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Reevaluating *Calculus bovis*: Modulating the liver cancer immune microenvironment *via* the Wnt/β-catenin pathway

Wang SY *et al*. CB modulates the liver cancer immunity

Abstract

In this article, we comment on the work by Huang *et al* published, which explores the mechanisms by which *Calculus bovis* (CB) modulates the liver cancer immune microenvironment *via* the Wnt/β-catenin signalling pathway. The study demonstrates that active components in CB effectively inhibit the activation of the Wnt/β-catenin pathway, significantly reducing the polarization of M2 tumor-associated macrophages. Both *in vivo* and *in vitro* experiments have validated the anti-tumour effects of CB, revealing its complex mechanisms of action through the modulation of immune cell functions within the tumour microenvironment. This article highlights CB's therapeutic potential in liver cancer treatment and calls for further investigations into its mechanisms and clinical applications to develop safer, more effective options for patients. The study also revealed that key components of CB, such as bilirubin and bile acids, inhibit tumour cell proliferation and promote apoptosis through multiple pathways. Future research should explore the mechanisms of action of CB and its potential integration with existing treatments to improve the therapeutic outcomes of liver cancer patients. With multidisciplinary collaboration and advanced research, CB could become a key component of comprehensive liver cancer treatment, offering new hope for patients.

Key Words: Liver cancer; *Calculus bovis*; Wnt/β-catenin signalling pathway; Tumour-associated macrophages; Tumour immune microenvironment; Anti-tumour therapy; Traditional Chinese medicine

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Core Tip: This study highlights the role of *Calculus bovis* (CB) in regulating the liver cancer immune microenvironment through the Wnt/β-catenin signalling pathway. Active components in CB inhibit Wnt/β-catenin activation, reduce M2 tumour-

associated macrophage polarization, and enhance anti-tumour immunity, suppressing liver cancer growth and spread. Multilevel experiments confirm CB's potential as an anti-tumour agent, offering a foundation for new liver cancer treatments based on traditional Chinese medicine. Future research should explore CB's effects across liver cancer subtypes and in combination with modern therapies to improve treatment efficacy and safety.

TO THE EDITOR

Primary liver cancer is a malignancy with high incidence and mortality rates. In 2022, there were 865269 new cases of liver cancer and 757948 deaths globally, making it the sixth most common malignancy and the third leading cause of cancer-related deaths worldwide[1]. Despite recent advancements in liver cancer treatment, significant challenges remain. First, the majority of liver cancer patients are diagnosed at an advanced stage of the disease, missing the optimal window for surgical and therapeutic interventions[2]. Additionally, existing screening tools, such as abdominal ultrasound, have low sensitivity for detecting early-stage liver cancer, particularly in obese patients and those with nonviral liver diseases[3]. Although the advent of immune checkpoint inhibitors and other novel systemic therapies has improved survival rates for advanced liver cancer patients, only approximately 30% of patients show an objective response to these treatments, and the three-year overall survival rate remains well below 50%[4,5]. Thus, improving early diagnosis rates, optimizing current treatment protocols, and developing chemoprevention strategies for patients with nonviral liver diseases are pressing issues[6].

In recent years, research on traditional Chinese medicine (TCM) for liver cancer treatment has made significant progress. TCM, with its multitarget, multipathway approach, often has unique advantages in enhancing efficacy, reducing side effects, and improving patients' quality of life[7,8]. *Calculus bovis* (CB), a valuable animal-derived medicinal substance, is widely used for treating various cancerous conditions[9]. CB, derived from dried gallstones obtained from cattle gallbladders or bile ducts, possesses

anti-inflammatory, antipyretic, detoxifying, sedative, and gallbladder-restorative properties[10]. Recent studies have shown that CB holds significant potential in liver cancer treatment, especially in exploring its anti-tumour mechanisms and optimizing combination therapies. Research focusing on the combined use of CB and musk has revealed that this combination effectively inhibits the proliferation and migration of the human liver cancer cell line SMMC-7721 and significantly impedes subcutaneous tumour growth in a liver cancer nude mouse model. The anticancer mechanism involves inhibiting the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of the rapamycin signalling pathway and regulating the expression of apoptosis-related proteins such as caspase-3, caspase-9, B-cell lymphoma 2 and Bax, thereby promoting tumour cell apoptosis[11]. Additionally, in-depth analysis of CB components revealed 11 compounds with anti-hepatocellular carcinoma activity, including oleanolic acid, ergosterol, and ursolic acid. These compounds interfere with key molecules and pathways, such as interleukin-6 (IL-6), mitogen-activated protein kinase 8, and vascular endothelial growth factor A (VEGFA), further elucidating the complex mechanism of action of CB in exerting anti-primary liver cancer effects through the induction of cell apoptosis and the modulation of immune-related pathways[12]. These findings provide scientific evidence for the application of CB in liver cancer treatment, further supporting its importance as a TCM therapeutic strategy.

Current research indicates that the Wnt/β-catenin signalling pathway plays a crucial role in the onset and progression of liver cancer. The Wnt/β-catenin pathway is a highly conserved signalling cascade that is essential for cell proliferation, differentiation, migration, and survival[13]. When this pathway is aberrantly activated, it can lead to uncontrolled cell proliferation and inhibited apoptosis, thereby promoting tumour initiation and progression[14]. Studies have shown that high expression of the Wnt/β-catenin pathway in liver cancer cells is closely associated with tumour aggressiveness and recurrence rates[15]. Moreover, this pathway plays a significant role in modulating the immune response within the tumour microenvironment (TME). Activation of the Wnt/β-catenin pathway can promote the polarization of tumour-

associated macrophages (TAMs) to the M2 phenotype, thereby suppressing anti-tumour immune responses and facilitating tumour growth and metastasis[16-18]. Thus, targeting the Wnt/β-catenin signalling pathway to inhibit its activity can potentially suppress tumour cell growth and enhance the body's anti-tumour immune response, representing a promising direction for liver cancer research.

This article discusses a study by Huang *et al* published in the *World Journal of Gastroenterology*, which demonstrates that CB can inhibit the polarization of M2-TAMs through modulation of the Wnt/β-catenin signalling pathway, thereby suppressing liver cancer growth. This study provides new insights, revealing the potential mechanisms by which CB regulates the tumour immune microenvironment. By inhibiting the polarization of M2-TAMs, CB can reduce tumour immune evasion and enhance anti-tumour immune responses, thereby inhibiting tumour growth and metastasis. These findings not only advance our understanding of the role of the Wnt/β-catenin signalling pathway in liver cancer but also provide a theoretical basis and practical guidance for the development of new liver cancer treatment strategies based on TCM. Future studies should further explore the specific actions of CB within the Wnt/β-catenin pathway and its clinical application prospects, which will help provide more effective treatment options for liver cancer patients.

LIVER CANCER TME AND IMMUNE REGULATION

The progression and deterioration of liver cancer largely depend on its unique TME, which is a complex system that not only supports the continuous proliferation and survival of tumour cells but also significantly impacts the efficacy of anti-tumour immune responses[19]. Among the key regulators of the liver cancer immune microenvironment are M2-TAMs. M2-TAMs secrete immunosuppressive cytokines and growth factors, such as IL-10 and transforming growth factor-beta (TGF-β), which promote angiogenesis and suppress the activity of anti-tumour immune cells, creating an environment that is conducive to liver cancer cell growth and spread[20].

Recent studies have revealed that the Wnt/β-catenin signalling pathway plays a critical role in regulating M2-TAM polarization. This pathway is aberrantly activated in various tumour types, facilitating β-catenin nuclear translocation and subsequent gene transcription, which drives the differentiation of M2 macrophages, exacerbating immunosuppression and promoting tumour immune evasion[13,21]. Additionally, cross-cancer research has shown that the Wnt/β-catenin signalling pathway plays a crucial role in modulating immune cell functions within the TME. Wei *et al*[22] demonstrated that B-cell CLL/lymphoma 9 inhibition significantly alters the composition of TAMs in colorectal cancer, introducing a TAM-to-natural killer score method to analyse the balance of immune cells within The Cancer Genome Atlas data and highlight the impact of this regulation on patient prognosis. These findings underscore the value of the Wnt signalling pathway as a cancer immunotherapy strategy.

In the context of lung cancer, Cui *et al*[23] identified sanguinarine as a modulator of the Wnt/β-catenin signalling pathway, effectively regulating M2 macrophage polarization and inhibiting angiogenesis. This discovery broadens our understanding of the anti-tumour mechanisms of natural products and suggests potential applications in comprehensive lung cancer treatment, particularly in combination with immunotherapy and antiangiogenic strategies. For triple-negative breast cancer, Wang *et al*[24] reported that VEGFA-mediated signalling plays a central role in the crosstalk between cancer stem cells and TAMs, providing a theoretical basis for combining immunotherapy and VEGFA-targeted therapy, which marks significant progress in overcoming this clinical challenge. Additionally, Sarode *et al*[25] highlighted the importance of the Wnt/β-catenin signalling pathway from another perspective and reported that the co-expression of β-catenin and Fos-like antigen 2 in lung cancer samples is closely associated with poor patient prognosis, further confirming the transcriptional regulatory role of this pathway in promoting tumour progression and metastasis.

Overall, the Wnt/β-catenin signalling pathway not only is a key mechanism that promotes tumour growth and immune evasion in liver cancer but also plays a widespread role in regulating the immune microenvironment and influencing disease progression in various malignancies. These studies collectively highlight the potential of the Wnt/β-catenin signalling pathway as a new target for cancer immunotherapy, particularly through regulating the function and phenotype of TAMs and their impact on tumour angiogenesis and immune surveillance.

The study discussed in this article used a multilayered experimental design to reveal the potential mechanisms of CB in liver cancer treatment. The experimental results showed that CB inhibits the polarization of M2-TAMs by suppressing the Wnt/β-catenin signalling pathway, thereby weakening tumour immune evasion, enhancing anti-tumour immune responses, and ultimately inhibiting liver cancer growth and metastasis. Specifically, this study identified the active components and targets of CB through network pharmacology, transcriptomics, and molecular docking and validated its anti-tumour effects *via* both *in vitro* and *in vivo* experiments. *In vitro*, CB-treated M2-TAMs significantly reduced the secretion of proinflammatory cytokines and growth factors, such as C-C motif chemokine ligand 22, arginase 1, TGF-β2, and IL-10, thereby diminishing tumour immune evasion and enhancing anti-tumour immune responses. Further *in vivo* experiments confirmed these findings, showing that CB treatment significantly inhibited tumour growth and metastasis in liver cancer nude mouse models. This study not only enhances our understanding of the role of the Wnt/β-catenin signalling pathway in liver cancer but also provides a theoretical basis and practical guidance for the development of new liver cancer treatment strategies based on TCM. CB, which modulates M2-TAMs in the tumour immune microenvironment, shows great promise as a potential anti-tumour drug. Future research should further explore the specific mechanisms of CB in the Wnt/β-catenin signalling pathway and its clinical application prospects to provide more effective treatment options for liver cancer patients.

Recent research has also suggested that CB-mediated inhibition of M2-TAM polarization might involve additional molecular interactions beyond the Wnt/β-catenin pathway[13,14]. For example, the active components of CB, such as bilirubin and bile acids, may interact with signalling molecules related to TAM differentiation, further decreasing the expression of M2 markers and promoting a shift towards an immune-activating microenvironment[10]. Studies have demonstrated that components such as bilirubin may reduce IL-10 and TGF-β levels, enhancing T-cell-mediated immune responses and improving the overall anti-tumour efficacy[17,18]. This multitargeted inhibition highlights CB's complex mechanism in counteracting the immunosuppressive environment fostered by M2-TAMs, offering a more robust immune-modulatory effect[19]. Further exploration into how CB influences these additional molecular pathways will deepen our understanding of its potential in modulating the liver cancer immune microenvironment.

ANTILIVER CANCER MECHANISMS OF CB

CB, a valuable traditional Chinese medicinal material, has various biological activities. Its main components include bilirubin, bile acids, amino acids, sterols, and other trace elements, with the composition varying among different sources of CB, such as natural CB, artificial CB, fed CB, and cultured CB. The combined effects of these components endow CB with extensive pharmacological effects and significant anti-tumour activity[10]. Studies indicate that while these sources share common bioactive components such as bilirubin and bile acids, the concentrations and ratios of these components can vary significantly depending on the source. For example, natural CB, derived directly from bovine gallstones, may contain a more complex profile of trace elements and amino acids than synthetic alternatives such as artificial CB or fed CB[9,10]. These compositional differences suggest that different types of CB may exhibit distinct pharmacological profiles, potentially leading to variations in their anti-tumour efficacy and immunomodulatory functions. However, current research comparing these

three types of CB is limited, and further studies are needed to better understand their specific effects and to optimize their therapeutic applications.

Among the primary active ingredients of CB, bilirubin and bile acids are considered its most important bioactive components. Bilirubin plays a crucial role in protecting the liver, combating oxidative stress, and mitigating inflammatory responses while also demonstrating significant anti-tumour activity. Studies have shown that bilirubin can induce apoptosis of tumour cells and inhibit their proliferation through various pathways. Additionally, bilirubin protects liver cells by reducing oxidative stress and alleviating inflammatory responses, which is important for preventing and managing liver cancer[26,27]. Bile acids, such as deoxycholic acid and cholic acid, also possess anti-tumour activities by regulating bile acid receptors and signalling pathways. Bile acids can induce apoptosis of tumour cells and inhibit cell proliferation, thereby enhancing the anti-liver cancer effects of CB[28]. Together, bilirubin and bile acids function as primary bioactive agents within CB, targeting liver cancer cells through direct cytotoxic mechanisms while also modulating immune pathways[27]. The complementary actions of CB in reducing oxidative stress, alleviating inflammation, and promoting apoptosis establish a robust foundation for its pharmacological efficacy against liver cancer[10]. These key components not only act against tumour cells but also shape the TME to reduce immune evasion, thereby augmenting the therapeutic potential of CB. In addition to these central components, CB also contains amino acids, such as taurine, which have protective effects on the cardiovascular and nervous systems. Taurine helps mitigate liver damage and inhibits tumour cell growth through antioxidant and anti-inflammatory actions. It not only directly inhibits tumour cell proliferation but also indirectly suppresses tumour growth and metastasis by enhancing immune system function[29,30]. Additionally, CB contains trace elements such as zinc, copper, and iron, which play vital roles in maintaining normal cellular functions and enhancing immunity. Zinc has antioxidant and anti-inflammatory properties, inhibits tumour cell proliferation and promotes apoptosis[31]. Copper and

iron contribute to anti-tumour processes through their involvement in redox reactions and gene regulation[32] (Figure 1).

These individual components in CB, including bilirubin, bile acids, amino acids such as taurine, and trace elements such as zinc and copper, do not merely function in isolation. Instead, they likely interact synergistically to amplify the overall therapeutic effects of CB. For example, the antioxidant properties of taurine may enhance the reduction in oxidative stress mediated by bilirubin, whereas trace elements such as zinc further support cellular stability and immune modulation[33,34]. Together, these complementary interactions reinforce the ability of CB to establish an anti-tumour environment that is both cytotoxic to cancer cells and supportive of immune defences. This synergy among CB components suggests a coordinated mechanism that enhances its pharmacological efficacy, making it a promising agent for liver cancer treatment.

This article highlights new perspectives on the traditional applications of CB and underscores its potential role in tumour immunotherapy. This study provides compelling evidence of how the complex chemical composition of CB - particularly bilirubin, bile acids, amino acids, and trace elements - interacts with the Wnt/β-catenin signalling pathway, a crucial mediator of liver cancer progression. This pathway not only promotes tumour cell proliferation but also induces M2-TAM polarization, fostering an immunosuppressive microenvironment. These findings demonstrate that the active ingredients of CB effectively inhibit β-catenin activation and its nuclear translocation, reducing its transcriptional activity and suppressing M2-TAM accumulation. This shift favours anti-tumour immune responses. Both *in vivo* and *in vitro* data confirm the dual mechanism of CB: It directly inhibits liver cancer cell proliferation and apoptosis while modifying the tumour immune microenvironment to reverse immune evasion. These insights enhance our understanding of the anti-tumour mechanisms of CB and suggest promising applications for combination therapies, particularly in combination with modern immunotherapy and chemotherapy.

CLINICAL SIGNIFICANCE AND APPLICATION PROSPECTS

The unique multicomponent and multitarget approach of CB highlights its clinical significance and application prospects. Compared with modern therapies such as immune checkpoint inhibitors and targeted therapies, CB offers unique advantages due to its approach[35]. While immune checkpoint inhibitors can be effective, their success is often limited by resistance and immune-related adverse events, with only a subset of patients experiencing durable responses[4,5]. In contrast, the complex composition of CB - including components such as bilirubin and bile acids - enables it to modulate the immune microenvironment through multiple pathways, potentially reducing the risk of resistance[10,12]. This multicomponent profile also supports a broader spectrum of immune responses, helping to sustain the anti-tumour effects of CB even when tumours develop mechanisms to evade single-target therapies[7]. Additionally, while targeted therapies typically focus on specific molecular targets, CB shows potential for synergistic action within the immune microenvironment. By inhibiting the Wnt/β-catenin pathway, CB may enhance T-cell-mediated anti-tumour responses, complementing immune checkpoint inhibitors and improving efficacy in cases of resistance[13,15]. This multitargeted immune modulation positions CB as a promising alternative, potentially preventing tumour cells from easily adapting or developing resistance[9,10].

CB shows broad prospects in personalized treatment because the diverse components that may yield varying efficacy against different liver cancer subtypes and genetic backgrounds. By using high-throughput technologies such as genomics and proteomics to classify patients, tailored treatment plans can be developed to achieve a truly personalized medicine[36]. Additionally, in-depth research into the active components and mechanisms of CB could lead to the development of novel small-molecule drugs that target specific signalling pathways, thereby increasing treatment safety and efficacy[37]. However, despite its promising potential in combination with modern immunotherapies, detailed exploration and preliminary data on specific combination strategies remain limited. Future multicentre longitudinal studies are necessary to evaluate optimal therapeutic regimens that integrate CB with immune checkpoint

inhibitors or targeted therapies, particularly in the context of liver cancer. Understanding the long-term effects of CB treatment - including potential side effects and resistance mechanisms - is crucial for a comprehensive assessment of its therapeutic viability. Systematic research addressing these aspects will provide a thorough evaluation of the efficacy and safety of CB in liver cancer therapy, ultimately guiding its integration into broader clinical practice.

CONCLUSION

This article highlights the innovative findings on the regulation of the liver cancer immune microenvironment by CB through the Wnt/β-catenin signalling pathway. Research has demonstrated that active components of CB, particularly bilirubin and bile acids, effectively reduce M2-type TAM polarization, enhance antitumour immune responses, and inhibit liver cancer cell proliferation and metastasis. These results increase our understanding of the role of the Wnt/β-catenin pathway in liver cancer and provide a foundation for integrating TCM into modern treatment strategies.

These findings suggest that CB could serve as a novel immune modulator, enhancing the effectiveness of existing immunotherapies and improving survival rates for patients with liver cancer. The multicomponent, multitarget properties of CB indicate potential for synergistic applications with immune checkpoint inhibitors and targeted therapies. Future research should focus on elucidating the specific mechanisms of CB across different liver cancer subtypes and employing modern technologies such as genomics for personalized treatment plans. Collaborative efforts are essential for advancing research and uncovering new therapeutic strategies, ultimately providing safer and more effective treatment options for liver cancer patients. Additionally, there are no preclinical studies or clinical trials investigating the combination of CB with immune checkpoint inhibitors; thus, future research should prioritize exploring the Wnt/β-catenin signalling pathway and M2 macrophages as potential targets for synergy with immune checkpoint therapies. This exploration could not only enhance our understanding of the intricate mechanisms underlying liver cancer treatment but also

pave the way for more effective, personalized therapeutic strategies that leverage the strengths of both traditional and modern medical approaches.

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