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ABOUT COVER

Editorial board member of *World Journal of Gastrointestinal Oncology*, Adriana Ciocalteu, MD, PhD, is a Researcher at the Research Center of Gastroenterology and Hepatology and a Teaching Assistant in the Department of Gastroenterology of the University of Medicine and Pharmacy, Craiova (Romania). Having received her Bachelor's degree from University of Medicine and Pharmacy of Craiova in 2011, she undertook her postgraduate training at the Clinical Emergency County Hospital Craiova in the Gastroenterology Unit, Copenhagen University Hospital, Herlev and at Aarhus University Hospital (Denmark), receiving her PhD in 2016. Upon return to Craiova, she began her practice as Specialist in Gastroenterology and Teaching Assistant in the Department of Gastroenterology at the University of Medicine and Pharmacy. Her ongoing research interests involve studying malignant pathology of the digestive system using state-of-the-art techniques. (L-Editor: Filipodia)

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Basic Study

Synergistic anti-liver cancer effects of curcumin and total ginsenosides

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Abstract

BACKGROUND

Liver cancer is the sixth most frequently occurring cancer in the world and the fourth most common cause of cancer mortality. The pathogenesis of liver cancer is closely associated with inflammation and immune response in the tumor microenvironment. New therapeutic agents for liver cancer, which can control inflammation and restore cellular immunity, are required. Curcumin (Cur) is a natural anti-inflammatory drug, and total ginsenosides (TG) are a commonly used immunoregulatory drug. Of note, both Cur and TG have been shown to exert anti-liver cancer effects.

AIM

To determine the synergistic immunomodulatory and anti-inflammatory effects of Cur combined with TG in a mouse model of subcutaneous liver cancer.

METHODS

A subcutaneous liver cancer model was established in BALB/c mice by a

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subcutaneous injection of hepatoma cell line. Animals were treated with Cur (200 mg/kg per day), TG (104 mg/kg per day or 520 mg/kg per day), the combination of Cur (200 mg/kg per day) and TG (104 mg/kg per day or 520 mg/kg per day), or 5-fluorouracil combined with cisplatin as a positive control for 21 d. Tumor volume was measured and the protein expression of programmed cell death 1 and programmed cell death 1 ligand 1 (PD-L1), inflammatory indicators Toll like receptor 4 (TLR4) and nuclear factor- κ B (NF- κ B), and vascular growth-related factors nitric oxide synthases (iNOS) and matrix metalloproteinase 9 were analyzed by Western blot analysis. CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) were counted by flow cytometry.

RESULTS

The combination therapy of Cur and TG significantly inhibited the growth of liver cancer, as compared to vehicle-treated animals, and TG showed dose dependence. Cur combined with TG-520 markedly decreased the protein expression of PD-L1 ($P < 0.0001$), while CD4⁺CD25⁺Foxp3⁺ Tregs regulated by the PD-L1 signaling pathway exhibited a positive correlation with PD-L1. Cur combined with TG-520 also inhibited the cascade action mediated by NF- κ B ($P < 0.0001$), thus inhibiting the TLR4/NF- κ B signalling pathway ($P = 0.0088$, $P < 0.0001$), which is associated with inflammation and acts on PD-L1. It also inhibited the NF- κ B-MMP9 signalling pathway ($P < 0.0001$), which is associated with tumor angiogenesis.

CONCLUSION

Cur combined with TG regulates immune escape through the PD-L1 pathway and inhibits liver cancer growth through NF- κ B-mediated inflammation and angiogenesis.

Key Words: Total ginsenosides; Curcumin; Liver cancer; Immune; Inflammation; Programmed cell death 1 ligand 1

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Core Tip: The occurrence and development of liver cancer can be driven by inflammation, and the imbalance of cell-mediated immune mechanism also plays an important role. Controlling inflammation and restoring cellular immunity are new targets for the treatment of liver cancer. Here, we combined curcumin, an effective drug that controls inflammation, with total ginsenosides, which enhance immune function. We confirmed that the two drugs had a synergistic anti-liver cancer effect in a mouse model of subcutaneous, and they played their roles mainly through programmed cell death 1 ligand 1 and NF- κ B signaling pathway. These findings provide a new idea for combined drug therapy for liver cancer.

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INTRODUCTION

Liver cancer has a high morbidity rate and is one of the leading causes of cancer-related mortality worldwide. The estimated global incidence rate of liver cancer per 100000 person-years was 9.3 while the corresponding mortality rate was 8.5^[1,2]. Chronic liver injury, inflammation caused by hepatitis B and C virus infection, excessive drinking, or non-alcoholic fatty liver disease can lead to liver cirrhosis and cancer^[3]. Currently, there is a lack of effective clinical treatment for liver cancer, and the 5-year survival rate is < 8%. The pathogenesis of liver cancer is not only driven by inflammation; cell-mediated immune mechanism imbalance also plays an important role. Controlling inflammation and restoring cellular immunity in the tumor microenvironment are new treatment targets for liver cancer^[4]. Curcumin (Cur), a

component of the turmeric plant, is a natural drug with anti-inflammatory, anti-cancer, anti-viral, and anti-thrombotic properties; it also exerts protective effects over the liver and heart, and is associated with the modulation of molecular pathways and cellular tumor targets^[5]. Recent studies have shown that Cur can inhibit the proliferation, invasion, and metastasis of liver cancer cells^[6]. Cur has been demonstrated to inhibit the proliferation of hepatoma cell line (HepG2 cells) in a dose- and time-dependent manner^[7]. The current preclinical model studies *in vivo* and *in vitro* have shown that Cur, along with other curcuminoids, has immense potential as a curative agent for liver cancer, based on its potent antioxidant and anti-inflammatory properties and its ability to regulate a variety of signaling mechanisms^[6]. However, most of its anti-tumor effects rely on the anti-inflammatory mechanism, and its effects on the immune system are not obvious. The combination of effective components of drugs that have anti-inflammatory and immunoregulatory effects could potentially be used to enhance anti-tumor efficacy. Total ginsenosides (TG) are the main active ingredient in ginseng roots, and possess anti-cancer, anti-oxidative, immune-enhancing, endocrine-regulating, and other pharmacological effects^[8,9]. TG can exert anti-cancer effects through the mediation of the cell immune system mechanism^[10-12].

In terms of immune mechanisms, cancer cells are sometimes able to evade the host immune system in the tumor microenvironment. One of the most critical checkpoint pathways in this system is tumor-induced immune suppression (immune checkpoint) mediated by the programmed cell death 1 (PD-1) and its ligand, programmed cell death 1 ligand 1 (PD-L1). Inhibiting the expression of PD-1 and PD-L1 can effectively inhibit the suppressive effect of cancer cells on immunity^[13-15]. Moreover, studies have shown that the expression level of PD-L1 on T lymphocytes is closely associated with regulatory T cells (Tregs)^[16,17], and that PD-L1 regulates and induces the development, maintenance, and function of Tregs^[18]. CD4⁺CD25⁺Foxp3⁺ Tregs are a group of immunoregulatory cells with an inhibitory effect; tumor cells can recruit Tregs to inhibit anti-tumor immunity and promote tumorigenesis^[19]. In terms of inflammatory mechanisms, Cur has been proven to have powerful anti-inflammatory properties^[20-22]. Cur mediated suppression of nuclear factor- κ B (NF- κ B), which is the master switch in the inflammatory cascade, is an extremely critical mechanism of its widespread therapeutic profile^[23]. The development of liver cancer is not only regulated by the immune system, but also closely associated with inflammation. A previous study proposed the theory of "hepatic inflammation-fibrosis-liver cancer axis" (IFC axis)^[24]. The formation of liver cancer at the end of the IFC axis is associated with NF- κ B signal transduction factors downstream of the pathway^[25,26], while Toll like receptor 4 (TLR4) can induce the activation of NF- κ B downstream of the pathway to produce inflammatory factors^[27,28], activate the IFC axis, and promote the development of liver cancer. Furthermore, NF- κ B-mediated cascades also act on the PD-L1 signaling pathway^[29]. Tumor angiogenesis is an important condition for tumor growth and metastasis^[30]. Meanwhile, this process is also regulated by the NF- κ B signaling pathway, which can modulate nitric oxide synthase (iNOS) or MMP9 expression to produce inflammatory cascades and promote tumor vascular development^[31]. Therefore, Cur, a drug that controls inflammation, was combined with TG, which regulates immune function, to explore whether they have synergistic effects in the suppression of liver cancer, and further investigate the molecular mechanism of inflammation and regulation of immunity in the liver cancer microenvironment.

MATERIALS AND METHODS

Chemicals

TG (cat No. 20140711) were purchased from Changsha Huirui Biotechnology Co., Ltd. (Changsha, China). The purity of TG was > 95% and all studied parameters met the requirements of quality. Cur (cat No. SLBN7214V, molecular weight 368.39) was purchased from Merck KGaA (Darmstadt, Germany). Cur is safe for use in large and small animal models, with daily doses up to 12000 mg^[32]. The initial dose of TG was calculated as two times of the usual dose of ginsenoside (10 g/d), which was converted to the low dose at the beginning. Twenty grams per day, which is two-fold ginseng's clinically-used dose, was taken as the initial dose for calculation. The dose of ginseng for mice converted according to the ratio of human *vs* mouse's surface areas is equivalent to be 2080 mg/kg per day. Based on the extraction rate of 4% from ginseng and the purity of 80% of ginsenosides finally obtained, the initial dose of ginsenosides was determined to be 104 mg/kg per day. The high dose was converted to 520 mg/kg per d at 5 times the low dose. 5-fluorouracil (5-Fu) injection (cat No. 20160037) and

cisplatin (DDP; cat No. 20160319) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China).

Cell culture and animals

HepG2 cells were purchased from Xiangya Hospital Type Culture Collection (Changsha, China) and routinely maintained in Dulbecco's modified Eagle's medium with high glucose (Thermo Fisher Scientific, Inc., Waltham, MA, United States) supplemented with 10% fetal calf serum (Thermo Fisher Scientific, Inc.), 50 mg/mL streptomycin, and 50 U/mL penicillin. Cells were grown in an incubator with 5% CO₂ at 37 °C. Male BALB/c-nu nude mice were provided by Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China) and maintained in the animal center of Xiangya Hospital Type Culture Collection. Since the incidence rates among men are two to three-fold higher than rates among women in liver cancer^[1], male nude mice were used. All animals were housed under specific pathogen-free conditions with *ad libitum* access to sterile food and water and a 12/12-h light/dark cycle. The experimental protocol and all animal treatments were approved by the Experimental Animal Welfare Ethics Committee of Central South University.

Liver cancer model and experimental design

Cur and TG were given in combination to test whether they have synergistic inhibitory effects on liver cancer. Meanwhile, Cur and TG were given separately as control groups. In addition, studies have shown that TG are dose-dependent^[33]. There has been no optimal dose of TG to treat tumors and no reported combination with Cur. To investigate the effect of TG dose on combined drug use, TG were given at a low dose and a high dose. In order to reduce the pain of the experimental mice and avoid the traumatic drug administration as far as possible, TG and Cur were given orally. However, chemotherapy drugs were not suitable for oral administration in mice, hence 5-Fu and cisplatin were intraperitoneally injected.

Male BALB/c-nu/nu mice ($n = 49$, 5 wk old, 18 ± 3 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. They were allowed to acclimatize for 1 wk prior to treatment. For treatments, HepG2 cells (1×10^7) in 0.1 mL saline were injected into the right flank of the test mice to form subcutaneous xenotransplantation tumors. When the mice developed tumors of a similar size, they were randomly divided into the following seven groups ($n = 7$ per group): Vehicle control group (daily oral dose of 0.2 mL of distilled water), TG 104 mg/kg per day group (TG-104; daily oral dose of 104 mg/kg TG), TG 520 mg/kg per day group (TG-520; daily oral dose of 520 mg/kg TG), Cur group (daily oral dose of 200 mg/kg Cur), Cur + TG-104 group (daily oral dose of 200 mg/kg Cur and 104 mg/kg TG), Cur + TG-520 group (daily oral dose of 200 mg/kg Cur and 520 mg/kg TG), and 5-Fu + DDP group (5-Fu + DDP, weekly intraperitoneal dose of 32 mg/kg 5-Fu and 4 mg/kg DDP). After 21 d of treatment, test mice were euthanized by cervical dislocation according to the ARRIVE guidelines. During the experiment, the tumor volume of mice was measured every 3 d to evaluate their growth. Tumor sizes were measured using calipers, and their volume was calculated using the following formula: $(L \times W^2)/2$, where L and W are the length and width of the tumor, respectively.

Western blot analysis

Tumor tissue specimens from mice were prepared and suspended in ice-cold RIPA lysis buffer (150 mmol/L NaCl, 20 mmol/L HEPES, 1% Triton X-100, 2 mmol/L EGTA, 20 mmol/L glycerol phosphate, 1 mmol/L EDTA, and 10% glycerol with protease; ApplyGene Inc). The protein levels of tumor tissues were examined by the BCA assay. Proteins (50 µg) were denatured and resolved by 4% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Following blocking with 5% milk in TBST, PVDF membranes (EMD Millipore) were blocked for 2 h in blocking buffer (5% 1 × TBST) prior to overnight incubation (at 4 °C). The proteins were transferred onto the PVDF membranes for 2 h, and incubated with the primary and secondary antibodies for 60 min at 37 °C prior to overnight incubation at 4 °C. The following primary antibodies were used: Anti-PD-1 and PD-L1 (cat Nos. 18106-1-AP and 66248-1-Ig, respectively; dilution, 1:500; polyclonal antibody; Proteintech Group, Inc.); anti-nuclear factor-κB (NF-κB; cat No. ab32536; dilution, 1:5000; monoclonal antibody; Abcam); anti-matrix metalloproteinase (MMP)-9 and nitric oxide synthase (iNOS; cat Nos. ab38898 and ab178945, respectively; dilution, 1:1000; polyclonal antibody; Abcam); an anti-actin antibody (cat No. 60008-1-Ig; dilution, 1:3000; monoclonal antibody; Proteintech Group, Inc.) was used as the protein-loading control. A horseradish peroxidase-conjugated polyclonal secondary antibody (dilution, 1:3000; Proteintech Group, Inc.)

was used for the assay. Protein signals were visualized by enhanced chemiluminescence using SuperECL Plus detection reagent (Thermo Fisher Scientific, Inc.) and exposure to Kodak Biomax XAR film (Kodak). The density of bands was quantified using Image J version 1.80 software (National Institutes of Health).

Flow cytometry and CD4⁺CD25⁺Foxp3⁺Treg isolation

Single cell suspensions were prepared from the tumor tissue of mice. Cells were stained with the following mouse-specific antibodies: FITC-conjugated CD4, APC-conjugated CD25, and PE-conjugated Foxp3 (cat Nos. 100406, 100910, and 320008, respectively; Biolegend Inc.). Analysis of cell surface markers was performed using FlowJo (FlowJo LLC) and gated according to surface markers and negative controls. Isotype-matched IgG staining was used as the negative control (Thermo Fisher Scientific, Inc.). Tregs were isolated from the tumor tissue of mice as follow single cell suspensions were made from the tumor tissues. These cells were analysed with fluorescence-conjugated antibodies and isotype-matched IgG controls. The cells were analysed on an FACScan flow cytometer.

Statistical analysis

Three biological replicates and two technical repetitions were performed for each assay, unless indicated otherwise. Data are presented as the mean \pm standard error of the mean. Data following a normal distribution were analysed using one-way analysis of variance, and statistical significance of the difference between two groups was determined using the least significant difference or Dunnett's T3 methods. Unless otherwise stated, $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Cur and TG suppress tumor growth in a subcutaneous liver cancer mouse model

Tumor volumes in the TG-520, Cur, Cur + TG (104 mg/kg and 520 mg/kg), and 5-Fu + DDP groups was reduced, as compared with that of the vehicle group (Figure 1A). The reduction of tumor volume of the Cur + TG-520 mg/kg group was more significant, as compared with the Cur or TG group. Graphs of *ex vivo* tumor growth further confirmed the effects of Cur and TG in tumor suppression (Figure 1B).

Cur and TG suppress the expression of PD-1/PD-L1 and Tregs

In order to study the anti-tumor mechanism of Cur and TG, the protein expression of PD-1 and PD-L1 in tumor tissues was determined by Western blot analysis (Figure 2A and C). As compared with the vehicle group, TG inhibited the expression of PD-1 and PD-L1 in a dose-dependent manner. No significant effect was observed in the Cur group, and enhanced PD-L1 expression was noted in the 5-Fu + DDP group ($P = 0.0021$). A more significant inhibitory effect on the expression of PD-L1 was observed in the Cur + TG-520 group than in either the TG (104 mg/kg or 520 mg/kg) or Cur group (Cur + TG-520 *vs* TG-104, $P < 0.0001$; Cur + TG-520 *vs* TG-520, $P = 0.0211$; Cur + TG-520 *vs* Cur, $P < 0.0001$). To further investigate whether the interaction of Cur and TG is mediated by the PD-L1 signaling pathway, CD4⁺CD25⁺Foxp3⁺ Tregs were evaluated by flow cytometry in tumor tissues. As illustrated in Figure 2A and D, the PD-L1 protein expression was positively correlated with the expression of CD25⁺Foxp3⁺ Tregs.

Cur and TG suppress the protein expression of NF- κ B

To determine the effects of Cur and TG on the inflammatory mechanisms in liver cancer, the protein expression of TLR4 and NF- κ B was further detected in tumor tissues. As shown in Figure 2B and C, compared with the vehicle group, both Cur and TG-520 inhibited the protein expression of NF- κ B ($P < 0.0001$), and the inhibitory effect was enhanced when they were combined (Cur + TG-520 *vs* TG-104, $P < 0.0001$; Cur + TG-520 *vs* TG-520, $P = 0.0002$; Cur + TG-520 *vs* Cur, $P = 0.0002$), and TG exerted the effect in a dose-dependent manner (TG-104 *vs* TG-520, $P = 0.0003$). However, the inhibition of the TLR4 protein expression was only observed when Cur and TG were used together (vehicle *vs* Cur + TG-104, $P = 0.0336$; vehicle *vs* Cur + TG-520, $P = 0.0088$). Compared with the protein expression of PD-L1 (Figure 2A), the expression of NF- κ B and PD-L1 in the Cur and TG-520 groups showed a positive correlation.

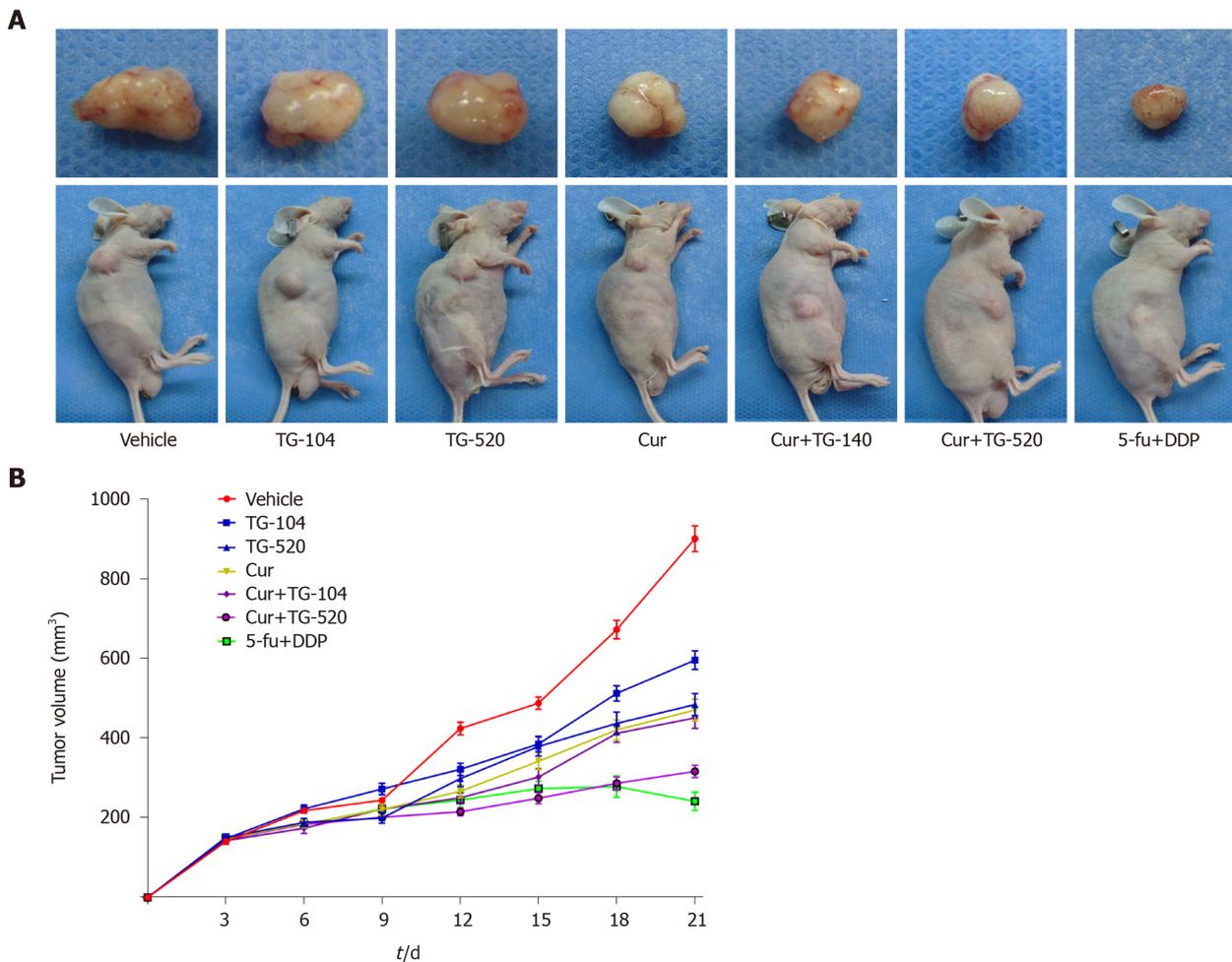


Figure 1 Curcumin and total ginsenosides inhibit liver cancer growth. A: Representative photographs of subcutaneous liver cancer following treatment with curcumin (200 mg/kg per day), total ginsenosides (104 mg/kg per day or 520 mg/kg per day), or the combination of the two for 21 d; B: The tumor volume of mice was measured. Data are presented as the mean \pm standard error of the mean. ^a $P < 0.05$ and ^b $P < 0.01$ between two groups using least significant difference or Dunnett's T3 method. Cur: Curcumin; TG: Total ginsenosides; 5-Fu: 5-fluorouracil; DDP: Cisplatin.

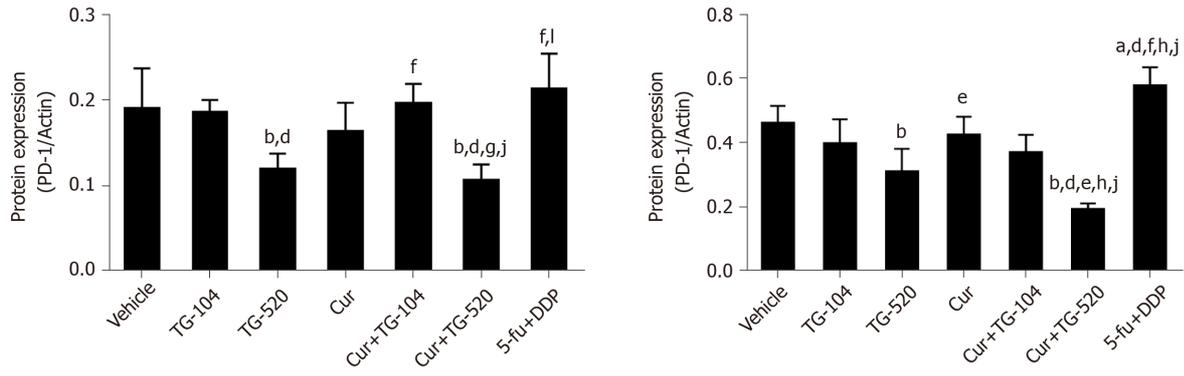
Cur and TG suppress the protein expression of MMP9

In order to determine whether Cur and TG inhibit tumor angiogenesis, the protein expression of iNOS and MMP9 was evaluated by Western blot analysis (Figure 3). As compared with the vehicle group, TG and chemotherapy could downregulate the expression of iNOS ($P < 0.0001$), but had no synergistic effects when combined with Cur. The Cur group has no obvious effect on the expression of iNOS (Figure 3A). Meanwhile, the protein expression of MMP9 was also decreased as did NF- κ B expression (Figure 3B and C). It was further explored whether the combination of Cur and TG has a synergistic effect on the regulation of the NF- κ B, MMP9 and iNOS protein expression. The combination of the two drugs increased the protein expression of MMP9 and NF- κ B, but not that of iNOS, as compared with the Cur or TG along group.

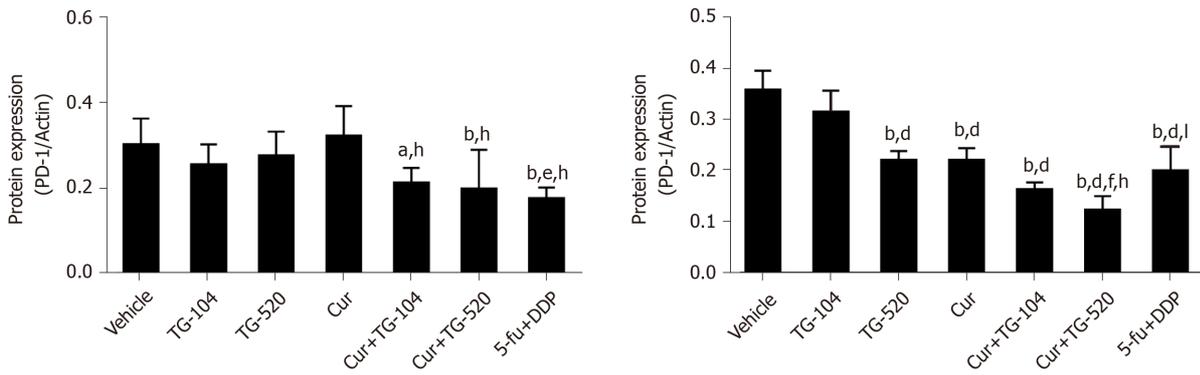
DISCUSSION

Liver cancer is one of the main causes of cancer-related mortality worldwide. The occurrence and development of liver cancer are driven by inflammation, but cell-mediated immune mechanism imbalance also plays an important role in this process^[4]. Cur is a natural phenolic pigment extracted from *Cur longa*, a type of rhizome that belongs to the ginger family. It can exert anti-tumor effects by modulating tumor microenvironment, such as inflammation, angiogenesis, and metastasis^[34]. In addition, ginseng is a herb used in adjuvant treatment of cancer. TG are one of the main components of ginseng that mainly exists in ginseng roots and has anti-tumor effects^[35], which it exerts by enhancing the body's immunity to inhibit

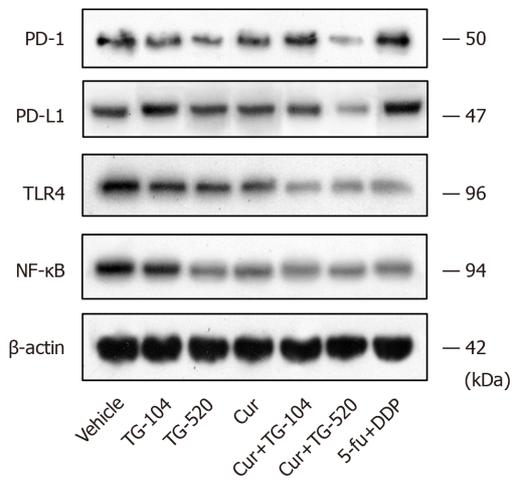
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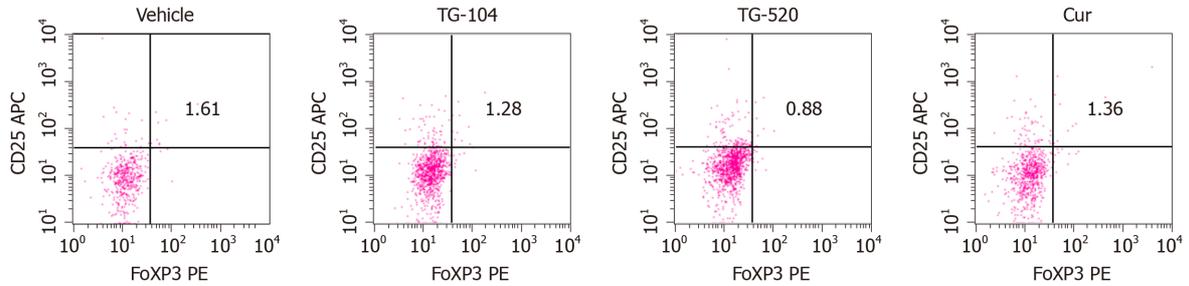
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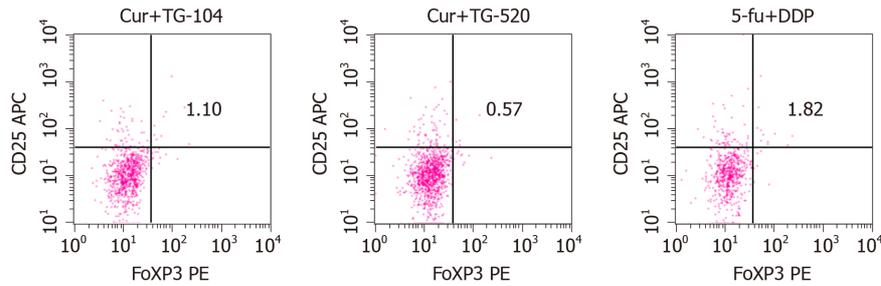


Figure 2 Curcumin and total ginsenosides inhibit the activation of programmed cell death 1/ programmed cell death 1 ligand 1 and Tregs. A subcutaneous liver cancer mouse model was treated with curcumin (Cur), total ginsenosides (TG), or a combination of the two for 21 d, and tumor tissues were harvested as indicated. A-C: The protein levels of programmed cell death 1, programmed cell death 1 ligand 1, Toll like receptor 4, and nuclear factor- κ B were evaluated by Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase was used as the loading control; D: CD4⁺CD25⁺Foxp3⁺ Tregs were evaluated by flow cytometry. Results show the proportion of CD4⁺CD25⁺Foxp3⁺ Tregs in HepG2 subcutaneous tumor tissues of BALB/c mice. Data are presented as the mean \pm standard error of the mean, and were compared between two groups using least significant difference or Dunnett's T3 method. ^a $P < 0.05$ and ^b $P < 0.01$ vs vehicle group; ^c $P < 0.05$ and ^d $P < 0.01$ vs TG-104 group; ^e $P < 0.05$ and ^f $P < 0.01$ vs TG-520 group; ^g $P < 0.05$ and ^h $P < 0.01$ vs Cur group; ⁱ $P < 0.05$ and ^j $P < 0.01$ vs Cur + TG-104 group; ^k $P < 0.05$ and ^l $P < 0.01$ vs Cur + TG-520 group. Cur: Curcumin; TG: Total ginsenosides; PD-1: Programmed cell death 1; PD-L1: Programmed cell death 1 ligand 1; Tregs: CD4⁺CD25⁺Foxp3⁺ regulatory T cells; TLR4: Toll like receptor 4; NF- κ B: Nuclear factor- κ B; DDP: Cisplatin; 5-Fu: 5-fluorouracil.

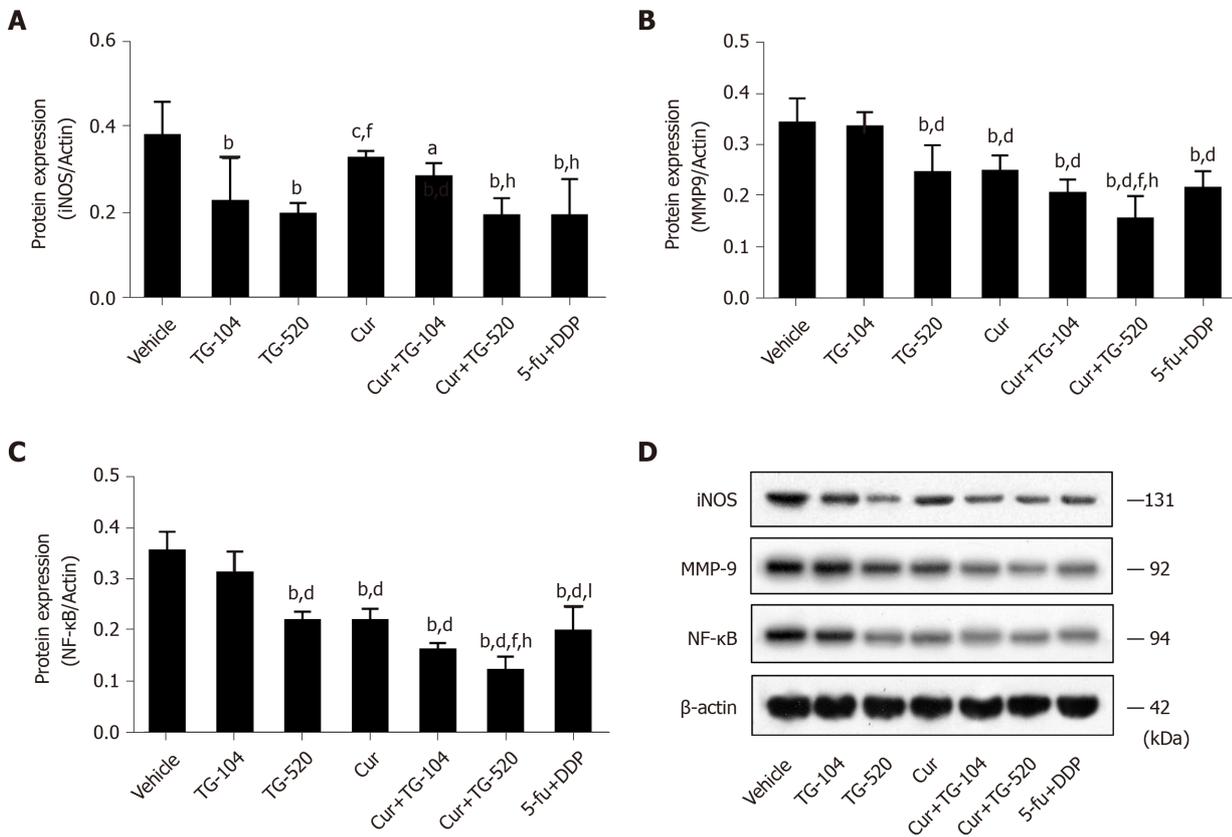


Figure 3 Curcumin and total ginsenosides inhibit the activation of matrix metalloproteinase 9 and nitric oxide synthase. A-D: The protein levels of nitric oxide synthase, matrix metalloproteinase 9, and nuclear factor- κ B were evaluated by Western blot analysis in tumor tissues from a subcutaneous liver cancer mouse model. Glyceraldehyde-3-phosphate dehydrogenase was used as the loading control. Data are presented as the mean \pm standard error of the mean, and were compared between two groups using least significant difference or Dunnett's T3 method. ^a $P < 0.05$ and ^b $P < 0.01$ vs vehicle group; ^c $P < 0.05$ and ^d $P < 0.01$ vs TG-104 group; ^e $P < 0.05$ and ^f $P < 0.01$ vs TG-520 group; ^g $P < 0.05$ and ^h $P < 0.01$ vs Cur group; ⁱ $P < 0.05$ and ^j $P < 0.01$ vs Cur + TG-104 group; ^k $P < 0.05$ and ^l $P < 0.01$ vs Cur + TG-520 group. Cur: Curcumin; TG: Total ginsenosides; DDP: Cisplatin; 5-Fu: 5-fluorouracil; NF- κ B: Nuclear factor- κ B.

tumor cell proliferation, invasion, and metastasis in a dose-dependent manner. Our present study combined Cur, which can control inflammation, with TG, which can adjust immunity, to investigate whether their combination could inhibit the growth of liver cancer and create synergy. The results demonstrated that Cur combined with TG inhibited tumor growth in mice with liver cancer, and had synergistic effects, while TG

showed dose dependence.

The participation of PD-1/PD-L1 in the immune response of the liver cancer microenvironment is involved in the occurrence and development of liver cancer^[36]. PD-L1 has a negative effect in regulating the immune response. After PD-L1 binding to PD-1, PD-1 transmits inhibitory signals, which can have a variety of biological effects, such as inhibiting the proliferation and activation of lymphocytes, the differentiation of CD4⁺ T cells into Th1 and Th17 cells, and the release of inflammatory cytokines^[37,38]. While PD-1 binding with PD-L1 is one of the important factors leading to cancer cells evading the immune system. Most studies in the field have found that, in animal models of liver cancer, a variety of cells express elevated levels of PD-1/PD-L1, leading to the inhibition of effector cells, which helps liver cancer cells escape immunity. Therefore, while administering radiotherapy, chemotherapy, and biotherapy, locally blocking the signalling pathway of PD-1/PD-L1 may be a promising treatment strategy for fighting liver cancer. In our study, we found that TG can block the PD-1/PD-L1 signaling pathway in a dose-dependent way, and have synergistic effects with Cur in inhibiting the PD-L1 expression, which indicates that the synergistic effects of Cur and TG are mainly mediated by the PD-L1 signaling pathways, and the combination of the two drugs may further inhibit the binding of PD-1 and PD-L1 to act on the immune system by inhibiting the expression of PD-L1. However, the mechanism through which the combination of Cur and TG reverses the immune escape of liver cancer cells requires further investigation.

CD4⁺CD25⁺Foxp3⁺ Tregs, a subset of T lymphocytes, could inhibit effector T lymphocyte proliferation and cytokine secretion, which is critical for tumor immune response^[39]. Foxp3⁺ Tregs have been found in many human tumors and play a key role in preventing immune suppression, escape, metastasis, and recurrence in tumors. They are also considered biomarkers and prognostic factors for human malignant tumors^[40-42]. It has been well documented that the infiltration of Foxp3⁺ Tregs in soft tissue sarcomas may lead to a poor patient prognosis and is considered as an important therapeutic target in human cancer immunotherapy^[43]. Increased Tregs infiltration has been reported to adversely affect tumor prognosis and proved to be an independent predictor of poor prognosis or high recurrence in liver cancer by preventing anti-tumor immune response^[44]. Furthermore, it was demonstrated that Foxp3⁺ Tregs are also a target of the PD-1 and PD-L1 protein binding in cancer cells^[45]. PD-L1⁺ T cells and Tregs can jointly inhibit anti-tumor immunity. The overexpression of PD-L1 in T cells is closely associated with the increasing number of Tregs^[16,46]. Consistent with this, it was also identified that the PD-1/PD-L1 signaling pathway can promote Tregs differentiation; the higher level of PD-L1 expressed by hepatic dendritic cells, the more Tregs can be induced^[47]. Specific blocking of PD-L1 with monoclonal antibodies or siRNA can reduce the production of CD4⁺CD25⁺Foxp3⁺ Tregs and induce Tregs apoptosis^[19,48]. Therefore, we speculated that the PD-1/PD-L1 signaling pathway can promote Tregs differentiation through PD-L1, and tested the expression of CD4⁺CD25⁺Foxp3⁺ Tregs in tumor tissues. Our results suggested that the combination of Cur and TG significantly deregulated CD4⁺CD25⁺Foxp3⁺ Tregs in liver cancer cells. Of note, it was observed that the low PD-L1 expression deregulates Tregs. We speculated that the combination of Cur and TG could further inhibit the expression of CD25⁺Foxp3⁺ cells by inhibiting the expression of PD-L1, to reduce its suppression of the immune response to liver cancer. Therefore, Tregs can be a malleable and attractive therapeutic target for liver cancer treatment.

In addition, inflammation is also an important factor to drive immune escape in liver cancer tumorigenesis and development^[4]. It has been long recognized that tumor cell immune escape driven by PD-1/PD-L1 has two parts: PD-1/PD-L1 can regulate lymphocytes and PD-L1 can be expressed on the surface of tumor-associated macrophages (TAM) and inhibit tumor-infiltrating lymphocytes (TIL) or induce TIL apoptosis to promote tumor immune escape^[49]. Inflammation is critical to PD-L1 overexpression. Chen *et al.*^[29] proposed that inflammatory cytokines produced by TAM can promote the expression of PD-L1 through the NF-κB signaling pathway. In the inflammatory signaling pathway, TLR4 is the upstream of NF-κB and can both mediate immune response and induce the release of inflammatory mediators^[24]. Blocking the TLR4/NF-κB pathway can inhibit the expression of PD-L1, thereby blocking the immune escape of tumor cells. Multiple studies have shown that the TLR4/NF-κB signaling pathway may become a new target for liver cancer treatment^[25]. Both Cur and TG have been reported to suppress the TLR4 or NF-κB expression^[6,50,51]. Our results also confirmed that both Cur and TG can inhibit the NF-κB-TLR4 signaling pathway in liver cancer cells. Furthermore, the combination of Cur and TG significantly and synergistically inhibited the TLR4 and PD-L1 expression. It can be speculated that the suppression of the TLR4/NF-κB/PD-L1 pathway by

combining Cur and TG could potentially inhibit inflammation-driven immune escape in liver cancer.

Cancer cells need to destroy extracellular matrix (ECM) components to escape from the primary site into blood and lymphatic vessels^[52]. The degradation of the ECM is one of the key steps of tumor cell invasion and metastasis. MMPs are important substances that degrade the ECM. MMP9 can degrade the major components of the ECM, the basement membrane collagen types IV and V. Therefore, its activity is closely associated with the angiogenesis, invasion, and metastasis of tumor cells^[53,54]. Cur can inhibit NF- κ B, as well as the activity of MMP-2 and MMP9, thereby exerting its effect against tumor metastasis and invasion^[55]. NF- κ B is thought to be the master switch of the inflammatory cascade^[23], and its activation can regulate several key inflammatory mediators. Jung *et al*^[56] found that the activated NF- κ B signaling pathway in liver cancer tissues can upregulate MMP-2 and MMP9, thereby promoting the metastasis of liver cancer. Other studies have shown that NF- κ B-induced tumor invasion and metastasis are achieved through MMP9, since NF- κ B contains MMP9 binding sites, and MMP9 upregulation is activated by NF- κ B^[57]. Therefore, inhibiting NF- κ B and upregulating the MMP9 pathway can help inhibit the invasion and metastasis of liver cancer cells. Our research data showed that NF- κ B was positively correlated with MMP9 protein expression but had no significant correlation with the iNOS expression. Therefore, we speculate that Cur combined with TG could inhibit the MMP9 expression in liver cancer cells through the NF- κ B signaling pathway, which plays an important role in suppressing vascular formation in liver cancer.

In summary, we demonstrated that TG inhibit the PD-1/PD-L1 protein expression, and that the combination of TG and Cur synergistically suppresses the protein expression of PD-L1 and NF- κ B by decreasing Foxp3⁺ Tregs expression, leading to a reduction in tumor growth. Furthermore, the combination improves immune escape in the liver cancer microenvironment through the suppression of PD-L1, and whether there is a synergistic downregulation of angiogenesis of tumor cells with MMP9 needs to be further investigated, to gain a further understanding of these mechanisms. Nonetheless, the current findings regarding the combination of Cur and TG have elucidated the molecular mechanism through the modulation of PD-L1, TLR4, NF- κ B, MMP9, and Tregs, and suggested that the combination of Cur and TG could potentially be important for the treatment of liver cancer. Therefore, Cur combined with TG regulates immune escape through the PD-L1 pathway and inhibits liver cancer growth through NF- κ B-mediated inflammation and angiogenesis. This study offers a potential combination of drugs that could improve the effectiveness of treatment for liver cancer.

Although curcumin is effective and safe, its low relative bioavailability is a major obstacle to clinical use. Many new approaches have been explored, including the use of liposomal, nanoparticles, curcumin phospholipid complexes, and structural analogues of curcumin (*e.g.*, EF-24) to enhance the bioavailability^[58]. In future studies, we will explore the use of liposomes or nanoparticles to enhance the medicinal value of curcumin.

ARTICLE HIGHLIGHTS

Research background

New therapeutic agents for liver cancer, which can control inflammation and restore cellular immunity, are required. Curcumin (Cur) is a natural anti-inflammatory drug and total ginsenosides (TG) are a commonly used immunoregulatory drug.

Research motivation

Both Cur and TG have been shown to exert anti-liver cancer effects. This study discussed the anti-tumor effect of Cur combined with TG in liver cancer and its molecular mechanism.

Research objectives

To determine the synergistic immunomodulatory and anti-inflammatory effects of Cur combined with TG in a mouse model of subcutaneous liver cancer.

Research methods

The changes in tumor volume and expression of relevant factors were compared in a subcutaneous liver cancer mouse model after treatment with Cur, TG, and the

combination of the two drugs. The protein expression of programmed cell death 1 (PD-1) and PD-1 ligand 1 (PD-L1), inflammatory indicators Toll like receptor 4 (TLR4) and nuclear factor- κ B (NF- κ B), and vascular growth-related factors nitric oxide synthases (iNOS) and matrix metalloproteinase 9 were analyzed by Western blot analysis. CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) were counted by flow cytometry.

Research results

The combination therapy of Cur and TG significantly inhibited the growth of liver cancer, and TG showed dose dependence. Cur combined with TG-520 markedly decreased the protein expression of PD-L1 ($P < 0.0001$), while CD4⁺CD25⁺Foxp3⁺ Tregs regulated by the PD-L1 signaling pathway exhibited a positive correlation with PD-L1. Cur combined with TG-520 also inhibited the cascade action mediated by NF- κ B ($P < 0.0001$), inhibiting the TLR4/NF- κ B signaling pathway ($P = 0.0088$, $P < 0.0001$), which is associated with inflammation and acts on PD-L1. It also inhibited the NF- κ B-MMP9 signaling pathway ($P < 0.0001$), which is associated with tumor angiogenesis.

Research conclusions

Cur combined with TG regulates immune escape through the PD-L1 pathway and inhibits liver cancer growth through NF- κ B-mediated inflammation and angiogenesis.

Research perspectives

This study offers a potential combination of drugs that could improve the effectiveness of treatment for liver cancer.

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