NFkB, MMP9 inhibitor as anticancer in HCC and therefore could boost the immune system, suppress the inflammation, and prevent chemoresistancy and metastasis in animal HCC model.

Methods : 46 Hep G2 xenograft nude mice were randomly divided into a model group of 7 mice, a 2.6mg/25g bodyweight low dose ginsenoside group of 7 mice, a 13mg/25g high dose ginsenoside group of 6 mice, a 5mg/25g curcumin group of 7 mice, a curcumin+low dose ginsenoside group of 6 mice, a curcumin+high dose ginsenoside group of 7 mice, and a 0.6mg/25g 5FU + 0.1mg/25g Cisplatin chemotherapy group of 6 mice., raised in SPF environment. HCC cells were transplant into mice subcutaneous. Curcumin and ginsenoside were given orally once every 2 days for 18 consecutive days, while 5FU and Cisplatin were injected intraperitoneal once a week. Tumor volume and weight were measured. PD-1 and NFkB expression were detected by western blotting. Data would be analyzed to proof the effectivity of these compound

Results : The tumor volume significantly inhibited by curcumin , ginsenoside, and combination of both, compared with the model group ($p<0.05$). There are no significant difference of tumor growth between combination of curcumin and ginsenoside with chemotherapy group ($p>0.05$). There are significant anti PD-1 effect of ginsenoside compared with other groups ($p<0.05$). There are significant NFkB inhibitory effect in the group treated with combination of curcumin and ginsenoside compared with other groups ($p<0.05$).

Conclusion : Ginsenoside and curcumin significantly inhibit tumor growth in HCC in vivo. Ginsenoside significantly downregulated the PD-1 expression in HCC. Combination of ginsenoside and curcumin have significant NFkB inhibitory effect. The effect ginsenoside and curcumin in vivo may be related to their mechanism to increase immune system, anti-inflammatory and prevent chemoresistancy

Keywords :Curcumin, Ginsenoside, , PD-1, NFkB ,

Abbreviations

HCC	Hepatocellular Carcinoma
PD-1	Programmed Death 1
NFkB	Nuclear Factor kappa-light-chain-enhancer of activated B cells

Introduction

Cancer is a chronic health problem and predicted to be a worldwide important cause of morbidity and mortality in the next few decades. HCC is largely a problem of less developed regions where 83% (50% in China alone) of the estimated 782,000 new cases worldwide occurred in 2012. It is the fifth most common cancer in men and the ninth of women. HCC is the second common cause of death from cancer worldwide, estimated to be responsible for nearly 746,000 deaths in 2012 (9.1% of the total). [1-6] By 2020 there will be approximately 24.6 million people worldwide will live with cancer with about 12.5% of all deaths attributable to cancer.[3-4,7] HCC invasiveness is strong and easy to relapse, thus it has a poor prognosis. The 5 year survival rate is less than 30% even for localized HCC, and the average 5 year survival rate for all stages is approximately 15%. Even after radical treatment, the recurrence rate within 5 years is more than 70%.[64,65] Most HCC patients lost the chance for surgical radical treatment because of vascular invasion or multiple metastases inside and outside liver. The expensive targeted therapy and severe chemotherapy with severe associated side effect is commonly administered with low response rate (2.3-8.7%).[8-10] Due to the unresponsiveness of HCC cells to most of the conventional therapies, clinical outcome of HCC treatment are often poor. Thus exploring new strategies for the treatment of HCC is necessary.[1] TCM has its features and advantages in the treatment of advanced HCC, e.g. reducing the toxicity of radiation and chemotherapy, improving general conditions of patient, improving patient's quality of life, alleviating cancer related symptoms, prolonging survival of patient, reducing recurrence rate, etc.[3,11-23, 62-63] Complementary and alternative medicine (CAM) is one of the possible strategies for approaching HCC treatment. Chinese Medicine (CM) derived from plants, animals, and minerals has been used for preventing and treating liver disease in China for centuries,[1,5,24-27, 70] and is now considered as an important component of the CAM system.[1,11,28]

It was in 1863 that Rudolf Virchow noted leucocytes in neoplastic tissue and made a connection between inflammation and cancer. The later study has proved that the microenvironment inflammatory of malignant tissues supported Virchow's hypothesis, and the links between cancer and inflammation are starting to have implication for cancer prevention and treatment. Many cytokines and chemokines are inducible by hypoxia, which is a major

physiological difference between tumor and normal tissue,[29] such as TNF (Tumor Necrosis Factor), IL-1β (Interleukin-1β), IL-6 (Interleukin 6), iNOS (inducible Nitric Oxyde Synthase), COX-2 (Cyclooxygenase-2), NFkB, and PGE-2 (Prostaglandin E2).[30] High level of TNF, IL-1β, IL-6 can stimulate angiogenesis and is related to tumor growth and spread. They can also induce chemoresistancy. Therefore can lead to poor prognosis and decrease the survival rate. TNF induces chemokines that promotes trafficking of the cells and accumulation of lymph nodes.[34] Reactive oxygen in the form of NO is often generated by inflammatory cytokine induction of NO synthase. NO can directly oxidize DNA, resulting in mutagenic changes and may damage some DNA repair proteins. iNOS can be detected in some kind of carcinomas. COX-2 is induced by cytokine and expressed both in inflammatory disease and cancer. NSAID (Non steroidal anti-inflammatory drugs) inhibit cyclooxygenase enzymes and angiogenesis.[31-35]

Recent studies have indicated that the tumor inflammatory microenvironment plays an essential role in the progression of HCC. The tumor microenvironment plays a critical role in modulating the process of liver fibrosis, hepatocarcinogenesis, epithelial-mesenchymal transition, tumor invasion, and metastasis.[71-77, 127] Many studies have proven that the pathogenesis of cancer is closely related to inflammation; and microenvironment immune in situ is considered to play the main role in cancer growth, invasion, and metastasis.HCC has become a worldwide burden because of the high morbidity and mortality rate, recurrence rate, relapse rate; with low survival rate.[36,37] And patients with HCC often come in advance unresectable stage, while the chemotherapy also have a high resistancy rate with severe side effects.

PD-1, a coinhibitory receptor expressed on the surface of activated T cells and B cells, has been linked to immune tolerance and therefore provides a possible mechanism of escape immune surveillance when tumor cells become capable of expressing PD-L1. PD-1 is mainly activated by interacting with its ligands PD-L1 and PD-L2. PD-L1 is widely distributed on diverse cell types in lymphoid and non lymphoid tissues, whereas PD-L2 is mainly expressed on dendritic cells and some macrophages. Once activated , PD-1 exerts a negative effect on immune responses by dephosphorylating key downstream proteins of the antigen receptor. Thus, the PD-1 pathway may serve as an important regulator for the induction and maintenance of peripheral immune tolerance.PD-1 expression by tumor infiltrating lymphocytes was proved to correlate with impaired immune responses and poor outcome in several tumor types. Tumor cells may

upregulate PD-L1 expression as a way to suppress the host immune response and therefore escape immune destruction.

Recently , PD-1 pathway has also proven to be important in cancer initiation and progression. Anti-PD-1 have been reported to enhance anticancer immune responses and induce cancer cell death. PD-L1 is induced on various cell types in response to certain inflammatory cytokines (primarily IFN-γ) which are produced during the immune response of T and NK cells. IFN-γ- stimulated neutrophils suppressed lymphocyte proliferation through expression of PD-L1. The PD-1/PD-L1 pathway has a crucial role in regulating immunosurveillance for tumors. Tumor cells expressing PD-1 can limit the activity of tumor antigen(TA)- specific CD8+ T cells, which reinforce their growth and invasiveness. PD-1 is upregulated by dysfunctional TA-specific CD8+ T cell, and PD-1 blockade enhances TA-specific T cell responses and inhibits tumor growth or partial tumor regression. PD-1 blockade also increases T-cell migration to tumors by elevating IFN-γ inducible chemokines, which augments T-cell mediated antitumor responses. PD-L1 is up-regulated in tumors by activation of key signaling pathway including PI3K, STAT3, IFNγ, and so on.

Blockade of PD-1 pathway may provide antitumor immunity, especially in PD-L1 positive tumors. Various cancer , such as melanoma, HCC, glioblastoma, lung, kidney, breast, ovarian, pancreatic, and esophageal cancers as well as hematological malignancies, have positive PD-L1 expression, and this expression has been correlated with poor prognosis. PD-L1 expression in HCC induces apoptosis in T cells. PD-L1 deficiency leads to hepatic accumulation and impaired apoptosis of T cells, and PD-1 deficiency leads to enhanced proliferation of effector cells in the liver during adenoviral infection. The expression of PD-1 was further suggested as a mechanism of immune evasion for tumors. Elevated expression of PD-L1 was found in different tumor entities. It could be shown that tumor cells expressing PD-L1 were able to induce apoptosis of T cells. Upregulation of circulating PD-L1/PD-1 was associated with poor prognosis of HCC.[84] Blockade of either PD-1 or its ligands (PD-L1) has shown consistent immune-potentiating effects, it can enhance or restore T cell effector function, including cytolitic activity against tumor cells.

Nuclear factor-kB (NF-kB) is the generic name for a family of dimers formed by several proteins. The different heterodimers bind to specific promoters to initiate transcription of a wide

range of genes that influence the inflammatory response as well as cell death and survival and tissue repair. [94,95] Activation of the NFkB has been related to a great number of benign and malignant tumours, for example , hormonal-associated prostate cancer, lung carcinoma, HCC, multiple myeloma, melanoma, glioblastoma, ovarian tumour, malignant lymphoma, leukemia, breast cancer, colorectal cancer, pancreatic cancer, squamous cell carcinoma, mesothelioma, nasopharyngeal carcinoma, biliary cancer cell, soft tissue sarcoma, and other tumours. The ikk inhibitor of the NFkB plays an important role in cancer cell death in HCC, a very aggressive malignant tumour. [95, 109] Substantial evidence indicates that NFkB regulates oncogenesis and tumour progression. NFkB activation suppresses the apoptotic potential of chemotherapeutic agents and contributes to resistance. NFkB inhibitors may be used to overcome chemoresistancy. Many anticancer agents induce NFkB nuclear translocation and activation of its target genes, which impinge on cellular resistance to anticancer agents. Many malignant tumours display constitutive NFkB activation that allows malignant cells to escape apoptosis. NFkB inhibition prevents tumour resistance to chemotherapeutic agents, thus NFkB inhibitor could increase the efficacy of many anticancer agents and enhance the cytotoxic effect of anticancer agents.The survival of hepatocytes and their progression to malignancy is regulated by NFkB. Moreover in a mouse model of colitis associated colon cancer , indicate that NFkB activation , which is often seen in inflammatory based disease, is associated with an increase incidence of cancer. The survival of hepatocytes and their progression to malignancy in Mdr 2- knockout mice is crucially regulated by NFkB. The inhibition of NFkB treatment in later stage of tumour development resulted in apoptosis of transformed hepatocytes and failure to progress to HCC. NFkB inhibitor could be a tool to draw out the pre malignant phase and inhibit tumour progression in chronic inflammatory disease with a high cancer risk. NFkB is known to inhibit apoptosis through induction of anti-apoptosis proteins and/or suppression of pro apoptotic gene. Several anticancer agents stimulate NFkB activation, which can potentially lead to chemoresistance. The NFkB pathway impinges on many aspects of cell growth and apoptosis. It seems that inhibition of NFkB activation can shift the death-survival balance towards apoptosis. [94]

NFkB mediates cell survival mechanism which guarantee cell viability against pathogenic stimuli. On the other hand , NFkB could also modulate pathogenic signaling pathways, leading to cell degeneration, aging, disease and death. [95] NFkB dependent regulation of cell adhesion molecules and cell surface proteases, such as matrix metalloproteinase(MMP) 2

and MMP 9, raises the possibility that NFkB is involved in the regulation of metastatic pathways. PGE_2 induces the activation of MMP2 and MMP9 in vivo.[94]

Generally, metastasis of cancer cells involves multiple processes, and various citophysiological changes, including altered adhesive capability between cells and the extracellular matrix (ECM) and damaged intercellular interaction. Degradation of ECM by cancer cells through proteases such as serine proteinase, matrix metalloproteinases (MMPs), cathepsins, and plasminogen activator (PA) may lead to the separation of the intercellular matrix to promote cancer cell mobility and may eventually lead to metastasis. Among the involved proteases, MMP2, MMP9, u-PA, and cathepsins reportedly play the most important roles in cancer invasion and metastasis. [96]

A subgroup of HCC characterized by up-regulated neurotensin (NTS) expression was accompanied by up-regulated inflammatory responses and epithelial mesenchymal transition (EMT). The levels of VEGF and MMP9 correlated with co-expression of NTS and IL-8 which significantly correlated with the clinical outcomes, and higher mortality rate.[93] Recent findings have shown that nanoparticle formulation of curcumin and sorafenib synergistically inhibits tumour growth and metastasis in an orthotopic model of HCC through down-regulated the expression of MMP9 via NFkB/p65 signalling pathway. [97]

Curcumin has in fact been shown to possess interesting anti inflammatory and anti tumor properties, which at last in part, appear be linked to its ability to suppress the activation of NFkB.[98, 124-126] Curcumin reduce cell migration and MMP9 production of the HCC cells. [99] Curcumin has been shown to have multiple anticancer effects, including inhibition of proliferation, induction of apoptosis, and inhibition of angiogenesis, but it also induce apoptosis independent cell death. The anticancer effect of curcumin and its structural derivatives are dependent on their capacity of modulating multiple molecular targets, including transcription factors, growth factors, kinases, inflammatory cytokines, adhesion molecules, apoptosis related protein, and signaling pathways such as NFkB, AKT, MAPK. One of the predominant target of curcumin is NFkB cell signaling pathway.[42-45,101,110]

Curcuma longa as TCM with *Huoxue Huayu* (活血化瘀) activity also plays important role in cancer treatment involving anti-proliferation, anti-inflammation, anti-oxidation, anti-angiogenesis, anti-thrombotic, immunomodulation, and so on.[3,21, 39-40, 46-48]

Many studies have also been done in Indonesia to explore the benefit of Curcumin in diseases. Curcumin can enhance sensivity of resistant MCF-7 cells to Doxorubicin through inhibition of HER2 and activation of NF-kB, therefore it could be applied in doxorubicin chemoresistant breast cancer patient.[49] Curcumin can avoid the effect of transport protein ATP-Binding Cassette (ABC) that can cause multi drug resistant (MDR) in breast cancer. Curcumin can also induce apoptosis and inhibit the proliferation of breast cancer cells.[50-52]

Study on effect of Curcumin in patients with acute coronary syndrome has proven low dose Curcumin can reduce total cholesterol and LDL cholesterol level.[53] Curcuminoid derived from *Curcuma longa* significantly suppresses the secretion of COX-2 enzyme by synovial fluid's monocytes of patients with knee osteoarthritis, and it was not significantly different compared to diclofenac sodium in suppressing the secretion of COX-2 enzyme.[41]

Curcuminoid has been proven as an anti-inflammatory agent on the hepatic microvascular response to endotoxin in LPS induced BALB/C mice.[38] Curcuminoids have been shown to be free radical scavengers that suppress the production of superoxide by macrophages and potent anti-inflammatory agents that inhibit the lipopolysacharide (LPS)-induced production of tumor necrosis factor alpha (TNF-α), Interleukin (IL)-1β, and the activation of nuclear factor (NF)-κB in human monocytic derived cells. Studies have also proven the efficacy of Curcuminoids in inhibiting the hepatic microvascular inflammatory response elicited by LPS. Curcuminoids are effective in suppressing the hepatic microvascular inflammatory response to LPS and may be a natural alternative anti-inflammatory substance. These suggest the possible prophylactic effect of Curcumin in HCC by inhibiting inflammation.[1,54-55]

Curcuma longa extract has proven to have anti-colon cancer effect in histopathologic assessment of DMBA induced BALB/C mice.[56] *Curcuma longa* can control body weight and abdominal fat weight by lowering body weight, visceral fat weight, and abdominal subcutaneous fat in male rats fed a diet high in carbohydrates and fat, so it can also be used to prevent weight gain by suppressing angiogenesis, affecting lipid metabolism in adipocytes, and suppressing pre-adiposite differentiation.[57]

Some studies results indicated that the deficiency of disease fighting ability, including the immune system deficiency, named *Zhengxu* （正虚） in CM terms, is the basic pathogenesis of cancer. In clinical trials and basic experiments, *Fuzheng* (扶正) herbs are found to possess the ability to improve the immunity, enhance the efficacy of chemo / radiotherapy and improve the quality of live of patients. It could inhibit the proliferation of tumor cells, induce apoptosis, improve the sensitivity to chemotherapy and modulate immune functions.[14,66-69] Ginsenoside is the principal bioactive chemical of Radix Ginseng (*Renshen* 人参); a Chinese herb that is commonly used as *Fuzheng* (扶正).[58, 61]

Ginseng, meaning "man-root" is a slow-growing root herb that has been used medicinally for more than 3000 years by practitioners of TCM. Touted by many TCM physician as the "root of longevity", ginseng is considered to be an adaptogen, a substance thought to enhance the body's ability to resist physical and mental stress; and considered to be a "general tonic" to protect the body against disease, much as one would expect from an immunostimulant. The main active components of ginseng are glycosidal saponins (glycosylated steroids) known as ginsenosides.[58] Many preclinical and clinical work on ginseng have been done on immune parameters. In vitro, ginseng activated macrophages to produce reactive nitrogen intermediates and become tumoricidal. Cancer is inherently immunosuppressive due to tumor-derived factors, and standard cancer treatments are likewise immunosuppressive. Herbal immunostimulants may be used by patients to attempt to overcome immunosuppression or counteract the infection that are of concern among patients with advance stage diseases. Ginseng may exert anticancer activity modulated by improvements in the cell mediated immune system, which is part of the body's anticancer defenses. [78.79, 80, 81, 87, 92] A number of molecular mechanisms have been proposed for such anti-cancer function, among which are inhibitory effects of NF-kB and AP-1 activity.[86]There is a further evidence that suggests the involvement of NF-kB, a transcription factor , in cell survival and/or apoptosis.[87,102]

Ginsenosides are reported to possess numerous biological activities, recent issues have arisen regarding their immunosuppressive and anti-inflammatory roles in inflammatory cells. Ginsenoside effectively inhibiting the production of inflammatory mediators through suppressing the activation of NFkB and its upst ream signaling cascade.[91, 122, 123]

Ginsenoside Rg3 treatment reduced the levels of vasculogenic mimicry in pancreatic cancer in vitro and in vivo through downregulation of MMP9 expression.[100] Ginsenosides inhibit COX2 expression attributed to inactivation of NFkB; a transcription factor whose activation inhibits the cell death signaling on oncogenic rats. [102]

Part 1. The effect of ginsenoside and curcumin on the growth of HCC in vivo

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are of concern among patients with advance stage diseases. Ginseng may exert anticancer activity modulated by improvements in the cell mediated immune system, which is part of the body's anticancer defenses. [78.79, 92]

A. Experimental Materials

1. Herbs and drugs

Ginsenoside and curcumin were identified and purchased by Natural Medicine Department of Hunan University of Chinese Medicine.

5FU and Cisplatin were purchased from Natural Medicine Department of Hunan University of Chinese Medicine.

1.1. Ginsenoside

Ginsenoside, 5g /vial, manufactured by Sigma.

1.2. Curcumin

Curcumin, 5g, manufactured by Sigma.

1.3 . Chemotherapy drugs

5FU injection, 5g/vial, manufactured by Shanghai source leaf biological technology Co.,Ltd.

Cisplatin injection, 250mg/vial, manufactured by Shanghai source leaf biological technology, Co.Ltd.

2. Experimental cells

Human hepatoma cell line HepG2 cells was purchased from Cell Center of Central South University Xiangya School of Medicine

3. Experimental animals

BALB / c (nu / nu) 4 to 6 weeks old male nude mice weighing 18-22 g were purchased from Animal Experiment Department of Central South University Xiangya School of

Medicine. Mice were housed under pathogen-free conditions, and fed with sterilized food and water ad libitum. Constant humidity (45-51%), constant temperature (26-28ºC), SPF level environment. Nude mice kept in the ultra-clean laminar flow within the plexiglass, fed with UV sterilized water and feed. Pre-experimental feeding 1 week.

B. Experimental Methods

1. Cell culture

1.1. Cell resuscitation

From the -80 ºC refrigerator with tweezers quickly remove the cryopreservation tube, quickly into the preheated 37 ºC warm water tank, and to speed up the shake to help dissolve. After dissolving, quickly remove the cryopreservation tube from the 37 ° C water bath, open the lid, suck out the cell suspension with a pipette, add to the centrifuge tube and add more than 10 times the culture medium, mix; -4 ºC centrifuge centrifugal 1000rpm × 5min, discard the supernatant, add 12% fetal bovine serum complete resuscitation of cells, count, adjust the cell density, inoculated to 75ml culture bottle, to prevent the constant temperature, humidity, 5% CO_2, 37 ºC culture Box, observe the cell condition, 4h until the cells adhere to the wall, replace the culture medium, and then continue to stand culture.

1.2. Cell Passage

If the cell growth is good, if the cell grows to 90% after the cell pass. Absorb or discard the old culture medium in the culture flask, then add 5-6mlPBS liquid cleaning three times, add a small amount of 0.25% trypsin to cover the bottom of the culture is appropriate, and then placed in 37 ℃ incubator for digestion, 3min After the microscope into the observation of cells, if digested completely, you can immediately stop digestion, and then pipettes in accordance with the order of repeated absorption of the cell culture medium, gently blowing the bottle wall cells, the adherent cells fall off and the formation of cell suspension And then with the counting plate count, and then inoculated in a new culture bottle, add 12% fetal bovine serum complete culture medium to drown the bottom of the bottle is appropriate, and then continue to stand in constant temperature, constant humidity, 5% CO_2, 37 ℃ Incubator continues to develop.

2. Animal modeling

2.1. Establishment of subcutaneous transplanted tumor of human liver cancer in nude mice

2.1.1. Take the logarithmic growth phase of HepG2 hepatocarcinoma cells, blot out or discard the old culture medium in the culture flask, add 5-6ml PBS to wash on both sides, add a small amount of 0.25% trypsin digestion for 3min, digest completely Use PBS to stop digestion.

2.1.2. with the pipette in order to repeatedly gently blow the bottle wall cells, the cells from the bottle wall off the formation of cell suspension, and then count the plate after counting.

2.1.3. The cell suspension was then transferred to a 15 ml centrifuge tube with a pipette, and the supernatant was discarded at 1000 r / min × 5 min to adjust the concentration to 5×10^7 cells / ml.

2.1.4. Before the inoculation, the cell suspension was completely blown away, and the cells of HepG2 cells were inoculated with 1 mL syringe. The cells were inoculated into the rear of the armpit in the nude mice, 0.2 mL in each part, In the subcutaneous needle into about 1cm, inoculation can be seen a small uplift, after inoculation with a cotton swab oppression pinhole moment, the nude mice back into the feeding box. Routine observation of nude mice, diet, defecation, activity, death and tumor growth and record.

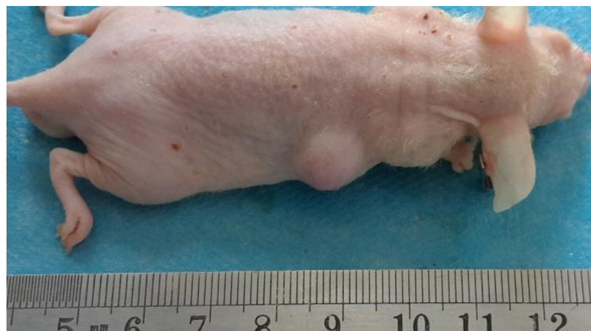


Figure 1 model of subcutaneous transplantation of nude mice

2.2. Drug formulation, animal grouping and drug intervention

2.2.1. Drug preparation

According to the human and animal according to the body surface area of the equivalent dose ratio conversion nude mice drug concentration.

The low dose ginsenoside dose was calculated based on human dose. Regular dose of ginseng for human is 1 g. Each gram of ginseng contain of 0.8 g ginsenoside. The dose for mice were calculated as 0.0026 x 0.08 which is 0.00208 g per 20 gram body weight. The mice in the experiment weighed 25 g each. Ginsenoside dose for each mice was 25/20 x 0.00208 which was 0.0026 g or 2,6 mg per 25 g body weight. The high dose ginsenoside were calculated five times higher than the low dose, which was 0.013g or 13 mg per 25 g body weight. The ginsenoside were given once daily through oral gavage.

The curcumin dose was calculated based on human dose. Regular dose of turmeric for human is 2.5 g. Each gram of turmeric contain of 0.63 g curcumin. The dose for mice were calculated as 0.0026 x 2.5 x 0.63 which is 0.004095 g per 20 gram body weight. The mice in the experiment weighed 25

g each. Curcumin dose for each mice was 25/20 x 0.004095 which was 0.0051 g or 5 mg per 25 g body weight.. The curcumin were given once daily through oral gavage.

5FU 1.25mg/mL were given 0.6 mg per 25 g body weight and Cisplatin 0.25mg/mL were given 0.1 mg per 25 g body weight. Chemotherapy drugs were injected intraperitoneally once a week.

2.2.2. Animal grouping and drug intervention

46 BALB / c (nu / nu) nude mice were randomly assigned to 7 groups according to different intervention. The specific grouping is shown in Table 1.

Table 1-1 Random distribution all animals into each experiment groups

Model	**Low Dose Ginsenoside 2.6mg/25g**	**High Dose Ginsenoside 13mg/25g**	**Curcumin 5mg/25g**	**Curcumin+ Low Dose Ginsenoside**	**Curcumin+ High Dose Ginsenoside**	**Chemotherapy 5FU 0.8mg/25g Cisplatin 0.1mg/25g**
7	7	6	7	6	7	6

Model group were given 0.9% sodium chloride solution. Ginsenoside and curcumin were administered once daily continuously for 17 days through oral gavage. Positive control group given 5FU and Cisplatin injection once a week intraperitoneally .

At present, there is no uniform formula for the volume of transplanted tumors. In this study, transplanted tumor volume is calculated by $V = ab^2 / 2$, where a and b are the long and short diameters in mm, V is the volume, the unit is mm^3.(a), short diameter (b), tumor volume, tumor volume = ab2 / 2 were measured with vernier caliper, and the animal diet, defecation, activity and death were observed from the first day of drug treatment.

2.3.Collect specimens and pretreatment

At 18 days after treatment with curcumin and ginsenoside, all the nude mice were sacrificed by cervical dislocation and the transplanted tumor was removed from nude mice, peeled off and measured its long and short diameter, and the specimen are saved according to different experimental requirements. Tumor specimen was deposited in liquid nitrogen, and the expression of PD-1, PD-L1, NFkB and MMP-9 was detected by western blot.

2.4.Determination of tumor inhibition rate in nude mice

After 17 days of drug intervention, the rats were sacrificed on the 18th day, and the subcutaneous transplanted tumor of liver cancer was dissected in the clean bench. The fascia was removed and the tumor weight was recorded. The vernier scale (a), short diameter (b), Respectively, to calculate the tumor volume and tumor growth inhibition rate. Tumor volume = ab2 / 2; tumor growth inhibition rate (%) = [(median tumor volume in the model group - median tumor volume in the administration group) / median tumor volume in the model group] × 100%.

C. Statistical Analysis

Data are summarized as mean ± standard deviation (SD). Tumor volume was analyzed by one way analysis of variance (ANOVA) and repeated measures using two way analysis of variance (ANOVA). Tumor weight were analyzed using one way analysis of variance (ANOVA). All data were analyzed using SPSS 23.0 and Graphpad Prism 6.0 statistical software. A P value< 0.05 was considered statistically significant.

D. Experimental result

46 normal nude mice for subcutaneous transplantation tumor modeling, since the first 14 days after modeling, we can see 40 nude mice modeling success, modeling success rate of 95.2%. Effects of curcumin on the growth of human hepatocellular carcinoma xenografts in

nude mice. On the 14th day after modeling, the tumor diameter was observed to be 5mm, and the mice were treated with drugs. The tumor length of the nude mice began to increase in different degrees.

In the whole process of curcumin and ginsenoside treated nude mice, we measured the volume of transplanted tumor every 6 days, the growth of HCC transplanted tumors in vivo was assessed. After a combination of observation and statistics, we found that the growth of HCC in nude mice was inhibited by curcumin and ginsenoside and showed stronger inhibition with combination of curcumin and ginsenoside. The tumor volume of ginsenoside and curcumin group were significantly reduce compared with model group. ($p<0.05$). The inhibition of growth in curcumin and ginsenoside combination group was essentially the same with the 5FU and Cisplatin group. ($p>0.05$)

Hep G2 transplanted tumors were treated with ginsenoside and curcumin, and the primary length (a) and short diameter (b), and calculate the volume (mm^3) with $V= ab^2/ 2$. Analysis of the data obtained from each experimental group found that: The changes of tumor volume are significantly different between each measurement in 6 days interval, but compared between groups the changes began to show significant difference in the curcumin group and it is more significant when high dose ginsenoside added to the curcumin in 12th day of measurement. The volume changes of the tumor become significantly difference in all groups compared with model group in the 18th days. ($P <0.05$). Combination of ginsenoside and curcumin have tumor inhibition effect significantly stronger then ginsenoside alone. ($p<0.05$). And the dose dependent effect was showed in the curcumin combined with high dose ginsenoside group; its inhibitory effect was essentially the same with the 5FU and Cisplatin group. ($p>0.05$).

At the 18th day the mice were sacrificed and the tumor were weighed using the digital scale. Tumor weight were significantly smaller compared to the model group. ($p<0.05$). Ginsenoside show significant dose dependent effect in tumor weight. Combined curcumin with ginsenoside had better result in tumor weight but no significant difference with high dose ginsenoside, curcumin, and even with 5FU and Cisplatin group. ($p>0.05$).

Table 1-2 The volume changes of Hep G2 xenografts after ginsenoside and curcumin treatment (mm^3) （$\overline{X} \pm S$）

Group	n	Days of treatment			
		0	6	12	18
Model	7	0 ± 0	95.82±40.18	339±124.29	751±270.55
Low Dose Ginsenoside	7	0 ± 0	123.75±65.99	236.55±70.72	447±55.96**
High Dose Ginsenoside	6	0 ± 0	61.39±36.29	230.6±103.65	335±170.17**
Curcumin	7	0 ± 0	72.29±50.65	193.24±119.82*	205±118.73**△△
Curcumin+Low Dose Ginsenoside	6	0 ± 0	73.14±45.73	153.79±68.65**	178±73.23**△△
Curcumin +High Dose Ginsenoside	7	0 ± 0	52.64±37.08	100.65±60.61** △▲	138±80.29**△△▲
Chemotherapy	6	0 ± 0	74.02±99.38	124.80±128.75**	129±179.95**△△▲

Note: Compared with model group * $p<0.05$, ** $p<0.01$; compared with low dose ginsenoside group, △ $P<0.05$, △△ $P<0.01$; compared with high dose ginsenoside group, ▲ $P<0.05$, ▲ ▲ $P<0.01$

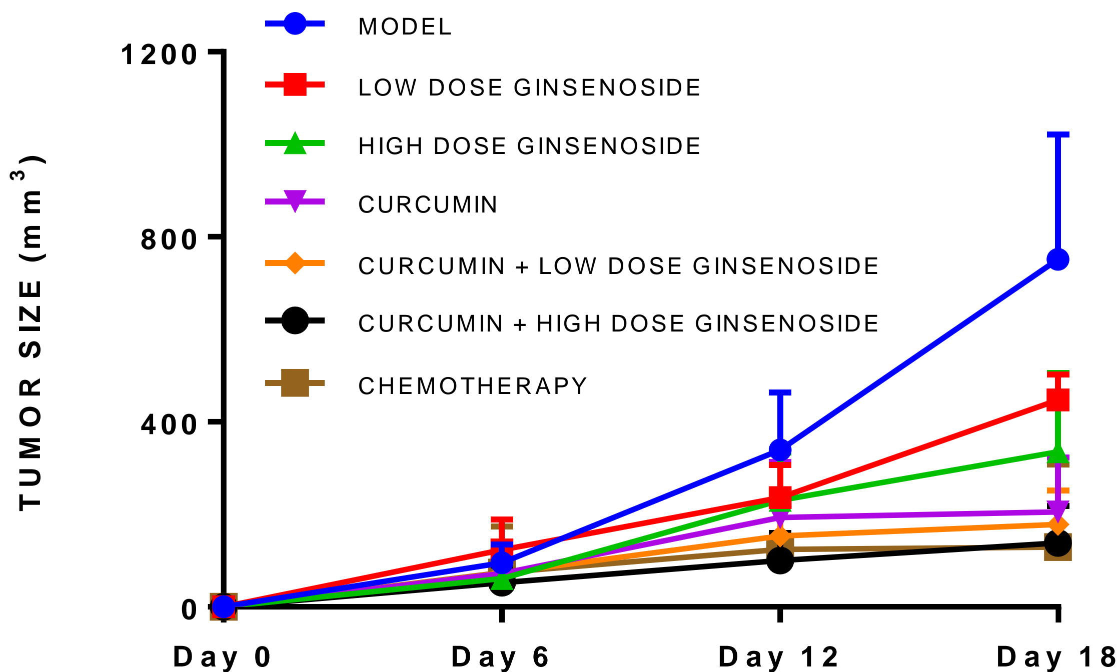


Figure 1-1 The growth curves of Hep G2 xenografts in vivo after ginsenoside and curcumin treatment

Table 1-3 The tumor weight of Hep G2 xenografts after ginsenoside and curcumin treatment (g) ($\overline{X} \pm S$)

Group	n	Tumor weight (g)
Model	7	1.09±0.32
Low Dose Ginsenoside	7	0.70±0.06**△△
High Dose Ginsenoside	6	0.38±0.16**△△
Curcumin	7	0.38±0.21**△△
Curcumin+Low Dose Ginsenoside	6	0.32±0.14**△△
Curcumin +High Dose Ginsenoside	7	0.30±0.12**△△
Chemotherapy	6	0.25±0.21**△△

Note: Compared with model group * p<0.05 , ** p<0.01; compared with low dose ginsenoside group, △ P <0.05, △△ P <0.01

Table 1-4 The antitumor effect ginsenoside and curcumin on Hep G2 xenografts

Group	N	Tumor weight (g) ($\overline{X} \pm S$)	Tumor volume (mm³) ($\overline{X} \pm S$)	Tumor inhibition rate (%)
Model	7	1.09±0.32	751±270.55	-
Low Dose Ginsenoside	7	0.70±0.06**△△	447±55.96**	29.64
High Dose Ginsenoside	6	0.38±0.16**△△	335±170.17**	50.67
Curcumin	7	0.38±0.21**△△	205±118.73**△△	67.10
Curcumin+Low Dose Ginsenoside	6	0.32±0.14**△△	178±73.23**△△	56.86
Curcumin +High Dose Ginsenoside	7	0.30±0.12**△△	138±80.29**△△▲	66.71
Chemotherapy	6	0.25±0.21**△△	129±179.95**△△▲	76.34

Note: Compared with model group * p<0.05 , ** p<0.01; compared with low dose ginsenoside group, △ P <0.05, △△ P <0.01; compared with high dose ginsenoside group, ▲ P <0.05, ▲ ▲ P <0.01

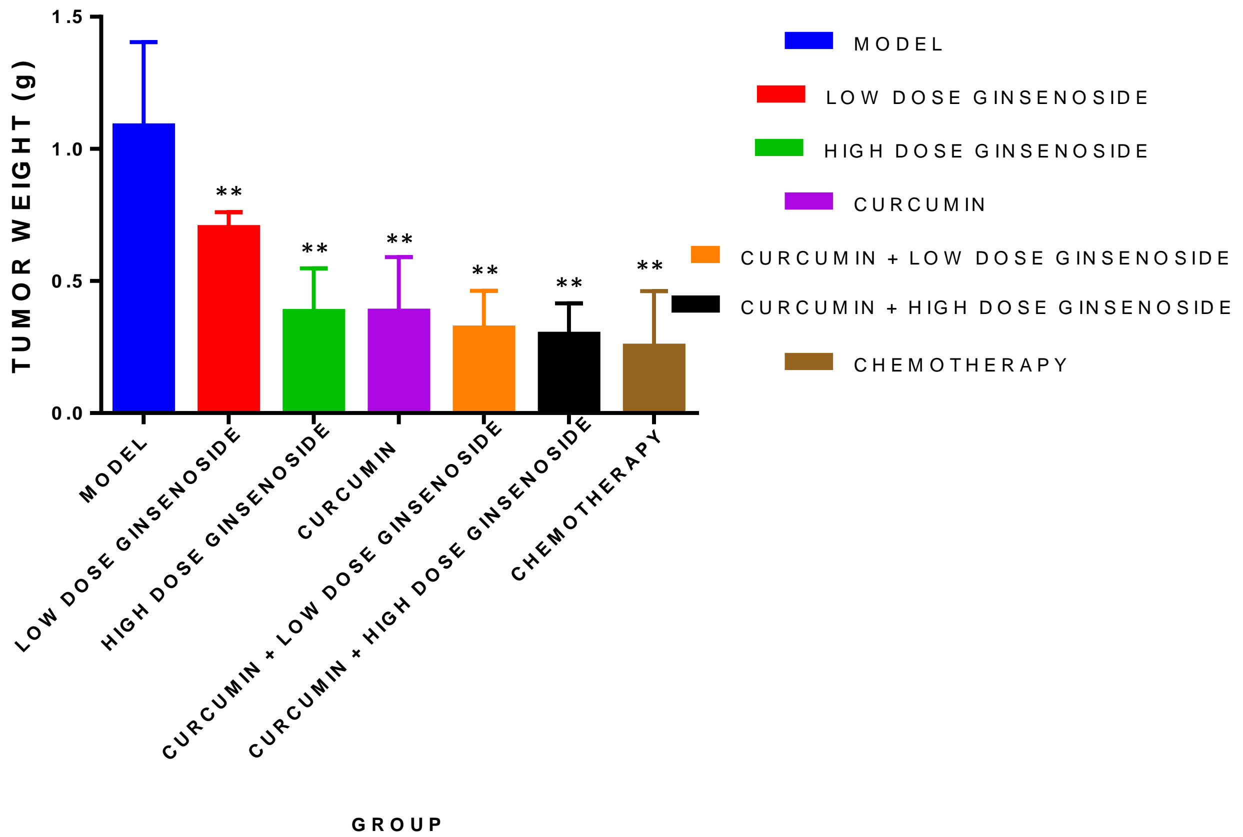
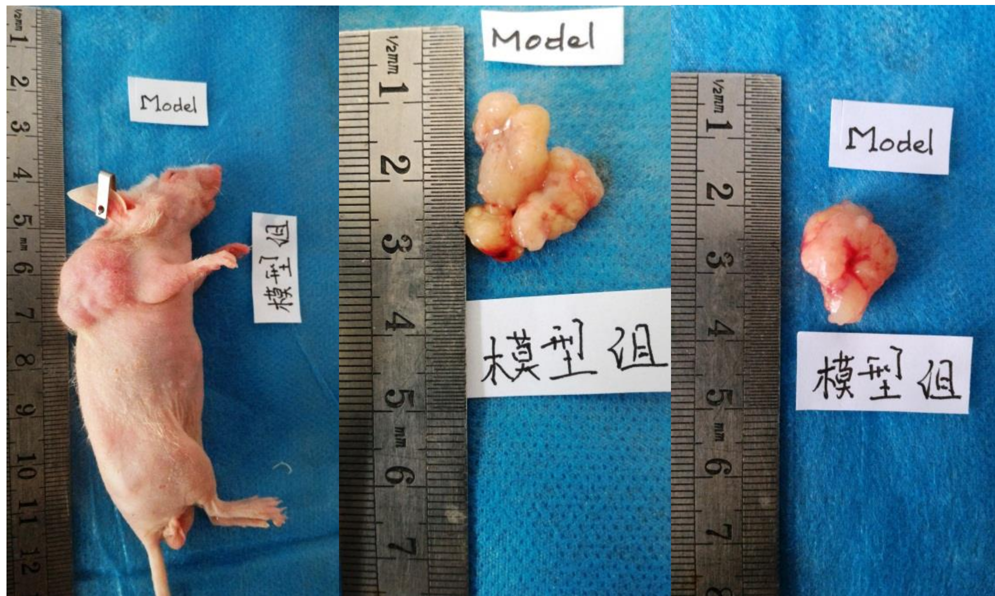
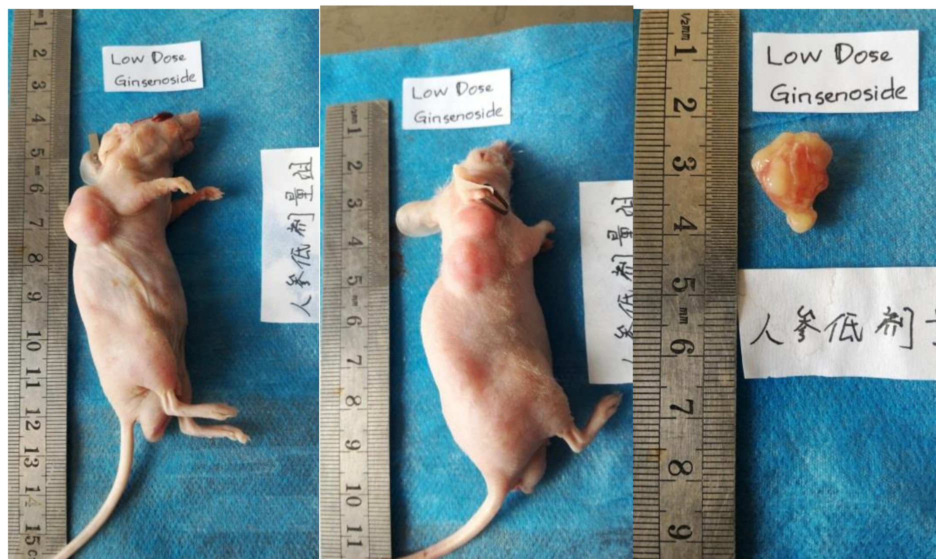


Figure 1-2 The tumor weight of Hep G2 xenografts in vivo after ginsenoside and curcumin treatment

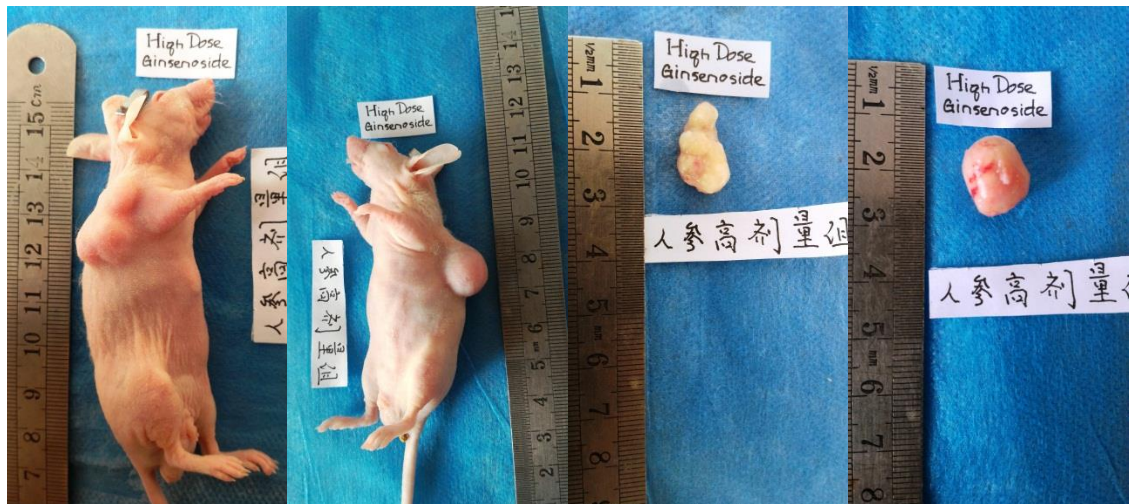
All numbers are shown as mean ± standard deviation, compared with the model group, * P <0.05, ** P <0.01.



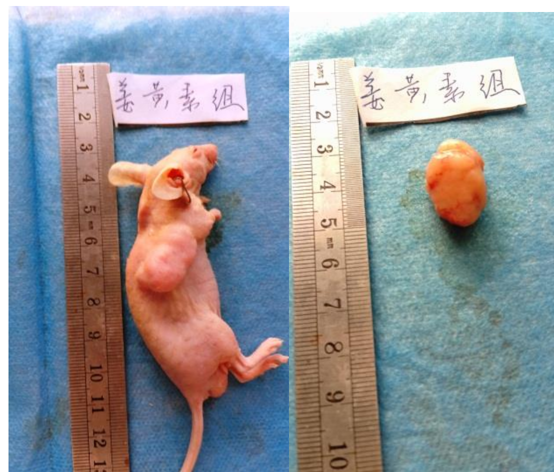
Model Group



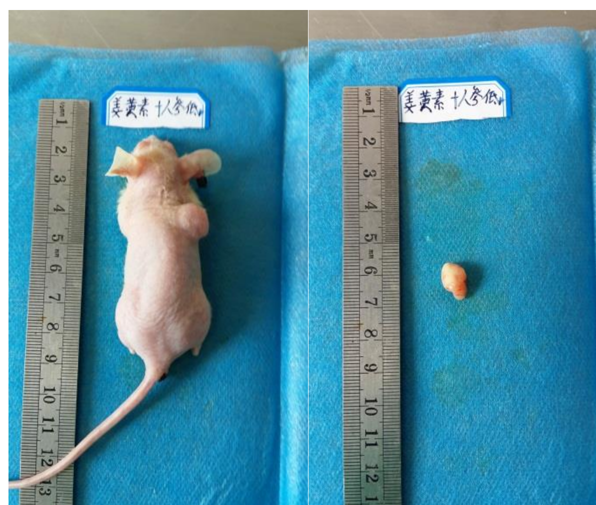
Low Dose Ginsenoside Group



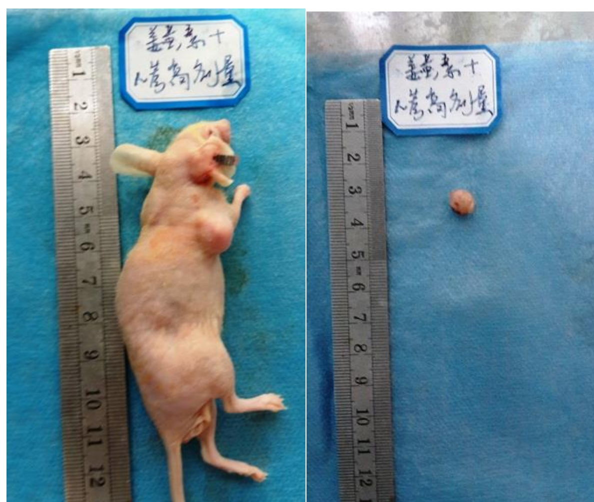
High Dose Ginsenoside Group



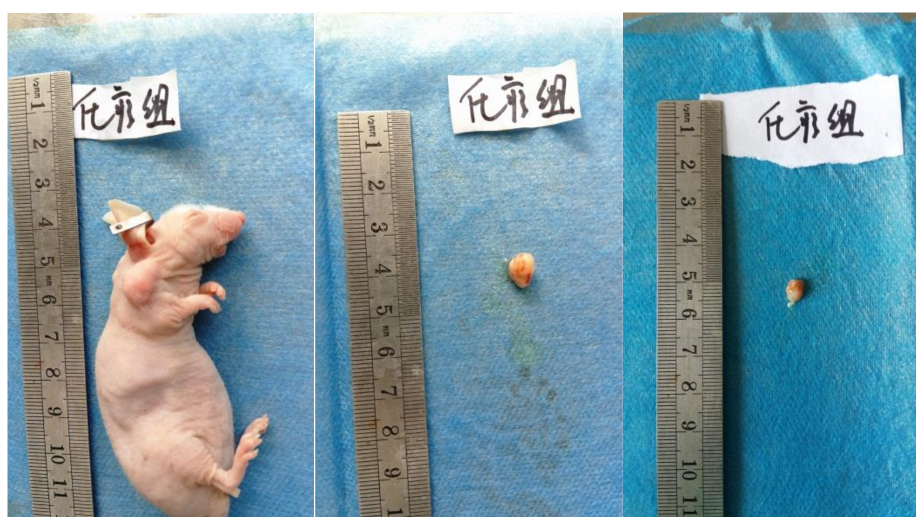
Curcumin Group



Curcumin + Low Dose Ginsenoside



Curcumin + High Dose Ginsenoside



Chemotherapy Group

Figure 1-3 The nude mice model of hepatocellular carcinoma and the specimen of xenografts in each group

E. Summary

1. HCC transplanted tumor grow slow after ginsenoside and curcumin treatment.
2. The growth of the HCC cells in nude mice can be inhibited by ginsenoside and curcumin, and the effect enhanced significantly with the combination and increase of the ginsenoside dose.
3. Curcumin and ginsenoside has synergic effect to inhibit the growth of HCC in nude mice

Part 2. The mechanism of ginsenoside and curcumin in HCC treatment

At present, the incidence of primary liver cancer in the world is increasing year by year trend, has become the most common high death rate of malignant tumors, but in China is significantly higher than the world level. Under normal circumstances, due to the onset of concealment and early symptoms are not obvious, the impact of daily life and work is small, it is difficult to be found at the beginning of the disease, most patients found in the late stage, the prognosis is poor. If you can find early in the incidence of HCC, the prognosis of patients is very favorable, also improve the effectiveness of clinical surgery and long-term efficacy after surgery. Surgical resection and liver transplantation are considered the best means of treating HCC , but postoperative metastases and recurrence is still the biggest obstacle to the survival of HCC.

PD-1 pathway has also proven to be important in cancer initiation and progression. Anti-PD-1 have been reported to enhance anticancer immune responses and induce cancer cell death. Tumor cells may upregulate PD-L1 expression as a way to suppress the host immune response and therefore escape immune destruction. The PD-1/PD-L1 pathway has a crucial role in regulating immunosurveillance for tumors. Blockade of PD-1 pathway may provide antitumor immunity, especially in PD-L1 positive tumors. Various cancer , such as melanoma, HCC, glioblastoma, lung, kidney, breast, ovarian, pancreatic, and esophageal cancers as well as hematological malignancies, have positive PD-L1 expression, and this expression has been correlated with poor prognosis.

Activation of the NFkB has been related to a great number of benign and malignant tumours, for example , hormonal-associated prostate cancer, lung carcinoma, HCC, multiple myeloma, melanoma, glioblastoma, ovarian tumour, malignant lymphoma, leukemia, breast cancer, colorectal cancer, pancreatic cancer, squamous cell carcinoma, mesothelioma, nasopharyngeal carcinoma, biliary cancer cell, soft tissue sarcoma, and other tumours. Substantial evidence indicates that NFkB regulates oncogenesis and tumour progression. NFkB activation suppresses the apoptotic potential of chemotherapeutic agents and contributes to resistance. NFkB inhibitors may be used to overcome chemoresistancy.

Tumor invasion and metastasis is a major sign of its degree of malignancy, degradation of extracellular matrix and vascular base destruction of the basement membrane is a common pathway experienced by malignancies during invasion and metastasis . The matrix metalloproteinase (MMPs) can hydrolyze the outer matrix components of the cells and the basement membrane of the blood vessels glycoprotein, MMP-9 is a member of the protease system, it can degrade IV, V-type collagen and gelatin extracellular matrix components, to prevent fibrosis, tumor cells for the possibility of transfer and invasion.

In recent years, plants, minerals and their derivatives study increased year by year, and from the plants found in the anti-tumor effect of the drug has been in clinical or clinically applied. Although a large number of studies have been largely elucidated that curcumin and ginsenoside inhibit HCC but the mechanism of action is not clear. Therefore this study will explore the impact of curcumin and ginsenoside on PD-1 and NFkB, which could be benefit as anticancer in HCC. Western Blot was used to detect the expression of PD-1 and NFkB.

A. Experimental Material

1. Tissue Specimen

Tissue specimens are HCC xenograft specimen collected from in vivo experiment in curcumin and ginsenoside treated nude mice.

2. Main Reagents

Conventional chemical reagents	Shanghai, China
Tris	United States Sigma
APS	United States Sigma
SDS	United States Sigma
TEMED	United States Sigma
Tween-20	United States Sigma
Acrylamide	United States Sigma
Glycine	United States Sigma
A crossed acrylamide	United States Sigma
HRP goat anti-mouse IgG	United States Proteintech
RIPA lysing solution	Beijing China

Protease inhibitor	Germany Merck
Protein Phosphatase Inhibitor	Switzerland Roche
HRP goat anti-rabbit IgG	United States Proteintech
Super ECL plus super sensitive luminescent liquid	United States Thermo pierce
Detergent liquid fixation	China Well Biology

3. Reagent Preparation Method

3.1.1.0 mol/L Tris.HCl

Tris (MW121.14) 30.29g and distilled water 200 mL. After dissolving then the pH adjusted to the desired point (6.8) with about 18 mL of concentrated hydrochloric acid, and finally set the volume to 200ml with distilled water and stored at room temperature.

3.2.1.74 mg/mL (10mmol/L) PMSF

PMSF 0.174g and Isopropyl alcohol 100mL. After dissolution , the mixture was dispensed into a 1.5mL centrifuge tube and stored at -20°C.

3.3.10% SDS

SDS 10g and distilled water to 100mL. 50 ^{o}C water bath dissolved , stored at room temperature. Such a precipitation in the long term preservation , the water bath after melting, can still be used.

3.4.10% persulfate (APS)

0.1g of sulfuric acid amine and ultra pure water 1.0mL. Dissolved, stored at 4°C, preserved for 1 week.

3.5.1.5 mol/L Tris.HCl (pH8.8)

Tris (MW121.14) 45.43g and ultra pure water 200 mL. After dissolving then the pH adjusted to the desired point (8.8) with concentrated hydrochloric acid, and finally set the volume to 250ml with ultra pure water and stored at room temperature.

3.6.0.5 mol/L Tris.HCl (pH6.8)

Tris (MW121.14) 15.14g and ultra pure water 200 mL. After dissolving then the pH adjusted to the desired point (6.8) with concentrated hydrochloric acid, and finally set the volume to 250ml with ultra pure water and stored at room temperature.

3.7.30% Acr/Bic

Acrylamide (Acr) 29 g, methylene bismaleamide (Bica) 1g, ultra pure water 100mL. Dissolved at 4 ºC after storage. Use to return to room temperature without precipitation.

3.8.Reduced 5XSDS loading buffer

0.5 mol/L Tris.HCl (pH6.8) 2.5 mL, Dithioglycol alcohol (DTT, MW 154.5) 0.39 g, SDS 0.5 g, Bromphenol blue 0.025 g, glycerin 2.5 mL. After mixing , the mixture was dispensed into a 1.5 mL centrifuge tube and stored at 4 ºC.

3.9.Electrophoretic buffer

Tris (MW121.14) 3.03 g, glycine (MW 75.07) 18.77 g, SDS 1 g, distilled water to 1000mL. Dissolved at room temperature after storage, the secondary solution can be reused 3 to 5 times.

3.10. Transfer membrane buffer

Glycine (MW 75.07) 2.9 g, Tris (MW 121.14) 5.8 g, SDS 0.37 g, methanol 200 mL, distilled water to 1000 mL. Dissolved at room temperature after storage, the secondary solution can be reused 3 to 5 times.

3.11. 10X Li Chunhong dye solution

Pichanghong S 2 g, Trichloroacetic acid 30 g, Sulfosalicylic acid 30 g, distilled water to100 mL. Use it to dilute it 10 times.

3.12. TBS buffer, TBST buffer

TBS buffer : 1 mol/L Tris.HCl (pH7.5) 10mL , NaCl 8.8 g, distilled water to 1000 mL. Could be prepared into 10X TBS buffer for preservation, diluted 10 times use. TBST buffer : 20% Tween 20 1.65 mL, TBS 700 mL. Could be used after mixed, the best used is within this time.

3.13. Close liquid

Skimmed milk powder 5 g, TBST 100 mL.

4. Experimental Instrument

Shaker	China Jiangsu Lin Lin Bell TS-92
Desktop refrigerated centrifuge	China Shenzhen dark horse TGL 18R
Electrophoresis instrument	US Bio-rad 164-5050
Electrophoresis tank	China Beijing six one DYCZ-24EN
Transfer film instrument	China Beijing six one DYCZ-40A
Whirl mixer	China Jiangsu Liner Bell QL-901
Magnetic stirrer	China Ronghua a85-1
Ordinary refrigerator	China Rongshida BCD-245F
Induction cooker	China United States MC-EP18
Precision PH meter	China lightning magnetic E-201-C
Electronic balance	China National Bridge FA-N
Electric glass homogenizer	Japan Xinzhi DY89-1

B. Experimental Method

Western Blot was used to detect the expression of PD-1, PD-L1, NFkB, and MMP-9 levels.

1. Sample preparation

1.1. Cut 0.25 g tissue , wash the tissue with ice pre-cooling PBS, add 300 ul of RIPA lysate in the homogenizer repeatedly grinding the sample until invisible tissue block seen.

1.2. Refrigerate, protein cleavage for 20 minutes

1.3. Centrifuge at 12°C and 12000 rpm for 15 minutes. (early to open the centrifuge pre-cooling)

1.4. After centrifugation of the supernatant transferred to the inverted 0.5mL centrifuge tube, part of the experiment used in the excess at -20°C

2. Protein concentration detection

   The protein concentration was determined according to the BCA Protein Quantification Kit (Wellbio) instructions.

2.1.Prepare the appropriate amount of BCA working fluid by 50 volumes of BCA reagent A plus 1 volume of BCA reagent B (50: 1) according to the number of samples. BCA working fluid is stable at room temperature for 24 hours.

2.2.Completely dissolve protein standard, the concentration of 2mg / ml. Protein samples in what solution, the standard is also appropriate to use what solution dilution.

2.3.Add the standard product to 0, 1, 2, 3, 4, 5, 6 μl to the standard wells of the 96-well plate and add 20 μl of the solution to the dilution standard.

2.4.Add the appropriate volume sample (part of the supernatant of step 4 in sample preparation) into the sample wells of a 96-well plate and add 20 μl of the solution to dilute the standard.

2.5.Add 200 μl of BCA working solution to each well and incubate at 37 ° C for 30 min.

2.6.Determination of A562,540-595nm wavelength between the acceptable. The protein concentration was calculated from the standard curve and the sample concentration ranged from 9 to 10 ug / ul. Only for the next experiment as a reference.

3. Western blot

3.1. Electrophoresis

3.1.1. With 10% separation glue, add TEMED immediately after the shake can be filling. After filling, with isopropanol sealant.

3.1.2. When there is a ray of water between the glue and glue, that glue has been condensed. And then wait 3min so that the plastic can be fully solidified to the upper layer of isopropyl alcohol and absorbent paper to dry.

3.1.3. With 4% of the concentrated glue, add TEMED immediately after the shake can be filling. Allow the remaining space to fill the concentrate and insert the comb into the concentrate.

3.1.4. Electrophoretic sample preparation, so that the total protein on each sample 50ug-100ug calculate the sample size required for each sample, and mix with 5 * loading buffer, boiling water for 5min, into the ice box in the cold.

3.1.5. According to the results of quantitative protein, 10 μl per empty sample has been denatured protein, began to electrophoresis. Concentration gel electrophoresis voltage of 80V, separation gel electrophoresis voltage of 120V. Electrophoresis

was terminated when the bromophenol blue was electrophoresed to the bottom of the gel.

3.2.Transfer film

3.2.1. Separate β-actin (42kDa), PD-1 (47kDa), NFkB (65kDa) respectively.

3.2.2. Prepare 6 sheets of filter paper and 1 PVDF film of the same size as the PVDF membrane. The PVDF membrane was first immersed in methanol and then placed in the membrane buffer with the filter paper until fully impregnated.

3.2.3. In accordance with the three filter paper, film, plastic, and the other three filter paper in the order in turn, requiring no bubbles in the middle.

3.2.4. Cover the instrument, turn on the power, constant current 300mA, transfer film β-actin and PD-1 for about 60 minutes, NFkB for about 80 minutes.

3.2.5. After film transfer, remove the membrane and place it in 1 * TBST for 5 min.

3.2.6. With Li Chunhong dye film, the efficiency of protein transfer film. Wash the cream with 1 * TBST.

3.3.Closed

5% skimmed milk powder was prepared with 1 * TBST, the membrane was immersed and allowed to stand at room temperature for 1 hour.

3.4.First resistance to incubation

Use 1 * TBST to dilute the primary antibody according to a certain percentage (see table below for details), incubate the membrane with the primary antibody, 4 degrees overnight. Incubate the end, 1 * TBST wash 3 times, each 15min. (Note: most CST antibodies need to be diluted with 5% BSA 1 * TBST)

Index name	First Antibody number	First Antibody source	Primary Antibody Dilution Ratio	Antibody Company
PD-1	18106-1-AP	Rabbit	1: 500	US proteintech
β-actin	60008-1-Ig	Mouse	1: 4000	US proteintech

Index name	First Antibody number	First Antibody source	Primary Antibody Dilution Ratio	Antibody Company
NFkB	Ab32536	Rabbit	1: 50000	UK abcam
β-actin	60008-1-Ig	Mouse	1: 4000	US proteintech

3.5.Secondary antibody incubation

HRP-labeled secondary antibody (Proteintech) was diluted with 1 * TBST, dilution ratio (M) 1: 4000, rabbit anti (R) 1: 6000, and the diluted secondary antibody was co-incubated with membrane for 45-60 min. Incubate the end, 1 * TBST wash 3 times, each 15min.

3.6.Color / exposure

ECL color exposure: incubate with ECL chemo-emissive liquid (Thermo) for 3 min, blot the liquid with absorbent paper, wrap the membrane with a wrap film, expose the film with X film for several seconds to several minutes in the cartridge; the ratio of the optical density of the measured indicators to the optical density of the internal reference yS-Actin quantitative values are analyzed.

C. Statistical analysis

Data are summarized as mean ± standard deviation (SD). Result were analyzed by one way ANOVA. All data were analyzed using SPSS 23 and Graphpad Prism 6.0 statistical package. A P value< 0.05 was considered statistically significant.

D. Experimental Result

Inhibitory effect of tumor growth by curcumin and ginsenoside was found in HepG2 xenograft animal model. Immune response has been important in cancer initiation and progression. Inflammation was considered as the main role of regulation in the role of curcumin and ginsenoside in the development of HCC. Chemoresistancy and metastasis are

problems that lead HCC to be a difficult kind of cancer to treated. PD-1 as immune response indexes, while NFkB as a chemoresistancy indicator.

1. **The effect of curcumin and ginsenoside on PD-1 expression on HepG2 xenograft animal model in vivo**

The result show the expression of PD-1 in ginsenoside group were significantly decreased compared with the model group ($p<0.05$) with no dose dependent effect. Ginsenoside have significant better effect then curcumin to block PD-1 expression ($p<0.05$). There were even significantly higher PD-1 expression in the combination of curcumin and ginsenoside group. The 5FU and Cisplatin group show significantly higher PD-1 expression compared with model and ginsenoside group.

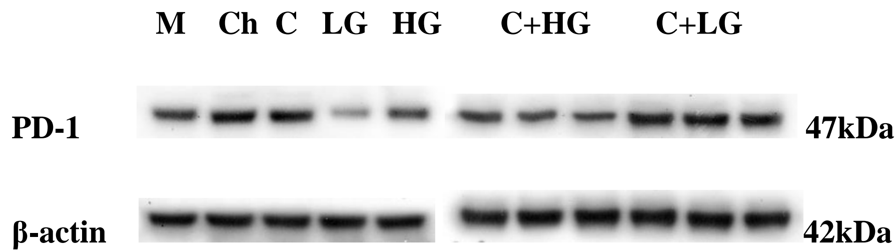
Table 2-1 The PD-1 of Hep G2 xenografts after ginsenoside and curcumin treatment ($\overline{X} \pm S$)

Group	n	PD-1/β-actin
Model	7	0.55±0.11
Low Dose Ginsenoside	7	0.33±0.06**
High Dose Ginsenoside	6	0.42±0.08*
Curcumin	7	0.63±0.14△△▲▲
Curcumin+Low Dose Ginsenoside	6	0.71±0.02*△△▲▲
Curcumin +High Dose Ginsenoside	7	0.48±0.04△○●●
Chemotherapy	6	0.69±0.14*△△▲▲□□

Note: Compared with model group * $p<0.05$, ** $p<0.01$; compared with low dose ginsenoside group, △ $P<0.05$, △△ $P<0.01$; compared with high dose ginsenoside group, ▲ $P<0.05$, ▲▲ $P<0.01$; compared with curcumin group, ○ $P<0.05$, ○○ $P<0.01$; compared with curcumin+low dose ginsenoside group, ● $P<0.05$, ●● $P<0.01$; compared with curcumin+high dose ginsenoside group, □ $P<0.05$, □□ $P<0.01$;

The result diagram

Grey scale analysis will be exposed after the film scanning , and the use of quantity of professional gray analysis software for analysis. The results are as follows.



M = Model

Ch = Chemotherapy

C = Curcumin

LG = Low Dose Ginsenoside

HG = High Dose Ginsenoside

C+LG = Curcumin + Low Dose Ginsenoside

C+HG = Curcumin + High Dose Ginsenoside

Figure 2-1 The relative expression of PD-1 in Hep G2 cell in each group

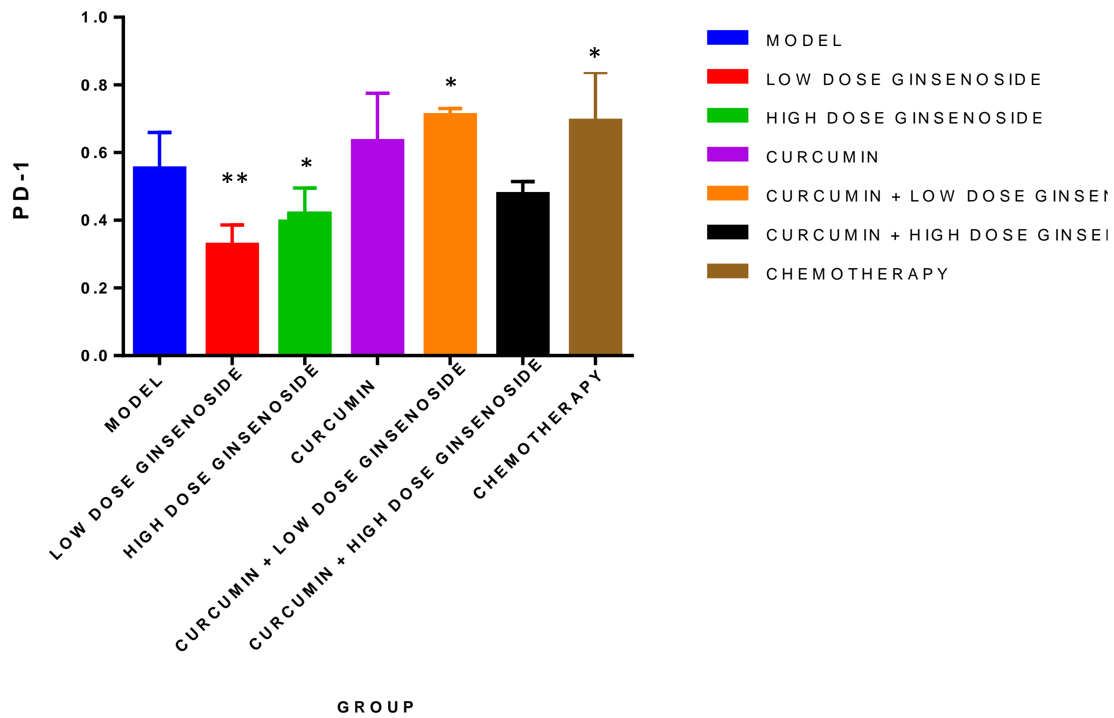


Figure 2-2 The relative expression of PD-1 in Hep G2 cell in each group (compared with control group (* P <0.05, ** P <0.01)

2. **The effect of curcumin and ginsenoside on NFkB expression on HepG2 xenograft animal model in vivo**

The expression of NFkB were not significantly inhibited by ginsenoside or curcumin alone. The inhibitory effect on NFkB significantly higher by combining curcumin and ginsenoside without no dose dependent manner and the inhibitory effect to NFkB even better although not significant with the 5FU and Cisplatin group.

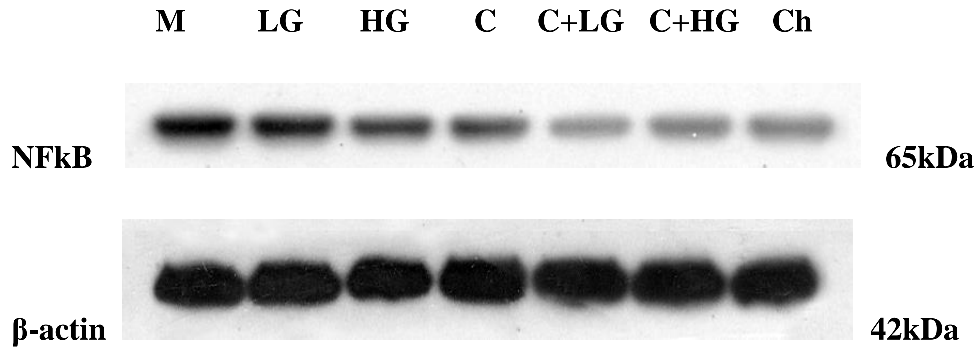
Table 2-2 The NFkB of Hep G2 xenografts after ginsenoside and curcumin treatment ($\overline{X} \pm S$)

Group	n	NFkB/β-actin
Model	7	0.36±0.04
Low Dose Ginsenoside	7	0.31±0.05
High Dose Ginsenoside	6	0.28±0.01
Curcumin	7	0.30±0.07
Curcumin+Low Dose Ginsenoside	6	0.13±0.04**△△▲▲○○
Curcumin +High Dose Ginsenoside	7	0.14±0.02**△△▲▲○○
Chemotherapy	6	0.2±0.05**△△▲○

Note: Compared with model group * p<0.05 , ** p<0.01; compared with low dose ginsenoside group, △ P <0.05, △△ P <0.01; compared with high dose ginsenoside group, ▲ P <0.05, ▲▲ P <0.01; compared with curcumin group, ○ P <0.05, ○○ P <0.01;

The result diagram

Grey scale analysis will be exposed after the film scanning , and the use of quantity of professional gray analysis software for analysis. The results are as follows.



M = Model

Ch = Chemotherapy

C = Curcumin

LG = Low Dose Ginsenoside

HG = High Dose Ginsenoside

C+LG = Curcumin + Low Dose Ginsenoside

C+HG = Curcumin + High Dose Ginsenoside

Figure 2-3 The relative expression of NFkB in Hep G2 cell in each group

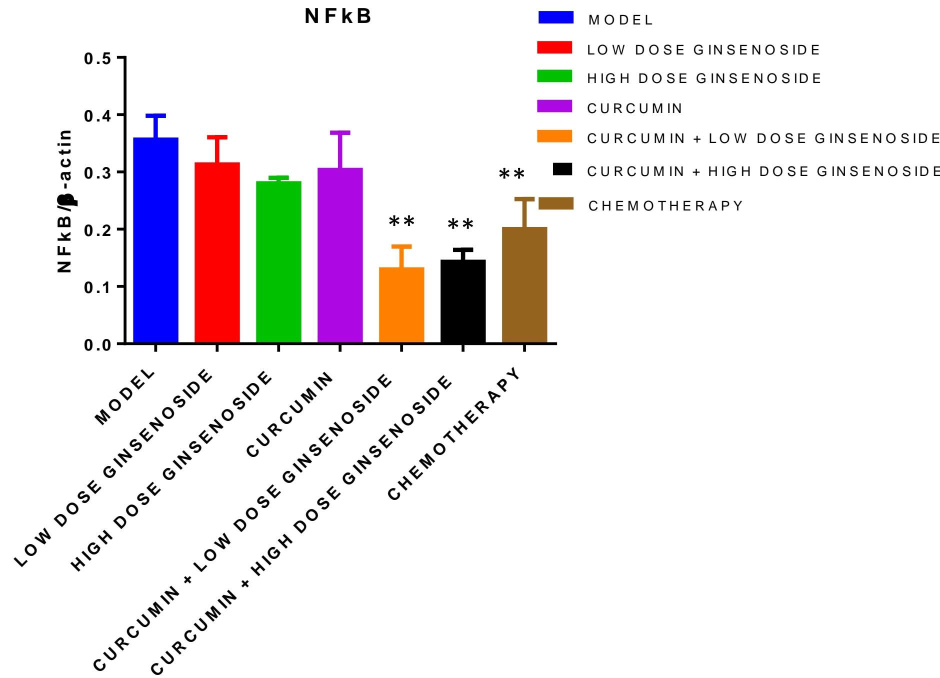


Figure 2-4 The relative expression of NFkB in Hep G2 cell in each group (compared with control group (* P <0.05, ** P <0.01)

E. Summary

1. Ginsenoside has a significant inhibitory effect to PD-1 expression
2. The inhibitory effect on NFkB significantly higher by combining curcumin and ginsenoside

Discussion

HCC is a large health problem in the world especially in China as the most high prevalence country. At present, the incidence of HCC in human malignancy have risen to 5.6%, most of which have occurred in China, and the incidence rate has increased year by year It is the fifth most common cancer in men and ninth in women; a second common cause of death from cancer worldwide. HCC strong invasiveness and easy to relaps lead to a poor prognosis. Most cases have a poor outcome because there was no chance for surgical treatment due to vascular invasion or multiple metastasis and also the problem of chemoresistancy to some chemotherapeutic agents.

Once diagnosed, HCC has a dismal prognosis. Small, localized tumors are potentially curable with surgery (resection & liver transplantation). Unfortunately, less than 20% of HCC patients are eligible for these procedures because most patients have advanced disease at diagnosis, have liver dysfunction limiting aggressive treatment, or have recurrent disease.HCC is notoriously resistant to chemotherapy and other systemic treatment modalities. The median survival for patients with advanced stage , unresectable HCC is less than 1 year. These reports underline the need for novel therapies for patients with this disease.

A number of other molecularly targeted approaches, all of which target signaling pathways activated in HCC are under investigation. However , the drug-metabolizing properties of the liver, in addition to elevated levels of multidrug resistance proteins expressed by HCC cells, likely contributes to the limited efficacy of chemotherapeutics and small molecule drugs in the treatment of HCC. Moreover, these agents typically have intrinsic hepatotoxicity that may further compromise liver function.

Immunotherapy represents an attractive alternative to these traditional therapies based on the sensitivity , specifity and self renewing capacity of the immune system.

In individual with persistent HBV infection, PD-1 expression by peripheral blood CD8+cytotoxic T lymphocytes (CTL) is upregulated with disease progression from cirrhosis to HCC. Abberantly activated monocytes that are enriched in HCC lessions have been shown to express high levels of the PD-L1 through a mechanism involving autocrine TNFα and IL-10 production. Antibody mediated blockade of PD-1 has demonstrated therapeutic benefit in the

setting of refractory solid tumors. Although single agent modalities have demonstrated some efficacy in the clinic, immunotherapy is expected to elicit synergistic anti tumor activity.[59-60, 115-116]

Because HCC has a rapid progress, easy to relapse after the solid tumor, so the prognosis is poor. Despite the HCC diagnosis and treatment have made great progress, but the prognosis of patients has not been significantly improved, the main reason is that the current drugs and technology can not completely solve the invasion, resistancy and metastasis of malignant tumors. According to the statistics, the overall 5-year survival rate of liver cancer is only about 7%, surgical resection and liver transplantation were not able to significantly improve the patients' survival rate. Although, in the study of the occurrence and development of liver cancer has made great progress, but the liver cancer treatment and prognosis, there are still a lot of problems to be solved. Many chemotherapeutic agents used has induced negative effect, such as induce resistancy, induce metastasis, and severe side effects. Further innovation needed to get better strategy toward better prognosis for HCC patients. Previous studies found some strategies that might be better in result with less side effect. Immunotherapy has been studied for this purpose as cancer induce immune system impairment. [82-85, 88-90, 106,114]

PD-1 appears to have a prominent role in modulating T cell activity in peripheral tissues via interaction with its ligands , PD-L1 and PD-L2. PD-1 is an immune checkpoint receptor that prevents overstimulation of immune responses and contributes to the maintenance of immune tolerance to self antigens. [104-105,118] An understanding of the checkpoint signaling pathway involving PD-1 receptor and its ligands (PD-L1/2) has clarified the role of these approaches in tumor-induced immune suppression and has been a critical advancement in immunotherapeutic drug development. Tumors that express PD-L1 can often be aggressive and carry a poor prognosis. The anti PD-1 and anti PD-L1 agents have a good safety profile and have resulted in durable responses in a variety of cancers. It appears that objective responds for anti PD-L1 antibodies may be lower that those with anti-PD1 antibodies, because the later blocks signaling via both the PD-L1 and PD-L2. Anti PD-1 and anti PD-L1 antibodies have yielded promising results with durable ressponses in several tumors and reasonable safety profile. PD-L1 expression has been shown to correlate with poor prognosis in many cancers. PD-L1 expression in tumors as a possible predictive biomarker of response to anti PD-1/PD-L1 drugs. [119]

Targetting only PD-L1 may be less toxicity but also maybe less effective than targeting PD-1 and thus blocking signaling via both PD-L1 and PD-L2. [120]

Expression of PD-L1 by tumor cells and immune infiltrates was significantly associated with expression of PD-1 on lymphocytes. PD-L2 was associated with PD-L1 expression. Tumor PD-L1 expression reflects an immune-active microenvironment while associated other immunosuppressive molecules, including PD-L1 and PD-L2, is the single factor most closely correlated with response to anti PD-1 blockade.[117,121]

One of the key checkpoint molecules that mediates tumor-induced immune suppression is PD-1. Blockade of PD-1/PD-L1 enhanced T-cell function and tumor cell lysis. Anti PD-1 has demonstrated antitumor activity in various solid cancers. PD-1/PD-L1 blockade could be a strategy for immunotherapy in HCC as PD-1 and PD-L1 found overexpressed in several kinds of malignancies, include in HCC.

NFkB plays a significant role in HCC. NFkB activation seen in inflammatory diseases are related to the increase incidence of cancer. It induces anti apoptotic protein and suppresses pro apoptotic gene. NFkB inhibition in the late stage of tumor development result in apoptosis of transformed hepatocytes and failure to progress to HCC; thus inhibit tumor progression in chronic inflammatory diseases with high cancer risk. Inflammation plays and important role in HCC occurrence and progression. Many anticancer agents induce NFkB translocation and activation and result in the development of chemoresistancy. NFkB inhibitor would be useful to prevent and treat chemoresistancy in cancer cell. [93,106,108]

Invasion and metastasis are the main features of malignant tumors, destruction of vascular basement membrane and degradation of extracellular matrix provides favorable conditions in the tumor cell invasion and metastasis. MMP-9 is a biodegradable extracellular matrix of IV,V-type collagen and gelatin matrix metalloproteinase (MMPs), in tumor metastasis to prevent fibrosis during the process of invasion, to provide a cell migration environment to inhibit tumor metastasis. The degradation of extracellular matrix through MMPs leads to the separation of the intercellular matrix and promote cancer cell mobility which caused metastasis.[103,107]

The inhibition of NFkB DNA binding activities could downregulate the MMP-9 expression. This could reverse multidrug resistance of cancer cells and prevent metastasis..

At present, in many treatment methods there is no effective technology and drugs could significantly improve the prognosis, recurrence and metastasis of HCC, and become one of the difficulties in clinical work. Advanced metastasis is the main threat in the life of patients with liver cancer, 5-year survival rate is very low. Therefore, the exploration of technology and drugs that can effectively prevent recurrence and inhibition of liver cancer transfer, would improve the patient's clinical symptoms and quality of life, to extend people's life has social and biomedical significance, and become the current need to address the social - biological - medical issues.

In recent years it has been no significant improvement in the prognosis and 5 year survival rate of HCC patients due to toxic side effects of chemical synthetic antitumor drugs. Some antitumor agent may upregulate the PD-1, PD-L1, and also NFkB and MMP-9 which could lead to the antiapoptosis, chemoresistancy and even metastasis which worsen the prognosis of HCC.

With the development of science and technology and the enhancement of health care consciousness, people's research on traditional Chinese medicine has also entered into a further study. A new exploratory study of the antitumor effects of plants and their derivatives has been carried out. Some anti-tumor plant extracts has been used as a drug for clinical treatment and achieved good results and found that the effective components of natural plants in the treatment of the tumor in patients have less adverse reactions, significant treatment effect. Seeking novel plants in natural plants and the exact effect of the compound has become the current anti-tumor drug research new goal, to improve people's understanding of the role of natural plant anti-tumor, and can bring a good socio- biological effects. Therefore, in recent years on the plant, minerals and their derivatives research continues to increase, some antitumor effects of plant extracts have been used as treatment for preclinical or clinical treatment.

Natural medicine, specifically Chinese medicine has been used empirically to treat malignancies. Previous studies shown that Chinese medicine has many benefit toward cancer treatment, it could be used as complementary medicine to improve patients quality of life, reduce the side effect of conventional cancer therapies, and prolong patients life. However there are still

limited scientific datas, especially about the mechanism of actions. Therefore this study was purposed to investigate this part.

Ginsenoside and curcumin has been used widely worldwide for many treatments of diseases include cancer. This research show that ginsenoside and curcumin could inhibit the growth of HCC in vivo. The inhibitory of tumor growth would be enhanced with the combination of both ginsenoside and curcumin, even similar to the chemotherapy.

Ginsenoside are commonly used to increase immune response and anti-inflammatory effect. HCC is one of many solid tumors with high expression of PD-1 and PD-L1 that induce immunosuppression. Ginsenoside in this research shows a significant anti PD-1 effect towards HCC cells while 5FU and cisplatin group has a high expression of PD-1 that could inhibit the antitumor effect. Previous studies declare that 5FU induce PD-1/PD-L1 expression in some cancer cells and develop resistance as a therapeutic challenge. Therefore combining anti PD-1 might help to overcome some of chemoresistance problem and enhance the anticancer activity of several chemotherapy drugs such as 5FU.[111-113]

In this study we found that the expression of PD-1 in chemotherapy group is higher then other groups similar to the previous studies that shown that 5FU could induce PD-1 expression. Ginsenosides blockade effect towards PD-1 expression could be used as a combination to chemotherapy agent to overcome their effect in PD-1 and therefore ginsenosides could be used as an immunotherapy for HCC patients.

Curcumin also has been studied for various diseases treatment, including as anticancer. The anti-inflammatory effect was such an important role in the anti HCC treatment. Curcumin downregulate the expression of MMP-9 via NFkB signaling pathway, therefore it could suppress tumor growth and metastasis. This research shown that the combination of ginsenoside and curcumin increased the inhibitory effect towards NFkB expression. Many anticancer agent induce NFkB activation , which could lead to chemoresistance. The combination of ginsenoside and curcumin could be an answer to significantly prevent and treat chemoresistancy in HCC patients.

Metastasis is the important issue to be treated in HCC and it could be monitored through the level of MMP-9 expression. Enhanced expression of MMP-9 will increase the metastasis of the

cancer cells. Ginsenoside and curcumin also proven to downregulate the expression of MMP-9 thus could prevent the metastasis of HCC and brought the patient toward a better prognosis.

Conclusion

1. Ginsenoside and curcumin single or combined has a significant effect toward tumor growth of HCC in vivo. The combination of both significantly increase the growth inhibitory effect toward HCC in vivo even similar to chemotherapy agent.
2. Ginsenoside inhibitory effect toward HCC in vivo belongs to its anti PD-1 and PD-L1 which were significantly stronger then other group. It could be suggested as anti PD-1 agent to be combined with chemotherapy agent especially in solid tumor with increased PD-1/PD-L1 expression as immunotherapy in HCC
3. Curcumin combined with ginsenoside has a significant NFkB inhibitory effect towards HCC in vivo. The inhibitory effect significantly stronger then using single curcumin or ginsenoside. This combination could be used as NFkB inhibitor to deal with chemoresistancy issue in HCC treatment that could be induced by some chemotherapy agents.
4. Curcumin and ginsenoside also act as MMP-9 inhibitor to prevent metastasis in HCC.

Research Prospects

Anticancer drug has been a hot topic in the field of biomedicine at present. Traditional Chinese medicine and its composition has become an important part. The antitumor effect of natural Chinese herb has been described in ancient literatures.

The results showed that ginsenoside and curcumin had significant inhibitory effect on the growth of HCC in vivo through many different mechanism. Ginsenoside and curcumin effect as PD-1 / PD-L1 blocker, NFkB and MMP-9 inhibitor would be a great innovation as anticancer and solve the problem of chemoresistance and metastasis in cancer treatment that could be induce by some chemotherapy agent. It could be further studied to found the effective dosage to be applied. This result could be implemented to make a better hope for HCC patients, and lead to a better prognosis in HCC.

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Literature Review

Recent Progress And Prospect of Curcumin and Ginsenoside in HCC treatment

HCC is typically an inflammation associated cancer and can be immunogenic. [128] Once diagnosed, HCC has a dismal prognosis. Small, localized tumors are potentially curable with surgery (resection & liver transplantation). Unfortunately, less than 20% of HCC patients are eligible for these procedures because most patients have advanced disease at diagnosis, have liver dysfunction limiting aggressive treatment, or have recurrent disease.HCC is notoriously resistant to chemotherapy and other systemic treatment modalities. The median survival for patients with advanced stage , unresectable HCC is less than 1 year. These reports underline the need for novel therapies for patients with this disease.

A number of other molecularly targeted approaches, all of which target signaling pathways activated in HCC are under investigation. However , the drug-metabolizing properties of the liver, in addition to elevated levels of multidrug resistance proteins expressed by HCC cells, likely contributes to the limited efficacy of chemotherapeutics and small molecule drugs in the treatment of HCC. Moreover, these agents typically have intrinsic hepatotoxicity that may further compromise liver function. Immunotherapy represents an attractive alternative to these traditional therapies based on the sensitivity , specifity and self renewing capacity of the immune system.

Although, in the study of the occurrence and development of liver cancer has made great progress, but the liver cancer treatment and prognosis, there are still a lot of problems to be solved. Many chemotherapeutic agents used has induced negative effect, such as induce resistancy, induce metastasis, and severe side effects. Further innovation needed to get better strategy toward better prognosis for HCC patients. Previous studies found some strategies that might be better in result with less side effect. Immunotherapy has been studied for this purpose as cancer induce immune system impairment. [114]

PD-1 appears to have a prominent role in modulating T cell activity in peripheral tissues via interaction with its ligands , PD-L1 and PD-L2. PD-1 is an immune checkpoint receptor that

prevents overstimulation of immune responses and contributes to the maintenance of immune tolerance to self antigens. [118] An understanding of the checkpoint signaling pathway involving PD-1 receptor and its ligands (PD-L1/2) has clarified the role of these approaches in tumor-induced immune suppression and has been a critical advancement in immunotherapeutic drug development. Tumors that express PD-L1 can often be aggressive and carry a poor prognosis. The anti PD-1 and anti PD-L1 agents have a good safety profile and have resulted in durable responses in a variety of cancers.

NFkB plays a significant role in HCC. NFkB activation seen in inflammatory diseases are related to the increase incidence of cancer. It induces anti apoptotic protein and suppresses pro apoptotic gene. NFkB inhibition in the late stage of tumor development result in apoptosis of transformed hepatocytes and failure to progress to HCC; thus inhibit tumor progression in chronic inflammatory diseases with high cancer risk. Inflammation plays and important role in HCC occurrence and progression. Many anticancer agents induce NFkB translocation and activation and result in the development of chemoresistancy. NFkB inhibitor would be useful to prevent and treat chemoresistancy in cancer cell.

Activation of the NFkB has been related to a great number of benign and malignant tumours, for example , hormonal-associated prostate cancer, lung carcinoma, HCC, multiple myeloma, melanoma, glioblastoma, ovarian tumour, malignant lymphoma, leukemia, breast cancer, colorectal cancer, pancreatic cancer, squamous cell carcinoma, mesothelioma, nasopharyngeal carcinoma, biliary cancer cell, soft tissue sarcoma, and other tumours. The ikk inhibitor of the NFkB plays an important role in HCC cancer cell death ,a very aggressive malignant tumour. [95]

Curcumin has in fact been shown to possess interesting anti inflammatory and anti tumor properties, which at last in part, appear be linked to its ability to suppress the activation of NFkB.[98] Curcumin reduce cell migration and MMP9 production of the HCC cells. [99] Curcumin has been shown to have multiple anticancer effects, including inhibition of proliferation, induction of apoptosis, and inhibition of angiogenesis, but it also induce apoptosis independent cell death. The anticancer effect of curcumin and its structural derivatives are dependent on their capacity of modulating multiple molecular targets, including transcription factors, growth factors, kinases, inflammatory cytokines, adhesion molecules, apoptosis related protein, and signaling

pathways such as NFkB, AKT, MAPK. One of the predominant target of curcumin is NFkB cell signaling pathway.[101]

Ginsenosides are reported to possess numerous biological activities, recent issues have arisen regarding their immunosuppressive and anti-inflammatory roles in inflammatory cells. Ginsenoside effectively inhibiting the production of inflammatory mediators through suppressing the activation of NFkB and its upst ream signaling cascade.[91] Ginseng upregulate T cell proliferation

Ginsenoside Rg3 treatment reduced the levels of vasculogenic mimicry in pancreatic cancer in vitro and in vivo through downregulation of MMP9 expression.[100] Ginsenosides inhibit COX2 expression attributed to inactivation of NFkB; a transcription factor whose activation inhibits the cell death signaling on oncogenic rats. [102]

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