Dahuang Zhechong pills inhibit liver cancer growth in a mouse model by reversing Treg/Th1 balance

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[ABSTRACT] The infiltration of immune cells into the hepatocellular carcinoma microenvironment is the main reason why hepatocellular carcinoma patients are prone to carcinoma recurrence and the disease are incurable. Notably, the infiltration of Treg cells is the main trigger. Dahuang Zhechong pill (DHZCP) is a traditional Chinese herbal compound successful in the treatment of hepatitis and hepatocellular carcinoma. DHZCP can heal and nourish while slowing the onset of the disease, thereby strengthening the body's immune function. It can localize tumors and ultimately achieve the goal of eliminating tumors. In this study, an orthotopic liver cancer model of mice was used to explore the mechanism of DHZCP enhancing anti-tumor immunity, which showed more Th1 cells in the peripheral blood and spleen after DHZCP treatment, while more IFN-γ was secreted to activate CD8⁺ T cells and Treg cell production was inhibited, thereby suppressing the growth of HCC. Finally, we also analyzed the potential components of DHZCP from the perspective of modern targets using network pharmacology methods and experimental results.

[KEY WORDS] Network pharmacology; Hepatocellular; Dahuang Zhechong pill; Balance; Th1-Treg


Introduction

Hepatocellular carcinoma (HCC) is a type of primary liver cancer that accounts for more than 90% of primary liver cancers and usually caused by chronic hepatitis, fibrosis, and cirrhosis [1]. A recent report from the National Cancer Center of China (NCCC) states that HCC remains the fourth most common cancer diagnosed in China, which is a serious, potentially fatal disease. In recent years, despite significant advances in immunotherapy for HCC, the prognosis for patients with immune escape is actually not very optimistic [2]. Tumor immune escape is a process where tumor cells survive in the body by evading recognition and attack by the immune system through multiple mechanisms [3]. Several studies have shown that depletion of CD8⁺ T cells and massive infiltration of regulatory T cells (Tregs) led to immune escape of HCC [4]. The number of Tregs in the peripheral blood and tumor tissues is closely related to the occurrence of liver cancer [5]. Treg cells are highly immunosuppressive CD4⁺ T cells, while the transcription factor Forkhead box P3 (FOXP3) is a key transcription factor that exerts immunosuppressive function. Treg cells in which CD25 is up-regulated deplete IL-2 through signaling and activate FOXP3 expression; they also inhibit the antitumor effects of effector T cells through direct contact of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) with antigen-presenting cells (APCs) or through secretion of inhibitory cytokines such as TGF-β, IL-10 and IL-35; and they express perforin and granzyme to kill effector T cells and APCs [6]. Animal experiments revealed that Treg clearance indeed led to tumor regression as well as alteration of infiltrating immune cells. However, the transient loss of Treg cells in model animals, followed by some serious systemic autoimmune diseases and inflammatory responses, complicating the subsequent treatment of the disease [7], is even a more worrisome result of these experiments. The above evidence suggests that Tregs are an important tar-
get for the treatment of HCC, which are crucial to develop a safe and effective therapeutic regimen for Tregs.

IL-12 is considered to be the most potent pro-inflammatory cytokine to trigger anti-tumor immune response [9], which plays a key role in the activation of innate immunity, induces the production of interferon-γ (IFN-γ) and contributes to T helper cell 1 (Th1) differentiation to promote the killing function of cytoxic T lymphocytes and natural killer cells. Therefore, IL-12 presents a powerful potential treatment of HCC [9].

Traditional Chinese medicine (TCM) has unique advantages in the treatment of cancer, including reducing the adverse reactions of chemotherapy, improving the overall efficacy of treatment, enhancing the integrity of the body and reducing the risk of cancer. TCM plays an important role in regulating the immune cells that infiltrate tumors. The Synopsis of the Golden Chamber written by ZHANG Zhong-Jing, a physician of the Han Dynasty, described 12 components about Dahuang Zhechong pill: Rheum officinale, Scutellaria baicalensis, Glycyrrhiza uralensis, Prunus persica, Gardenia jasminoides, Paeonia lactiflora, Rehmannia glutinosa, Toxicodendron vernicifluum, Eupolyphaga sinensis, Arylotus Seu Tabanus, Prunus armeniaca, and Holotrichia diomphalia, which can invigorate blood circulation and break up stasis, clear the meridians and collaterals and eliminate symptoms. Recently, a toal of 137 chemical components in Dahuang Zhechong pill (DHZCP) prescription have been identified based on UHPLC-Q-TOF-MS/MS technology and Peakview analysis software in our team our team identified based on UHPLC-Q-TOF-MS/MS technology and Peakview analysis software in our team our team [10]. According to the Synopsis of the Golden Chamber, DHZCP has not only the effect of directly killing tumor cells, but also the effect of "Bu xu". By enhancing the body's immune function, it can limit tumors and eventually eliminate them. Recently, some studies have proposed that DHZCP reduced the levels of serum TGF-β and considerably suppressed immune cell infiltration [11]. At the same time, DHZCP markedly lowered the expression of CCL2, reduced macrophage recruitment and attenuated M2 polarization in tumor-bearing mouse livers [12]. These findings suggest that DHZCP has significant immunomodulatory effects on the immune system. However, the effects of DHZCP on Treg regulation and other helper T cell alterations in HCC have not been reported. In the current study, an orthotopic liver cancer model of mice was used to explore the mechanism by which DHZCP enhanced anti-tumor immunity in mice. Results showed that T1 cells were generated in the peripheral blood and spleen after DHZCP treatment, while more IFN-γ was secreted to activate CD8⁺ T cells and Treg cell production was inhibited, thereby suppressing the growth of HCC. Finally, we also analyzed the potential components of DHZCP from the perspective of modern targets using network pharmacology methods and experimental results.

Materials and Methods

The compound-target-disease network of DHZCP

The chemical components in DHZCP were obtained from TCMSP (http://tcmspw.com/tcmsp.php) and TCMID (http://119.3.4.228:8000/tcmid/). TCMSP is a unique systems pharmacology platform of Chinese herbal medicines that describes the relationships between drugs, targets, and diseases. Natural compounds were selected based on oral bioavailability (OB) ≥ 30%, DL ≥ 0.18. TCMID is a comprehensive database that provides information for drugs, diseases, and components. In this study, we selected natural compounds from databases and literature. Based on the structural and functional similarity of the drugs, the efficient target hits for bioactive components of DHZCP were predicted using SwissTargetPrediction (http://www.swisstargetprediction.ch/), and the targets hitting top 100 probabilities were selected for further investigation. The STRING database (https://string-db.org/) was used to analyze the intersecting protein-protein interactions (PPIs), and the compound-target-disease network was determined using Cytoscape 3.6.0.

Bioinformatics annotation of compound-target-disease network of DHZCP

Proteins with overlapping expression patterns and high-level functions were evaluated using the STRING database and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (https://www.kegg.jp/). Bubble chart was annotated using OmicShare (https://www.omicshare.com/tools/).

Experimental animals and design

Six- to eight-week-old healthy male C57BL/6N mice were purchased from Charles River (Beijing Vital River Laboratory Animal Technology). The mice were acclimated in accordance with the National Institutes of Health Guidelines for Laboratory Animals and approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine.

Establishment of a HCC orthotopic model and grouping

Orthotopic tumors derived from mouse can be introduced with Hepa1-6 cells (10⁷/mL) (Cat#: LZQ0023, RRID: CVCL_0327) by direct intrahepatic injection. Treg depletion in mice was achieved by injecting 100 μg of anti-CD25 three days before tumor injection and ten days after tumor injection. In the HCC group, conventional dose DHZCP group, high-dose group, and anti-CD25⁺ DHZCP (conventional dose) group, the mice were orally given 200 μL of DHZCP once daily for 21 d, while those in the control group were treated with the same volume of stroke-physiological saline solution daily for 21 d.

Reagents

DHZCP [2.4 g kg⁻¹ (high-dose) and 1.2 g kg⁻¹ (conventional dose)] was obtained from Beijing Tong Ren Tang (lot number: 16013144). In vivo mAb anti-mouse CD25 (IL-2Ra) (clone: PC-61.5.3, Cat#: BE0012, BioXcell, West Lebanon, USA RRID: AB_1107619), FITC anti-mouse CD3 antibody (BioLegend, San Diego, California, USA, Cat#: 100203,
Triton and ruptured cell heated for repair, fixed with 0.3% and hydrated with a gradient of ethanol. Tissue antigens were slides. The slides were dewaxed in xylene at 65 °C overnight.

Immunohistochemical staining (IHC) was performed with PE-conjugated Abs. while anti-IL-4 and anti-foxp3 cell surface molecule staining and anti-IL-17 Abs, for 30 min. For further intracellular cytokine staining, the incubated with a FITC-conjugated anti-CD4 antibody at 4 °C. CD8 cells were stained with fluorophore-conjugated antibody (Abs) or isotype control Abs at 4 °C for 30 min. The prepared CD8 cells were analyzed by flow cytometry. Finally, the percentages of helper T (Th) 1, Th17, Th2, Treg, CD4, and CD8 cells were examined.

Flow cytometry

On day 27 after injection, peripheral blood samples were collected from C57BL/6 mice, while the spleen and thymus were collected from each mouse. Lymphocytes were isolated from the mouse lymphocyte cell separation solution, centrifuged, and cultured at 37 °C in CO2 for 6 h. Subsequently, the percentages of helper T (Th) 1, Th17, Th2, Treg, CD4, and CD8 cells were analyzed by flow cytometry. Finally, the dynamic changes in Treg, Th1, Th17, and Th2 cells after treatment were examined.

For surface markers, Th1, Th17, Treg, Th2, CD4, and CD8 cells were stained with fluorophore-conjugated antibodies (Abs) or isotype control Abs at 4 °C for 30 min. The prepared Th1, Th17, Treg, and Th2 cells were transferred and incubated with a FITC-conjugated anti-CD4 antibody at 4 °C for 30 min. For further intracellular cytokine staining, the cells were incubated with anti-IFN-γ and anti-IL-17 Abs, while anti-IL-4 and anti-foxp3 cell surface molecule staining was performed with PE-conjugated Abs.

Hematoxylin and eosin staining (H&E)

Liver (tumor) tissues fixed in 10% formaldehyde were embedded in paraffin, cut into 5-μm sections, and mounted on slides. The slides were stained with H&E for histopathological examination and distinguishing tumor tissue from normal tissue.

Immunohistochemical staining (IHC)

Liver tissues fixed in 10% formaldehyde were embedded in paraffin, cut into 3-μm sections, and mounted on slides. The slides were dewaxed in xylene at 65 °C overnight and hydrated with a gradient of ethanol. Tissue antigens were heated for repair, fixed with 0.3% Triton and ruptured cell nuclear membranes, followed by incubation with primary overnight and secondary antibodies for 1 h. Finally, DAB color developer was added dropwise for target antibody labeling.

The staining intensity was determined according to the protocol in Handala et al. [13]. Given the heterogeneity of staining of the proteins, tumor specimens were scored in IHC Profiler in Image J. The percentages were grouped as follows: 0 (0%), 1 (1%–10%), 2 (11%–50%), and 3 (> 50%). The staining intensity was categorized as follows: 1, negative; 2, low positive; 3, positive; and 4, high positive. A final score was obtained for each case by multiplying the percentage and the intensity score.

Quantification of mRNA by RT-PCR

Total mRNA was extracted from sciatic nerve tissue using TRIzol, according to the manufacturer’s instructions. The purity of RNA was determined by measuring the absorbance at 260 and 280 nm (A260/A280). Reverse transcription of RNA was performed using Hiscrpt II QRT SuperMix according to the manufacturer’s instructions. RNA quantification was performed by qPCR using the ChamQ SYBR Master Mix. The program conditions were as follows: pre-incubation at 95 °C for 30 s, amplification at 95 °C for 10 s, annealing at 60 °C for 30 s for 40 cycles, separation at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. After amplification, standard curves were generated for target genes, which were normalized against the endogenous reference gene β-actin. Relative expression of target genes was determined using the 2−ΔΔCt method. Primer sequences were as follows: β-actin, forward: 5′-CAATAGTGATGACCTGGCCGT-3′; reverse: 5′-TTCTCCTACTGATCTTGTTC-3′; reverse: 5′-AGCAGCTTCTTTGTATCATCAC-3′; TGF-β, forward: 5′-CCTGGTCTGCAATGG-3′, reverse: 5′-GGATGGCGGTGAATCCAAA-3′; IFN-γ, forward: 5′-TCTGAGTGCAATGTGGAAGA-3′, reverse: 5′-GGTGGCGCTAATCGAAA-3′; IL-2, forward: 5′-GGATGGCGGTGAATCCAAA-3′; reverse: 5′-GAGGTCAATGGATCTTGAAGA-3′; IL-10, forward: 5′-GAGGTCAATGGATCTTGAAGA-3′; reverse: 5′-AACCTTGAGGGAAGTGGAGG-3′.

Statistical analysis

Results are presented as means ± SEM. To compare means among multiple groups, one-way ANOVA followed by the student’s t multiple comparison test was performed. Each experiment was repeated three times. Statistical analyses were performed using GraphPad software (GraphPad Prism, RRID: SCR_002798) to perform a two-tailed t-test, and P-values < 0.05 were considered statistically significant.

Results

Effect of DHZCP on tumors in mice with orthotopic liver cancer

The inhibitory effects of DHZCP on the growth of liver cancer was assessed by B-mode ultrasound and histopatholog-
Ultrasonography showed that an irregular mass with an uneven low echo and an unclear boundary appeared in the liver 7 days after injection of Hepal-6, indicating that the model was successfully established (Fig. 1A). Then, 28 days after injection, hard and gray-white masses were observed, and histopathologic findings showed irregular or oval cells with abnormal nuclei and a nested or glandular distribution of cancer cells invading the hepatic sinusoids in the livers of model mice. In addition, the connections between the tumor and its adjacent normal tissues were unclear in the model mice (Figs. 1B−1D). Three weeks after administration of DHZCP, echo attenuation, and lesion diameter reduction in B-mode ultrasound were detected, indicating that tumor growth was substantially suppressed compared with the model group (Fig. 1C, \( P < 0.01 \)). H&E staining showed that cancer cells were necrotic, and inflammatory cell infiltration in the DHZCP groups compared with the model group (Fig. 1D). These results indicated that DHZCP can inhibit the growth of HCC.

**Effect of DHZCP on the proportions of T-lymphocyte subgroups in HCC mice**

To investigate the effects of DHZCP on the immune system of tumor-bearing mice, we analyzed the number and proportion of T-lymphocyte subgroups in blood and spleen samples. The results showed that the number of CD4\(^+\) T (Fig. 2A, \( P < 0.01 \)) and CD8\(^+\) T cells (Fig. 2A, \( P < 0.05 \)) in peripheral blood mononuclear cells (PBMCs) was significantly reduced, and the balance of CD4/CD8 was lower in model mice (Fig. 2B). DHZCP dramatically increased the levels of CD4\(^+\) T (Fig. 2A, \( P < 0.05 \)) and CD8\(^+\) T cells (Fig. 2A, \( P < 0.05 \) for the DHZCP-L group and \( P < 0.01 \) for the DHZCP-H group). Obviously, the decreased CD4/CD8 ratio was restored (Fig. 2B). In contrast, similar results were obtained in the splenic lymphocyte-subgroup analysis, in which DHZCP increased the levels of CD4\(^+\) T and CD8\(^+\) T cells (Figs. 2C and 2E, \( P < 0.01 \)) and restored the balance of

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**Fig. 1** Effect of DHZCP on tumors in mice with orthotopic liver cancer. (A) Experimental progress diagrams. (B) B-ultrasonic picture of the liver, where yellow arrow pointed to the occupied area. (C) Statistical measurement of tumor size by B-mode ultrasound, and the top right image is representative of the measurements, where a and b are the long diameter and short diameter of the tumor, respectively. (D) Macroscopic and histological appearance of liver cancer. Data are shown as means ± SD (\( n = 3 \)). **P < 0.01 vs model**
According to the findings mentioned above, DHZCP significantly reduced the proportion of Treg cells. Thus, we used anti-CD25 antibody in vivo to deeply investigate the mechanism of DHZCP by neutralizing Treg cells (Fig. 4A). Seven days after inoculation, a significant increase in Treg cell levels was observed in the peripheral blood of the model group (Fig. 4B, \( P < 0.05 \)), but the levels of Treg cells decreased in the neutralization group (Fig. 4B). The effect of DHZCP on other subpopulations of CD4\(^+\) T cells was further analyzed. The results showed that Th1, Th2, and Th17 levels were the same in the control, model, and DHZCP groups. Compared with the DHZCP group, there was no statistically significant difference in Th1 levels in the neutralization group (Figs. 4B and 4C). Similarly, the changes in Th2 and Th17 levels were not significant. IL-12 and IL-2 are important cytokines that induce Th1 and CD4\(^+\) T cell differentiation. Thus, we measured the mRNA expression of IL-12 and IL-2 in the peripheral blood (Figs. 4D and 4E). The results showed that the levels of IL-2 and IL-12 were reduced in the neutralization group compared with the control group, while low doses of IL-12 indicated that Th1 differentiation was suppressed. However, DHZCP reversed this phenomenon, restored the levels of IL-2 to normal and increased the proportion of Th1 by elevating IL-12. This may indicate that DHZCP can activate Th1 cells and restore the body’s normal Treg cell ratio. Comparing the combination group with the DHZCP group, the levels of IL-2 and IL-12 were elevated, without significant differences in mice (\( P = 0.085 \)), which indicated that after Treg neutralization, the body produced IL-2 to promote Th1 differentiation, but DHZCP maintained the number of Treg cells at a normal level (Figs. 4D and 4E). This is reflected in the feedback activation of helper T cells by DHZCP after Treg removal. Thus, DHZCP can promote Th1 differentiation and thus CD8\(^+\) T cell activation by stimulating IL-2 to reduce Treg levels and produce IL-12.

Regulation of Treg infiltration by DHZCP in tumors

The immune status of the peripheral blood of tumor patients is different from immune cells in the tumor microenvironment, so immunohistochemistry was used to analyze the local CD4\(^+\), CD8\(^+\) and Treg cells respectively. In the model group, there were no significant changes in CD4\(^+\), but CD8\(^+\)
cells were less infiltrated and the number of Treg cells significantly was increased. On the contrary, the low- and high-dose groups of DHZCP significantly decreased the number of Treg cells and increased CD8⁺ proportion. These findings indicated that DHZCP not only improves the systemic immunity, but also remodels the immune microenvironment of liver cancer (Figs. 5A−5C).

Potential components of DHZCP to regulate Treg and Th1 from the perspective of modern targets by network pharmacology

We screened selected compounds in the TCMSP and TCMID databases based on absorption, distribution, metabolism, and extraction (ADME) (See experimental method for details). In addition, a number of animal drugs were added based on literature review and preliminary tests, obtaining 707 chemical compounds. The liver cancer-associated disease targets were obtained using GeneCards and based on relevance score > 10, yielding the first 969 gene symbols associated with liver cancer. Thus, a Venn diagram of the disease-drug was plotted, with 153 targets for drug action (Fig. 6A). STRING protein network interactions were assessed using these genes (Fig. 6B). Immune-related pathways identified by KEGG pathway analysis included Th17 cell differentiation, Th1 and Th2 cell differentiation and chemokine signalling pathways (Fig. 6C). Cytoscape was used to establish a composite target disease network of DHZCP (Fig. 6D), which also verified that DHZCP changed the CD4⁺ T subgroups in this experiment. Herein, we made a preliminary summary of the material components that exerted effects on Th1 differentiation. The components that significantly contributed to Th1 differentiation include the following (Table S1), and these components were included in our consideration. Long-chain (LCFA), medium-chain (MCFA) and short-chain fatty acids (SCFA) affecting the differentiation of helper T cells were reported by Aiden Haghikia et al. [14]. SCFA increased the proliferation and differentiation of Treg, while MCFA and LCFA enhanced the differentiation of Th1 cells [14]. We also found lauric acid and linoleic acid in DHZCP. Natural flavonoids such as wogonin, rhein, kaempferol, 5, 2′-dihydroxy-6, 7, 8-trimethoxyflavonoids and other compounds affected the activation of mast cells and T cells [15-19]. These components functioned mainly through NF-κB, MAPK and other classic pro-inflammatory related pathways. This information provides us with a basis for exploring the properties of compounds.

Discussion

Treg cells are a subset of CD4⁺ T cells that specifically
Fig. 4  Effect of DHZCP on Treg cell differentiation. (A) Experimental progress diagram. (B) Treg cell proportion in the control, model, DHZCP and neutralization groups in the peripheral blood. (C) Representative plots of stained Treg, Th1, Th2 and Th17 cells in PBMCs. (D)Th1, Th2, Th17 cell proportion were obtained by flow cytometry in the peripheral blood. (E) The mRNA expression of IL-2, and IL-12 in peripheral blood lymphocytes in the control, model, DHZCP and neutralization groups in the peripheral blood. *P < 0.05 vs control, **P < 0.01 vs model

It is known that Treg cells express the transcription factor FOXP3. Typically, the secretion of chemokines and cytokines by the tumor microenvironment (TME) to enrich peripheral Treg infiltrate the tumor locally, thereby inhibiting the killing effect of CD8+ T cells. Th1 cells, the earliest subpopulation of CD4+ T cells, can activate the cellular immune response through secretion of pro-inflammatory factors in tumors (e.g., IFN-γ) that activate effector T cell function.

The pathogenesis of liver cancer is complex, with extremely high recurrence and mortality. For a long time, renowned medical experts have considered the efficacy of DHZCP in liver fibrosis, cirrhosis and hepatocellular carcinoma. The immunomodulatory effects of DHZCP were explored in our experiments using an in situ hepatocellular carcinoma model, especially the modulation of CD4+ T subpopulation, which was tentatively interpreted and corroborated using network pharmacology. Interestingly, in this study, we observed that DHZCP stimulated the proliferation and differentiation of CD4+ T cells via IL-12 and IL-2. The group's previous theories based on the elimination of masses and blood stasis showed that DHZCP metabolized and inhibited HCC growth through SMMC-7721. According to network pharmacology and current studies, we speculate that DHZCP acts in two ways, one as a direct intervention of the drug in the differentiation of Naïve CD4+ T and/or Treg cells, and the other as an immunosuppressive intervention of the drug after killing tumor cells to relieve the immunosuppression of tumor cells.

Therefore, in subsequent studies, we need to consider not only the direct immunomodulation of drugs, but also the indirect effects of tumors. First, for the direct effects of drugs, we found from online pharmacology that triterpenoid saponins as well as fatty acids, and flavonoids have a good regulatory effect on CD4+ T differentiation. Second, HCC has a unique microenvironment that affects the metabolism of immune cells and remodels these infiltrating immune cells due to low oxygen, low PH and metabolic waste. Previous studies showed that DHZCP inhibited HCC growth by influencing metabolic levels. Whether DHZCP indirectly altered immune cells in TME by influencing tumor cells, among other factors, is unclear. Because of the differences in immunity between the peripheral blood and the tumor, the mechanism of action of DHZCP needs to be further explored. Finally, we have not yet performed pharmacokinetic studies to assess plasma and anti-tumor drug levels. We intend to develop a subcutaneous tumor model in future studies to compare the possible role of network pharmacology with the components contained in tissues to identify specific possible compounds that regulate Treg and Th1 differentiation. In in vitro studies, we should also extensively review and consider the bioavailability of each component, serum levels [including peak plasma levels (cmax)], maximum tolerance to DHZCP doses, and especially liver metabolism.

**Conclusion**

In general, DHZCP promotes Th1 differentiation and IFN-γ secretion by stimulating IL-2 and IL-12 levels, while
reducing local Treg infiltration, thereby activating CD8^+ T and relieving tumor immune escape. We plan to explore localized/targeted drug concentrations, which directly influence tumor-infiltrating immune cells.
Supplementary Material

Supplementary information can be acquired by e-mail to corresponding author.

References


