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Basic Study

Calculus bovis inhibits M2 tumor-associated macrophage polarization *via* Wnt/β-catenin pathway modulation to suppress liver cancer

Zhen Huang *et al. Anti-Liver cancer Mechanism of Calculus bovis*

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Abstract

BACKGROUND

Calculus bovis (CB), utilized in Traditional Chinese medicine (TCM), has exhibited anti-tumor effects in various cancer models. It also constitutes an integral component in the compound formulations known as Pien Tze Huang, which is indicated for the treatment of liver cancer. However, its impact on the liver cancer tumor microenvironment (TME), particularly concerning tumor-associated macrophages (TAMs), is not well understood.

AIM

This study aims to elucidate the anti-liver cancer effect of CB by inhibiting M2-TAM polarization *via* Wnt/β-catenin pathway modulation.

METHODS

This study identifies active components in CB using ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS),

evaluates its anti-neoplastic effects in a nude mouse model, and elucidates mechanisms *via* network pharmacology, transcriptomics, and molecular docking. *In vitro* assays investigate CBS effects on HepG2 cells and M2-TAMs, with validation of Wnt pathway modulation by RT-qPCR and Western blot.

RESULTS

The study identified a total of 22 active components in CB, with 11 of them being detected in the bloodstream. Preclinical investigations demonstrated CB's ability to effectively inhibit liver tumor growth. An integrated approach employing network pharmacology, transcriptomics, and molecular docking implicated the Wnt signaling pathway as a target of CB's antineoplastic activity by suppressing M2-TAM polarization. *In vitro* and *in vivo* experiments further confirmed that CB significantly hinder M2-TAM polarization and suppress Wnt/β-catenin pathway activation. The inhibitory effect of CB on M2-TAM was reversed when treated with the Wnt agonist SKL2001, thus confirming the pathway specificity.

CONCLUSION

The research demonstrated that CB-mediated inhibition of M2-TAM polarization through Wnt/β-catenin pathway, contributing to the suppression of liver cancer growth.

Key Words: *Calculus bovis*; M2 tumor-associated macrophage polarization; Liver cancer; Wnt/β-catenin pathway;The tumor microenvironment

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Core Tip: CB, a valued herb in traditional Chinese medicine (TCM), has shown definite anti-liver cancer effects *in vivo* experiments. Analyzing the composition of CB and using network pharmacology for target prediction, CB exhibits anti-liver cancer effects by affecting immune related pathways in the tumor microenvironment. Through transcriptome sequencing, we further showed that regulating M2-type polarization of TAMs is a more specific reason for the effect of CB. *In vitro* studies, the Wnt/β-catenin pathway is a crucial mechanism by which CB regulates M2-type polarization of TAMs. The entire study provides evidence to the research of anti-liver cancer drugs.

INTRODUCTION

Liver cancer, a global health concern, ranks the sixth in prevalence and the fourth in cancer mortality worldwide[1]. Liver cancer is highly malignant, progresses rapidly, has a poor prognosis. The 5-year survival rate for liver cancer patients is only 3%, severely impacting their quality of life[2]. The primary treatment options for liver cancer include surgical interventions such as liver resection or transplantation, interventional therapies, local ablation treatments, and targeted and immunotherapies[3-6]. However, the application of these treatments has limitations, and the survival rate for some patients remains low even after treatment[7,8]. Therefore, there is an urgent need for novel and more effective therapeutic strategies for the treatment of liver cancer. Traditional Chinese medicine (TCM) is characterized by its multi-component and multi-target approach[9,10]. Studies have shown that TCM can inhibit liver cancer by regulating the tumor microenvironment (TME)[11], offering a new perspective for liver cancer treatment. Within the multifaceted TME, macrophage exhibits a crucial role in liver cancer progression[12]. Tumor-associated macrophages (TAMs), derived from circulating monocytes, can adopt M1 or M2 phenotypes in response to various TME cytokines and growth factors[13]. While M1-type TAMs exhibit anti-tumor properties driven by cytokines such as IFN-γ and TNF-α[14], M2-TAMs support tumor growth and metastasis through pathways like NF-κB, IL-6/STAT3, and Wnt/β-catenin[15-17]. These pathways also facilitate TAM-mediated

tumor proliferation, invasion, and angiogenesis[18,19]. Therefore, reversing TAMs polarization and targeting these pathways present promising therapeutic approaches for liver cancer.

Traditional Chinese medicine values *Calculus bovis* (CB) for its anti-tumor potential across various models[20]. Combinations of CB with Moschus have been shown to induce liver cancer cells apoptosis, like SMMC-7721 and HepG2[21,22]. Prior research has documented CB's mechanisms against liver cancer[23] and its ability to enhance macrophage phagocytosis while suppressing pro-inflammatory cytokine secretion, alleviating liver inflammation and injury[24]. However, the full extent of CB's influence on the liver cancer TME, particularly on TAMs, remains elusive.

This investigation revealed that CB's antineoplastic effects may involve inhibiting M2-polarized TAMs differentiation through modulation of key molecular pathways. Our comprehensive *in vitro* and *in vivo* analyses aimed to elucidate mechanisms clarifying CB's anti-liver cancer activity, including its active constituents, cellular targets, and the signaling pathways they affect. The findings demonstrated CB's regulatory role in macrophage phenotypic plasticity and interaction with Wnt/β-catenin pathway within TME. This study lays a foundation for developing CB-derived antineoplastic therapeutic strategies, inhibiting M2-TAM polarization by targeting Wnt/β-catenin pathway to suppress liver cancer.

MATERIALS AND METHODS

Chemicals and reagents

CB (20160526-2) was sourced from Hunan Sanxiang Herbal Pieces Co., LTD. (Hunan, China). Sorafenib was obtained from GLPbio, Inc. (Montclair, USA). HepG2 cells, FBS and DMEM were acquired from Meisen Cell Technology Co., Ltd. (Zhejiang, China). HepG2 cells authenticated using short tandem repeats (STR). The THP-1 cells were provided by the State Key Laboratory of Macau University. *Phorbol 12-myristate 13-acetate* (PMA), TRIzol Kit (15596026, Thermo Fisher Scientific, Massachusetts, USA) were procured from Sigma (Darmstadt, USA), and the cytokines for cell induction,

including IL-4 and IL-13 were also obtained from Sigma. Cell CCK-8 Kit was procured from Biosharp (Anhui, China). Annexin V-FITC/PI Apoptosis Kit was purchased from APExBIO (Houston, USA). Type I collagenase was procured from Biofroxx (Santiago, Germany). Protein Assay Kit was purchased from CWBIO (Jiangsu, China). NovoStart®SYBR qPCR SuperMix Plus and NovoScript®Plus All-in-one 1st Strand cDNA Synthesis SuperMix were procured from Novoprotein Inc. (Jiangsu, China). The primary antibodies against Wnt5B (#55184-1-AP), Beta-catenin (#66379-1-Ig), Axin2 (#20540-1-AP), and GADPH (#66009-1-Ig), as well as HRP-conjugated affinipure goat anti-rabbit IgG (#SA00001-2), and recombinant secondary antibody multi-rab HRP-goat anti-mouse (#RGAMΦ01), were acquired from Proteintech (Wuhan, China).

In vivo and in vitro analysis of CB components

Sample preparation: For the preparation of the CB extract, 2.0 g of CB was mixed with 30 mL of 100% methanol, followed by ultrasonication for 30 min at 250 W and 40 kHz. Following ultrasonication, the mixture was allowed to settle. Then, 2 mL of the mixture was drawn and centrifuged at 8,000 *g* at 25°C for 5 min. The supernatant was then filtered *via* 0.22 μm microporous membrane, and 1 milliliter of this filtrate was reserved in an injection vial for subsequent analysis.

For the serum sample preparation, 2 mL of CB-enriched serum and 2 mL of blank serum were taken separately and each mixed with 6 mL of 100% methanol. The mixtures were chilled in ice bath, then centrifuged at 12,000 g at 25℃for 5 min. Supernatants were dried utilizing a centrifugal evaporator. Then dried samples were re-dissolved within 100 μL methanol and again centrifuged under the same conditions to ensure clarity. Finally, the purified supernatants were filtered *via* a 0.22 μm microporous membrane for further examination.

Chromatography and mass spectrometry conditions: The chromatographic analysis was conducted using an Agilent ZORBAX Eclipse Plus C18 column, applying a gradient elution method with mobile phases of acetonitrile and either 1 mL/L formic acid in water for positive ion mode or 5 mmol/L ammonium acetate in water for

negative ion mode. The sample injection volume was 2 μL. Subsequently, mass spectrometry was performed with both positive and negative ESI settings, utilizing multi-reaction monitoring scanning mode, and calibrated with ESI-L Low Concentration Tuning Mix. The MS spanned m/z 100 to 1500 with a resolution of 30,000, employing nitrogen as the nebulizing gas, and maintained a drying temperature of 325°C, capillary voltage of 4.0 kV, fragmentation voltage of 110 V, and sheath gas temperature of 350°C.

Animal experiments

Subcutaneous transplantation tumor experiment: The male 6-wk-old BALB/c nude mice and SD rats, supplied by Hunan Slake Jinda Laboratory Animal Company Limited (Changsha, China), were fed in specific pathogen-free conditions. All animals were maintained in an environment with a controlled 12-hour dark/Light cycle, at 21 ± 2°C temperature, and 50 ± 10% relative humidity.

The nude mice xenograft tumors were induced *via* subcutaneous injection of 5 × 10^6 HepG2 cells into the posterior side of the right forelimb. Upon reaching a tumor volume of 100 mm^3, the mice were allocated into: A control group receiving 9 g/L saline (0.2 mL/d orally), low-dose (L-CB), medium-dose (M-CB), and high-dose (H-CB) CB groups receiving 45.5 mg/(kg•d), 113.75 mg/(kg•d), 227.5 mg/(kg•d) of CB, and a Sorafenib group administered 20 mg/(kg•d) of Sorafenib. Body weight and tumor volume were monitored at 3-d intervals. This study complied with the Animals (Scientific Procedures) Act in United Kingdom, 1986, ensuring the ethical treatment of laboratory animals. Approval for the study was granted by the Ethical Review Committee of Experimental Animal Welfare of Slacker Jingda Laboratory, Changsha, Hunan, China (Approval No. IACUC-SJA2022105).

Blank and drug-enriched serum preparation: In this research, the rats were allocated into: A control group receiving 5 g/L CMC-Na and a CB-treated group administered a 157.5 mg/kg CB suspension. Both regimens were administered bi-daily for one wk. After a fasting period of 12 h, the rats underwent anesthesia utilizing a 30 g/L solution

of pentobarbital sodium at a 30 mg/kg dosage, facilitating the subsequent collection of serum.

Network pharmacological analysis

Collection of potential targets and construction of component-target-disease networks: In this study, we identified 22 unique chemicals in CB through UPLC-EIS-Q-TOF-MS analysis. Subsequently, the TCMSP and SwissTargetPrediction tools were employed to predict their biological targets, which were further refined using Uniprot database[25]. In order to identify liver cancer-related targets, a comprehensive search was conducted across multiple databases including DrugBank, OMIM, PharmGKB, and TTD. The obtained data underwent refinement to eliminate redundancies and false positives. Utilizing R software, an intersection analysis was performed between the predicted targets of CB's bioactive compounds and those present in the liver cancer target database. This enabled us to identify potential therapeutic targets for CB against liver cancer. Finally, a "component-target-disease" interaction network was constructed using Cytoscape.

Protein interaction network construction: The PPI network for the identified anti-liver cancer targets of CB was established *via* the STRING database. Following this, R language software was employed to quantify the frequency of these target interactions, with the results presented in a histogram format for clearer analysis and interpretation.

Analysis of GO function and KEGG pathway enrichment: The anti-liver cancer targets obtained from screening of CB were imported into Metascape database for GO function analysis. Using R language as a platform, KEGG enrichment analysis was carried out by inputting the corresponding script commands to clarify the pathways involved in the genes.

Molecular docking: To investigate the interaction between liver cancer targets and active components of CB, we employed bioinformatics tools and databases. The structures of target proteins Wnt5B, β-catenin, and Axin2 were obtained from UniProt. These protein frameworks were prepared for molecular modeling using PyMOL

software by removing water molecules and small ligands, with the refined structures saved in PDB format. The 'Component-Target-Disease' network focused on 17 primary active components of CB against liver cancer was explored using PubChem to retrieve their 2D structures. Chemical structures were further refined and optimized with Chem3D software to ensure accuracy in subsequent studies. Molecular docking simulations were applied with AutoDock Vina software to assess binding affinity between CB components and target proteins based on calculated binding energies. Finally, results from molecular docking were comprehensively visualized and analyzed using PyMOL software to understand the interactions between active compounds of CB and respective liver cancer protein targets.

RNA extraction, RNA sequencing and bioinformatics analysis

TRIzol method was utilized to extract total RNA and quality assessment *via* electrophoresis and bioanalyzer. Following RNA extration, the sequencing of cDNA libraries was conducted on the Illumina NovaSeq 6000 platform, with reproducibility evaluated by Pearson's correlation coefficient. RNA expression levels were quantified utilizing Subread software and normalized for gene length and sequencing depth. Differential gene expression analysis was conducted through DESeq2, followed by GO and KEGG pathway enrichment analyses facilitated by ClusterProfiler.

Cell culture

HepG2 cells were cultured in DMEM medium enriched with 100 mL/L FBS and antibiotic at 37°C, 50 mL/L CO_2, while THP-1 monocytes were similarly cultivated in RPMI-1640. For experimental purposes, PMA at a concentration of 100 ng/mL was applied for 48 h to induce THP-1 monocytes to differentiate into Mφ macrophages. To model the tumor-promoting M2 macrophages, these Mφ macrophages were further incubated with IL-4 and IL-13, each at a concentration of 20 ng/mL, for an additonal 48 h.

Preparation of Conditioned medium

Mφ and M2 + 10%(100 mL/L) BS conditioned medium: Differentiated Mφ macrophages and M2 macrophages were cultured in DMEM medium enriched containing 10%(100 mL/L) BS and 1%(10 mL/L) penicillin-streptomycin; M2 + (0%, 5%, 10%, 20% respectively equal to (0 mL/L, 50 mL/L, 100 mL/L, 200 mL/L) CBS conditioned medium: Differentiated M2 macrophages cultured in DMEM medium enriched with [0%(0 mL/L), 5%(50 mL/L), 10%(100 mL/L), 20%(200 mL/L)] CBS and 1%(10 mL/L) penicillin-streptomycin. All of the above conditional medium were supernatant collected after culture at 37°Cin 50 mL/L CO_2 atmosphere for 24 h.

Cytotoxicity was determined by CCK-8 assay

HepG2 cells (5×10^5 cells/mL) and (2.5×10^4 cells/mL) were plated in 96-well plates. After a 24-hour cultivation period, media containing various concentrations of CBS (0%, 5%, 10%, and 20%, respectively equal to 0 mL/L, 50 mL/L, 100 mL/L, 200 mL/L) was administered to the wells for THP-1 cells treatment. HepG2 cells are maintained in different Conditioned medium. The cultures were incubated for an additional 24 h to assess the treatment impact. Then 10 µL CCK-8 solution was added to each well. To facilitate color development, the plates underwent a further two-hour incubation. Optical density was subsequently tested at 450 nm utilizing Spark multimode microplate reader (TECAN, Switzerland).

Flow cytometry

Cell apoptosis detection: HepG2 cells (5×10^5 cells/mL) were allocated into 96-well plates and incubated for 24 h. Concurrently, THP-1 monocytes, transitioned to Mφ macrophages or subsequently to M2 macrophages, were incubated in either 10% (100 mL/L) control serum or 10% (100 mL/L) CB-enriched serum. Thereafter, the HepG2 cells received treatments with media conditioned by these macrophages for an additional day. Following this period, cells were harvested utilizing 800 µL of non-EDTA trypsin for collection. The analysis of cell apoptosis and viability involved

staining with Propidium Iodide (5 µL)and Annexin V (5 µL). Stained cells were then examined *via* a CytoFLEX flow cytometer (Beckman, USA). **Macrophage polarization detection:** Tumor tissues were minced into small pieces and treated with type I collagenase, then incubated for 1.5 h at 37°C to facilitate digestion. Cell suspension was layered gently over a density gradient consisting of 300 mL/L and 700 mL/L Percoll solutions and centrifuged to separate lymphocytes, which were harvested from the interface between these two layers. For the generation of M2-TAM *in vitro*, cells were dissociated using trypsin without EDTA and collected. Before antibody staining, cells were blocked with anti-mouse CD16/32 antibody (TruStain FcX™) to inhibit non-specific binding, followed by staining with primary antibodies against CD45, CD11b, and F4/80 [all diluted 1: 100 in 1% BSA (10 g/L BSA in PBS)] for 30 min at 4°C. the cells were fixed and permeabilized according to the BD Cytofix/Cytoperm kit protocol, followed by staining with an anti-CD206 antibody for intracellular marker detection. The stained samples were then examined on a CytoFLEX (Beckman, USA). Data were further evaluated and visualized using FlowJo software (version 10).

Cell scratch assay

HepG2 cells were allocated into six-well plates, underwent transfection, then received various treatments. After 24 h, a pipette tip was utilizing to draw a straight line across the cell layer to create a scratch, simulating a wound. The wells were then exposed to media supplemented with different levels [0%(0 mL/L), 5%(50 mL/L), 10%(100 mL/L), 20%(200 mL/L)] of CB-enriched serum. Cell migration was monitored and recorded under an inverted microscope at the start (0 h) and after 24 h. Scratch area was quantified by Image J software.

Transwell invasion

Invasion and migration assays were conducted by 24 well Transwell chambers with 8 µm pores. A mixture of Matrigel and DMEM was allocated into upper chamber

containing HepG2 cells, while lower chamber contained DMEM with 100 mL/L FBS as an attractant. After 24 h, non-migrated cells were expunged, whereas the migratory cells on the lower surface underwent fixation, staining, and imaging with Image J software for quantifying migration and invasion rates.

RT-qPCR assay

Total RNA isolation was completed utilizing TRIzol reagent (Vazyme, China). The cDNA was then reverse-transcribed employing the All-in-one 1st Strand cDNA Synthesis SuperMix (gDNA Purge). RT-qPCR experiments were conducted with the help of NovoStart® SYBR qPCR SuperMix Plus in conjunction with the LightCycler® 96™ Real-Time PCR System (Roche Inc., Switzerland). Primers specifically designed for the gene sequences of interest were synthesized based on sequences available from GenBank (Table 1). The $2^{-\Delta\Delta Ct}$ method was utilized to test relative expression of each gene.

Western blot analysis

THP-1 monocytes were incubated with 10%(100 mL/L) CB-enriched serum for 24 h and then processed for protein isolation using RIPA buffer (Applygen, China). Protein levels were assessed by the BCA assay, then 50 μg sample protein was used to Western blot analysis on a 100 mL/L SDS-PAGE gel. Membranes underwent an overnight incubation at 4°C with primary antibodies targeting Wnt5B, Axin2, Beta-catenin, and GAPDH, followed by HRP-tagged secondary antibodies. Signals were detected by an ECL kit, purchased from Biosharp Inc., Anhui, China, followed by imaging on an Amersham Imager 600. Image Pro Plus software was utilized to conduct band intensities analysis.

Statistical analysis

SPSS version 26.0 was utilized to execute Statistical analysis. Data normality was verified through the Shapiro-Wilk test. Representation of normally-distributed datasets was achieved by the mean ± SD (mean ± SD). Analysis of Variance (ANOVA) were

employed to evaluated Group differences, followed by either SNK post-hoc or Tukey's tests depending on suitability. For two-group comparisons, the independent samples *t* test was applied. The threshold for statistical significance was designated as a *p* value below 0.05.

RESULTS

Analysis of pharmacodynamic material basis of CB

To examine the pharmacological components of CB, we utilized UPLC-Q-TOF MS for an extensive analysis. This led to the identification of 22 chemical constituents, such as *bilirubin*, *bile acids*, *cholesterols*, and *acid esters*, detailed in Table 2 and illustrated in Figure 1. *In vivo* studies further revealed 11 components and their metabolites present in the blood of rats treated with CB, as shown in Figure 1-1D and Table 2. Standardized comparisons and quantitative assessments were conducted for select components (Figure 1C and Table 3 and 4). Notably, *lithocholic acid* was found to be the most abundant component in the CB extract, reaching a concentration of 0.7066 mmol/L, whereas *Glycohyodeoxycholic Acid* was most prevalent in the CB-enriched serum, with a concentration of 0.2759 mmol/L.

CB suppresses liver cancer growth in vivo

Then, the anti-tumor effects of CB were evaluated in animal models. Treatments with low, medium, and high doses of CB (L-CB, M-CB, H-CB) and Sorafenib significantly reduced both the size and weight of tumors relative to the controls (Figure 2A-C). Interestingly, there was no notable impact on the body weight of mice across all experimental groups. (Figure 2D)

The anti-cancer efficacy of CB was further validated by histological analysis. As illustrated in Figure 2E, hematoxylin and eosin (H&E) staining of tumor pathology indicated distinct morphological indications of tumor regression following treatment with medium and high doses of CB, as well as with Sorafenib. Observed signs of regression included degenerative changes in tumor cells, reduced nucleolar staining

intensity, and the presence of nuclear fragments, which are indicative of a diminished malignancy post-treatment. These findings suggest that CB, particularly at medium and high dosages, significantly impedes liver cancer growth.

Cyberpharmacology analysis of CB components for anti-liver cancer study utilizing UPLC-Q-TOF-MS

Construction of a "component-target-disease" network: Following database searching, 392 possible chemical targets associated with CB's components were discovered. Concurrently, liver cancer-related targets were compiled from comprehensive databases, including DrugBank, OMIM, PharmGKB, and TTD, resulting in 6,307 targets associated with liver cancer. By utilizing R language software for data analysis, we matched the targets related to CB's active components against those associated with liver cancer. This comparative approach led to the identification of 168 potential anti-liver cancer targets through intersection analysis (Figure 3A). The constructed "component-target-disease" interaction network (Figure 3B) visualizes the potential efficacy of CB against liver cancer, encompassing 463 edges and 184 nodes with an average degree value of 5.04. Notably, compounds such as *Gelsevirine*, *Nisinic Acid*, *Methyl cholate*, *7-Ketolithocholic acid*, *Glycocholic Acid*, *Deoxyloganin*, *Bilirubin*, *Hyodeoxycholic acid*, *Campechic acid A*, *Glycohyodeoxycholic Acid*, and *Phorbol 12,13-Dimyristate* were identified with degree values equal to or greater than 10, highlighting their significant roles in CB's anti-liver cancer activity[26].

GO analysis: In our study, we utilized PPI network analysis to identify primary targets of CB against liver cancer. This analysis revealed TNF and IL-6 as central targets (Figure 3C), highlighting their significant roles in the immune response associated with liver cancer. Further, GO functional analysis was conducted on these key targets to categorize their roles in biological processes, molecular functions, and cellular components. The visualization of the top 20 items was conducted with statistical significance at $P < 0.01$ (Figure 3D). Our findings demonstrated that biological processes engaged in liver cancer pathogenesis mainly included regulation of MAPK cascade

signaling pathway, hormonal responses, positive regulation of cell migration and death as well as secretory regulation. Cellular components associated with liver cancer pathogenesis predominantly comprised membrane rafts, receptor complexes, and postsynaptic membranes, along with cellular projection membranes.

KEGG analysis: Moreover, the core targets were analyzed, and the signaling pathways with a $P < 0.01$ were chosen for visualization (Figure 3E). KEGG analysis revealed significant pathways, including PI3K-Akt, Wnt pathway, Rap1 pathway, and Ras, among others, as depicted in Figure 3E. The Wnt pathway, known for its involvement in cell proliferation, differentiation, and apoptosis, is notably linked to cancer progression and immune system modulation. Based on these insights, further detailed investigation into the Wnt pathway was prioritized to better understand CB's mechanism against liver cancer, supported by findings from Pai *et al*[27].

Molecular docking results: The study excluded *isolinolic acid* from further analysis due to its absence of a two-dimensional (2D) structure in the database. In order to investigate the interactions between the target proteins associated with the Wnt signaling pathway and active components of CB, molecular docking was conducted on the remaining 16 active components, including Wnt5B, β-catenin, and Axin2. A binding energy threshold of -5.0 kcal/mol was set as an indicator of strong affinity between the active ingredients and their target proteins. Our findings revealed that *Bilirubin* and bile acid-like compounds such as *Glycocholic Acid, Taurodeoxycholic Acid, Glycohyodeoxycholic Acid, Hyodeoxycholic Acid, and 7-Ketolithocholic Acid* exhibited binding energies equal to or less than -6.5 kcal/mol with Wnt5B protein, β-catenin protein, and Axin2 protein respectively. This is depicted in Figures 3F and 3G which demonstrate a stable binding conformation. These results suggest that CB has potential for inhibiting liver cancer progression by modulating the Wnt pathway.

Wnt pathway and its target molecules engaged in CB's effects on M2-TAM revealed by transcriptome sequencing analysis

Transcriptome analysis was utilized to investigate CB's impact on gene functionality during cell migration more closely. This analysis identified significant changes in 820 genes, comprising 359 genes with increased expression and 461 with decreased expression (Figure 4A). To deepen biological insights, GO and KEGG enrichment were performed on differentially expressed genes, indicating that the Wnt pathway is a key target of CB-mediated inhibition in liver cancer. Furthermore, CB appeared to affect mRNA differential expression in mouse tumor tissues by targeting molecular functions and biological processes related to the Wnt pathway (Figure 4 B-D). Notably, among the genes in this pathway, CCL22 and TGF-β, both known for their association with M2-TAM polarization, were significantly enriched. Therefore, our results support the hypothesis that CB may exert its anti-liver cancer effects through modulation of the Wnt pathway and subsequent alteration of M2-TAM polarization.

CBS inhibits M2 macrophage polarization in vitro

To assess the cytotoxic effects of CBS on THP-1 cells, we initially performed a CCK8 assay using varying concentrations [5%(50 mL/L), 10%(100 mL/L), and 20%(200 mL/L)]. The results in Figure 5A showed no significant impact on cell viability after exposure to CBS. Subsequently, we investigated the potential of CBS to inhibit M2 macrophage polarization stimulated by IL-13 and IL-4. Flow cytometry measured CD86 and CD206 surface markers on THP-1 cells. As shown in Figure 5B, stimulation with IL-13 and IL-4 resulted in a substantial upregulation in CD206 expression, confirming successful M2-TAM polarization. Furthermore, we evaluated the influence of different concentrations of blank serum and CBS on mRNA expression levels of CD206 in M2-TAM using RT-qPCR analysis. Across blank serum concentration, CD206 expression remained stable with no significant alterations. However, notable differences in CD206 mRNA expression were detected when comparing groups treated with 10% (100 mL/L) CBS to those receiving 10% (100 mL/L) blank serum, as well as between the 20% (200 mL/L) CBS group and the corresponding blank serum group ($P < 0.01$). Treatment with both 10% and 20% (100 and 200 mL/L) CBS led to a statistically downregulation of

CD206 mRNA expression contrasted with concentration of 5% (50 mL/L) CBS; however, there was no remarkable difference found between these two higher concentrations (Figure 5C). Consequently, a concentration of 10% (100 mL/L) CBS was selected for subsequent experiments. Flow cytometry analysis demonstrated that treatment with this chosen concentration effectively reduced CD206 expression in M2-TAM (Figure 5D). Additionally, RT-qPCR analysis revealed significant reductions in mRNA levels of CCL22, Arg-1, TGF-β2 and IL-10 (Figure 5E). The findings collectively indicated that CBS effectively inhibits M2-TAM polarization.

CBS mitigates the proliferative and migrative properties of liver cancer cells by suppressing TAMS polarization to M2

We utilized the CCK-8 assay to evaluate the effects of CBS at various concentrations on HepG2 cell proliferation within a medium conditioned by M2 macrophages, revealing notable inhibitory effects (Figure 6A). Flow cytometry analysis indicated an enhancement in the proliferation of liver cancer cells comparing to Mφ macrophages. Notably, enhanced apoptosis was observed in the group treated with M2 + 10%(100 mL/L) CBS conditioned medium, in stark contrast to the Mφ + 10%(100 mL/L) BS and M2 + 10%(100 mL/L) BS conditioned medium groups (Figure 6B). These results confirm that CBS not only impedes the M2-TAM-induced proliferation of liver cancer cells but also significantly promotes their apoptosis. In the 24-hour wound healing assay, the migratory capacity of HepG2 cells was increased when cultured with medium from the M2 + 10%(100 mL/L) BS group, compared to the Mφ + 10%(100 mL/L) BS group. However, this enhanced migratory behavior was significantly reduced following treatment with an M2 + 10%(100 mL/L) CBS-conditioned medium (Figure 6C). Similarly, the transwell invasion assay revealed that the M2 + 10%(100 mL/L) CBS-conditioned medium effectively decreased the migration rate of HepG2 cells (Figure 6D). Collectively, these findings indicate that CBS-treated M2-TAM medium considerably diminishes the cells invasion and migration capabilities in liver cancer, underscoring its potential therapeutic value in halting the progression of liver cancer.

CBS inhibits TAMS to M2 polarization through the Wnt pathway against liver cancer

RT-qPCR and Western blot assays showed an upregulated expression of Wnt5B and β-catenin, along with a reduction in Axin2 Levels, confirming the activation of the Wnt pathway (Figures 7A, B). Significantly, the inclusion of 10% (100 mL/L) CBS markedly mitigated the IL-13/IL-4 induced alterations, suggesting Wnt pathway modulation in the CBS-mediated inhibition of macrophage M2 polarization. To further elucidate CBS's impact on the Wnt signaling within macrophages, we utilized SKL2001, a recognized activator of this pathway, in our experimental framework. As depicted in Figure 7C, THP-1 cells co-treated with 10% (100 mL/L) CBS and SKL2001 manifested a significant elevation in β-catenin levels compared to cells treated with 10% (100 mL/L) CBS alone. Additionally, flow cytometry analysis demonstrated that 10% (100 mL/L) CBS treatment resulted in a reduction of CD206 expression to around 40%. Notably, the simultaneous administration of SKL2001 with CBS abrogated the CBS-induced suppression of CD206 expression (Figure 7D). These findings highlight that CBS's inhibitory effect on M2-TAM polarization predominantly operates through the downregulation of the Wnt pathway.

CB regulates M2 polarization via the Wnt pathway in vivo to combat liver cancer

In subsequent *in vivo* experiments, we aimed to confirm on the effect of Wnt pathway. Consistent with our earlier findings, CB treatment resulted in a significant downregulation of Wnt5B and β-catenin, alongside an increased expression of Axin2 (Figure 8A, B). These findings suggested that CB suppresses the Wnt pathway activity. Moreover, flow cytometry analysis indicated that CB effectively reduced the expression of the M2 macrophage marker CD206, with the most significant decrease observed in the high-dose group (Figure 8C).

DISCUSSION

Despite recent advances in treatment methods that have reduced liver cancer mortality, prognosis remains poor due to high rates of metastasis and chemotherapy resistance. TCM can inhibit liver cancer by regulating TME, providing a new perspective for liver cancer treatment. CB, a classic herb in TCM with ancient medical texts and thousands of years of use, is a foundational substance in compound formulations like Xihuang Pills and Pien Tze Huang for treating tumors, and its immunomodulatory and anti-inflammatory effects are well-documented, highlighting its medicinal significance in traditional Chinese therapeutic practices[28-30].We hypothesized that CB inhibits tumor progression by modulating macrophages within the tumor microenvironment, and subsequently investigated this effect.

Antitumor experiments conducted *in vivo* and *in vitro* have demonstrated that CB significantly inhibits the progression of liver cancer. Network pharmacology and transcriptome sequencing analysis indicated enrichment of the Wnt/β-catenin signaling pathway, suggesting that CB's mechanism of action may involve this pathway. Previous studies have established that the Wnt signaling pathway is crucial in the pathogenesis of liver cancer[31-33], regulating the proliferation of liver cancer stem cells and promoting tumor growth. Our research illustrated the mechanism through which CB impedes progression of liver cancer by alerting M2-TAM polarization *via* Wnt/β-catenin pathway modulation. Detailed serum analysis revealed 11 bioactive compounds, including *bilirubin*, which exhibits antioxidant, anticancer, and anti-inflammatory effects as documented by Yu *et al*[22]. Bile acid-like components and acid esters, recognized for their roles in enterohepatic circulation and antitumor properties[34], also contribute to CB's hepatoprotective profile. These findings verified the multiple therapeutic effects of CB in targeting liver cancer through an interaction of its active constituents.

In the further analysis to the Wnt pathway, a significant upregulation of CCL22 and TGF-β2 was observed among downstream genes. The stimulation of TGF-β and Wnt/β-catenin pathways is known to facilitate M2 macrophage polarization, which has a significant impact on connecting inflammatory and oncogenic processes. This

polarization further enhances the invasion and migration in liver cancer[35]. It has been demonstrated that CCL22 exacerbates autoimmune diseases by recruiting macrophages and enhancing their effector functions. Neutralizing CCL22 Leads to an altered cytokine profile within macrophages, characterized by reduced TNF-α levels and increased IL-10 Levels, aligning with the characteristics of M2-TAM[13, 36]. CB may impede liver cancer progression by obstructing M2-TAM polarization *via* the Wnt/β-catenin pathway. *In vitro*, CB-supplemented serum reduced M2-TAM markers and cytokines in THP-1 cells activated with IL-4/IL-13 for 24 h. Identification of M2-TAM relies on their responsiveness to IL-4, IL-10, IL-13 and TGF-β resulting in elevated levels of CD206 and Arg-1 expression[37]. Moreover, TAMs tend to secrete lower amounts TNF-α、IL-1β while exhibiting higher concentrations of TGF-β and IL-10[37-39]. These observations support the findings obtained from this study.

Research has shown that the Wnt pathway is essential to drive liver cancer progression, particularly through upregulating CCL22 and TGF-β2, which are associated with M2 macrophage polarization[40-42]. It has been demonstrated that CB-enriched serum can inhibit M2-TAM polarization stimulated by IL-13/IL-4, reversing switch to an M2 phenotype and reducing the invasive and migratory capabilities in liver cancer both *in vitro* and *in vivo*. Meanwhile, the presence of SKL2001 indicates that CB's inhibitory effects on M2-TAM activation can be modulated by the Wnt/β-catenin pathway. The weak direct killing effect on tumor cells exhibited by CB *in vitro* experiments could not perfectly explain its anti-tumor effects *in vivo*. Therefore, we speculate that immunomodulation may be an important pathway for anti-tumor activity of CB. Our study firstly demonstrate that CB inhibits liver cancer development by regulating TAM polarization, enhancing our understanding of CB's pharmacological role and offering a promising and effective treatment option for liver cancer. On the other hand, T-cell infiltration, NK cell activation and PD-1/PD-L1 expression are the factors affecting the TME, and it is not clear whether CB has the regulatory effect on them, which needs to be paid attention to in future studies.

We found that there are as many as 22 constituents of CB. However, it is not clear which compound plays a role in regulating macrophage polarization, which needs to be clarified in future studies. Further, the synergistic effect of these active components with clinical antitumor drugs should be focused. In addition, new drug development strategies, such as structural modification and targeting system construction, should be utilized to enhance the antitumor effects of the active ingredients of CB and to improve their targeting properties, making them potential antitumor drugs.

CONCLUSION

CB has long been valued in Traditional Chinese herbal medicine, utilizing its against inflammatory and immunomodulatory properties to treat a variety of tumors. The influential anti-inflammatory and immunomodulatory properties of CB make it a potential therapeutic agent against liver cancer by modulating the TME and inhibiting M2-TAM macrophage polarization through Wnt/β-catenin pathway. Its suppression effects and capability of targeting the Wnt pathway offer a novel approach to cancer therapy, potentially leading to progressive advances in malignancy treatment.

ACKNOWLEDGEMENTS

Research background

Calculus bovis (CB) has long been valued in Traditional Chinese herbal medicine, utilizing its against inflammatory and immunomodulatory properties to treat a variety of tumors. However, its impact on the liver cancer tumor microenvironment (TME), particularly concerning tumor-associated macrophages (TAMs), is not well understood.

Research motivation

The full extent of CB's influence on the liver cancer TME, particularly on TAMs, remains elusive. We revealed that CB's antineoplastic effects may involve inhibiting M2-polarized TAMs differentiation through modulation of key molecular pathways. Our

study lays a foundation for developing CB-derived antineoplastic therapeutic strategies, inhibiting M2-TAM polarization.

Research objectives

The objective of this research is to clarify the anti-liver cancer mechanism of CB *via* the inhibition of M2-TAM polarization through modulation of the Wnt/β-catenin pathway.

Research methods

The research was initiated by identifying the active constituents in CB and in CB-containing serum (CBS) using UPLC-QTOF-MS. The anti-neoplastic effects of CB were evaluated in nude mice of subcutaneous liver cancer model. The interaction and mechanisms underlying the anti-liver cancer activity of CB was elucidated through network pharmacology, transcriptome sequencing, and molecular docking studies. *In vitro* experiments utilized CBS to modulate M2-TAMs, assessing its impact on HepG2 cell proliferation, migration, M2-TAM polarization, and apoptosis *via* CCK8, scratch assay, flow cytometry, transwell assay and RT-qPCR. Furthermore, this study investigated the influence of CB on Wnt pathway-related genes and proteins through *in vitro* and *in vivo* experiments as well as RT-qPCR and Western blot analysis. Specificity was confirmed using Wnt pathway agonists.

Research results

We identified a total of 22 active components in CB, with 11 of them being detected in the bloodstream. Preclinical investigations demonstrated CB's ability to effectively inhibit liver tumor growth. An integrated approach employing network pharmacology, transcriptomics, and molecular docking implicated the Wnt signaling pathway as a target of CB's antineoplastic activity by suppressing M2-TAM polarization. *In vitro* and *in vivo* experiments further confirmed that CB significantly hinder M2-TAM polarization and suppress Wnt/β-catenin pathway activation. The inhibitory effect of

CB on M2-TAM was reversed when treated with the Wnt agonist SKL2001, thus confirming the pathway specificity.

Research conclusions

CB-mediated inhibition of M2-TAM polarization through Wnt/β-catenin pathway, contributing to the suppression of liver cancer growth.

Research perspectives

CB's effects on tumor-associated macrophage polarization have been confirmed, its suppression effects and capability of targeting the Wnt pathway offer a novel approach to cancer therapy, potentially leading to progressive advances in malignancy treatment.

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