Original Article
Matrine improves the hepatic microenvironment and reverses epithelial-mesenchymal transition in hepatocellular carcinoma by inhibiting the Wnt-1/β-catenin signaling pathway

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Abstract: Objective: Hepatocellular carcinoma (HCC) is a malignant tumor with high morbidity and mortality. Despite rapid progress in targeted therapy and immunotherapy for HCC over the past 10 years, the overall efficacy remains unsatisfactory. This is mainly due to the presence of an intrahepatic microenvironment of cirrhosis in HCC patients, leading to cancer recurrence and drug resistance. Methods: In this study, we investigated the correlations between the Wnt-1/β-catenin signaling pathway and the prognosis as well as liver function of HCC patients. Additionally, we conducted in vitro experiments using different concentrations of matrine on HuH-7 cells. Furthermore, we verified the associations between the Wnt-1/β-catenin signaling pathway, inflammation, and epithelial-mesenchymal transition (EMT) in a rat model of pre-hepatocellular carcinoma. Finally, matrine was employed to treat pre-hepatocellular carcinoma in rats and patients with advanced hepatocellular carcinoma. Results: The results demonstrated the activation of the Wnt-1/β-catenin signaling pathway, the occurrence of EMT, and exacerbated inflammation in human HCC tissues. In HuH-7 cell experiments, matrine effectively downregulated the Wnt-1/β-catenin pathway, reversed EMT, and suppressed migration and invasion of HCC cells. In the rat model of pre-hepatocellular carcinoma, matrine dose-dependently inhibited the activation of the Wnt-1/β-catenin signaling pathway, reversed the occurrence of EMT, and alleviated liver inflammation. Matrine analogues exhibited promising hepatoprotective effects in patients with advanced HCC. Conclusions: Matrine can reverse EMT, alleviate intrahepatic inflammation, and counteract immune depletion by inhibiting the Wnt-1/β-catenin signaling pathway in HCC.

Keywords: Matrine, Wnt-1/β-catenin signaling pathway, hepatocellular carcinoma (HCC), epithelial-mesenchymal transition (EMT), cirrhosis, Solt-Farber rat model

Introduction

Hepatocellular carcinoma (HCC) is the second-leading cause of cancer-related death worldwide, with a 5-year survival rate of less than 10% at all stages [1, 2]. Due to its insidious onset, approximately 80% of HCC patients have middle or late-stage disease at the time of diagnosis, making them unsuitable for therapeutic surgery and resulting in a poor prognosis [3, 4]. Even if patients undergo surgery to remove the cancer, the prognosis remains unfavorable. The main reason for this is the persistence of inflammation, fibrosis, cirrhosis,

and other adverse factors in the liver microenvironment. Studies have confirmed that many cases of postoperative recurrence of liver cancer do not originate from the primary lesion but rather from the emergence of new lesions unrelated to the original lesion [5]. Therefore, disrupting the continuous deterioration related to liver cirrhosis and decreasing immune function in patients seems to be more important than simply removing the cancer.

The Wnt/β-catenin signaling pathway is the classical Wnt signaling pathway and is involved in hepatitis, hepatic fibrosis, and HCC. Under

abnormal conditions, Wnt-1 and other ligands can bind to LRP-5/6 and Dishevelled on cell membranes to inhibit the phosphorylation and degradation of β-catenin. Normally, β-catenin is phosphorylated and degraded by the β-catenin destruction complex composed of Axin, APC, GSK-3β, and CK-1. However, under abnormal conditions, a large amount of free β-catenin accumulates in the cytosol and is transported into the nucleus, where it binds with the transcription factors TCF/LEF to form the β-catenin-TCF/LEF transcription complex. This complex specifically activates the transcription of downstream target genes such as c-Myc, cyclin D1, and VEGF, promoting the occurrence and development of tumors [6, 7]. In hepatic fibrosis and cirrhosis, the Wnt/β-catenin signaling pathway can promote the activation of hepatic stellate cells (HSCs) and exacerbate hepatic damage and cirrhosis [8, 9]. HCC, being the ultimate complication of chronic liver disease, is closely related to abnormal activation of the Wnt/β-catenin signaling pathway. In HCC, the mutation rate of β-catenin, the main protein of the Wnt/β-catenin signaling pathway, is as high as 20-40% [10]. Additionally, the mutation rate of Axin-1 is 10%, Axin-2 is 3-4%, and APC is 1%-2% [11, 12]. Abnormal activation of the Wnt/β-catenin signaling pathway can activate other downstream genes, such as CCND1 and c-Myc, which exacerbate inflammation in HCC, promote tumor invasion and metastasis, and confer resistance to targeted drugs [6, 7].

To a certain extent, the Wnt/β-catenin signaling pathway promotes metastasis and progression of HCC by inducing epithelial-mesenchymal transition (EMT) in the HCC microenvironment. Activation of this pathway leads to abnormal phosphorylation of β-catenin, preventing it from binding to E-cadherin on the cell membrane and disrupting the formation of a zipper structure, thereby inducing EMT. During EMT, the expression of E-cadherin decreases, while the expression of N-cadherin and vimentin increases, accompanied by an increase in cytoplasmic and nuclear β-catenin. These changes promote invasion and metastasis of HCC [13, 14]. Furthermore, abnormal activity of the Wnt/β-catenin signaling pathway is closely associated with changes in the immune microenvironment. It can directly inhibit CD8+ T cells and impair the immune activity of T cells, contributing to immunotherapy resistance in HCC

patients and promoting tumor progression [15-17]. Abnormal activation of the Wnt-1/β-catenin signaling pathway leads to the continuous deterioration associated with intrahepatic inflammation, fibrosis, immune depletion, and other adverse factors in the microenvironment, which is the main cause of HCC recurrence and death. Inhibiting the overactivation of the Wnt/β-catenin signaling pathway may improve the prognosis of HCC patients.

Matrine ($C_{15}H_{24}N_2O$) is an effective alkaloid extracted from the traditional Chinese medicine *Sophora flavescens*. It is known for its affordability and minimal side effects. Numerous studies have demonstrated the various beneficial properties of matrine, including its anti-hepatitis B virus activity [18], its ability to alleviate liver fibrosis [19], and its anti-HCC effects [20]. Previous research has also revealed that matrine can exert its antitumor effects by modulating the Wnt/β-catenin signaling pathway. For instance, Li et al. discovered that matrine mitigated 5-FU resistance in NSCLC stem cells by downregulating β-catenin gene expression in a let-7b-dependent manner [21]. Xiao et al. found that matrine inhibited the growth of breast cancer cells, induced apoptosis, suppressed the expression of vascular endothelial growth factor, and downregulated the activity of the Wnt/β-catenin signaling pathway [22]. Similarly, Liu et al. demonstrated that matrine induced apoptosis and inhibited the proliferation of colon cancer cells by blocking the Wnt/β-catenin signaling pathway [23]. Furthermore, recent studies have highlighted the immune-enhancing effects of matrine in patients with various diseases. For example, Peng et al. observed that matrine increased the number of T lymphocytes in the peripheral blood of patients with COVID-19, thereby enhancing their immunity and antiviral effects [24].

In this study, we aimed to verify the impact of the Wnt-1/β-catenin signaling pathway on the liver function and prognosis of HCC patients. Additionally, we sought to validate the inhibitory effects of matrine on the Wnt-1/β-catenin signaling pathway, its ability to reverse EMT, and its ability to improve liver function while exploring a novel treatment approach for HCC through animal experiments and human studies.

Table 1. Clinical characteristics in 66 patients with hepatic cirrhosis with HCC

Variables	
Gender: Male/Female	54/12
Age (y): <50/≥50	25/41
Tumor diameter (cm): >5/≤5	47/19
Child-Pugh: A/B	57/9
Vascular invasion: With/Without	11/55
BCLC stage: A/B/C	16/29/21
Histological Differentiation: Well/Moderate/Poor	9/24/33
Peritumor metastasis: With/Without	19/47
AFP (ng/ml): >400/100-400/<100	32/10/24
HbsAg: Positive/Negative	52/14
HbeAb: Positive/Negative	26/40
Pres1: Positive/Negative	38/28
ALBI grade: 1/2/3	32/32/2
Follow-up: Completion/loss	63/3

HCC: Hepatocellular carcinoma; BCLC stage: Barcelona clinical liver cancer stage; AFP: Alpha Fetoprotein; ALBI grade: Albumin-bilirubin grade.

Materials and methods

Patient tissue samples

Sixty-six HCC tissues and sixty-six adjacent cirrhotic tissues were collected from the Department of Hepatobiliary Surgery, the Fourth Hospital of Hebei Medical University, between January 2012 and December 2015. The inclusion criteria were as follows: 1) diagnosis of HCC and 2) undergoing the first surgery. The exclusion criteria were: 1) prior treatment with targeted therapy, immunotherapy, or any interventional procedures, 2) presence of multiple organ dysfunction, such as heart, brain, or lung dysfunction, 3) presence of sarcoma hepatoid adenocarcinoma, neuroendocrine carcinoma, or other malignant tumors, 4) incomplete clinical data, and 5) refusal by the patient or their family. All patients were followed up until December 2015. Detailed clinical data of the patients are presented in **Table 1**. The study received approval from the ethics committee of the Fourth Hospital of Hebei Medical University (certificate No. 2021KY103).

Cell culture and cell proliferation assay

The HuH-7 cells were purchased from the Cell Laboratory of Central South University (Changsha, China). Cells were maintained in DMEM + 10% fetal bovine serum (Solarbio, 12100) in a humidified incubator (5% CO_2 at 37°C, FBS, DMSO = 5:4:1). Counting Kit-8 was used to quantify proliferation of HCC cells, according to manufacturer's instruction. 96-well plates were used to seed (5×10^3 cells/well). Various concentrations of matrine (batch No. 0301010007160301, Purity: 99%, Ningxia bauhinia pharmaceutical co. LTD., Yinchun, China) were used to treat cells for 48 h and 72 h. After washing cells with PBS, 10 µL of CCK-8 (cat. No. BS350B; Biosharp, China) was added to each well, mixed, and incubated for 2 hours. Optical density (OD) value at 450 nm 50% inhibitory concentration (IC_{50}) was determined by using a microplate reader (Thermo Scientific, USA) in triplicate.

Colony formation assay

HCC cells were seeded into a 12-well plate and allowed to attach overnight. After treatment with matrine for 48 hours, an equal number of treated cells (400 cells/well) were reseeded for culture. Fresh medium was replaced for continuous culture for a duration of 13 days. At the end of the culture period, the cells were stained with 0.2% crystal violet, and the HCC cell colonies were counted under a microscope.

Cell invasion

Matrigel transwell chambers (Corning, New York, USA) were used following the manufacturer's instructions to evaluate the invasion of HCC cells. Cells were pre-treated with vehicle (0.2% DMSO) or matrine for 48 hours. Treated cells, at a density of 1×10^4 per well, were suspended in serum-free media and added to the upper chamber, while medium containing 10% FBS was added to the lower chamber. After removing the cells remaining in the upper chamber, crystal violet staining was performed. Migrated cells were visualized and imaged using an inverted microscope (OLYMPUS, IX71).

Wound healing assay

Three equidistant horizontal lines on the back of a 6-well plate were drawn. HCC cells were seeded into the wells at a density of 2×10^5 cells per well and allowed to grow until they reached

80% confluence. A 200 µl micropipette tip was then used to scratch the cell monolayer and create a wound gap with a width of 1 mm. Subsequently, the cells were washed with PBS and incubated with various concentrations of either the vehicle (0.2% DMSO) or matrine for 0 and 48 hours. Images of the wound were captured using an inverted microscope (OLYMPUS, IX71), and the area of the wound was analyzed using ImageJ software (NIH, USA).

Western blotting

Protein was measured with a BCA kit. Equal amounts of protein were loaded in 10% SDS-polyacrylamide gels, separated by electrophoresis and electro-transferred onto a methanol-equilibrated nitrocellulose membrane. The membrane was incubated with primary antibodies specific to Wnt-1 (cat. No. ab109437; 1:1000, Abcam, Cambridge, MA, US), β-catenin (cat. No. ab32572; 1:1000, Abcam, Cambridge, MA, US), Axin-1 (cat. No. DF12847; 1:1000, Beijing Affinity Biosciences, US), E-cadherin (cat. No. ab76319; 1:1000, Abcam, Cambridge, MA, US), N-cadherin (cat. No. ab76011; 1:1000, Abcam, Cambridge, MA, US) and vimentin (cat. No. ab92547; 1:1000, Abcam, Cambridge, MA, US). β-actin (cat. No. BS-0061R; 1:1,000; Bioss, Beijing, China) acted as the reference protein for loading control. Each reaction was run in triplicate.

Animals and grouping

A total of 48 male Sprague-Dawley rats (clean grade; 6 weeks old; average weight, 120±20 g) were purchased from the Laboratory Animal Center of Hebei Medical University. The rats were randomly divided into four groups: the control group, model group, low-matrine group, and high-matrine group. The model group, low-matrine group, and high-matrine group were used to construct a Solt-Farber model (partial hepatectomy [HP] + diethylnitrosamine [DEN] + 2-acetylaminofluorene [AAF]) in order to generate preneoplastic lesions. Starting from the day of DEN injection (PCode: 1002279831, Sigma-Aldrich; Merck KGaA, US), the low- and high-matrine groups received matrine intragastrically at a dosage of 1.25 mg/kg and 12.5 mg/kg, respectively, twice per day at 8 am and 5 pm. This administration continued until the rats were sacrificed. In the model group, the rats were administered an equal volume of saline.

Rats in the control group did not receive DEN injection, 2-AAF (PCode: 1002176257, Sigma-Aldrich; Merck KGaA, US) supplementation, or hepatectomy. The rats were sacrificed at 2, 4, and 7 weeks after hepatectomy (4 rats per group, per time). Subsequently, the liver was immediately isolated and fixed in 10% formaldehyde. Liver tissue was collected for H&E staining and immunohistochemical detection. Total blood samples were collected for serum analysis. All animal experiments for this project were approved and supported by the Laboratory Animal Ethics Committee of the Fourth Hospital of Hebei Medical University (certificate No. 2017026).

Liver biological activities

Serum samples were used to evaluate liver status by determining the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (GGT) at the Research Center of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China).

Haematoxylin and eosin staining (H&E staining)

Liver specimens that had been fixed with a 10% formaldehyde solution were removed and subjected to dehydration using a gradient of ethanol concentrations (60%, 70%, 80%, 90%, 95%, and 100%) for 1 hour. Subsequently, the slides were incubated in xylene twice for 30 minutes each time and then immersed in wax for over 2 hours for paraffin embedding. Afterward, the tissue sections were prepared with a thickness of 4-6 µm. The paraffin sections were dried in an oven at 67°C for 24 hours and then dewaxed twice with xylene for 10 minutes each. To facilitate rehydration, the sections were briefly immersed in a gradient of ethanol (100% to 60%) for 1 minute each, followed by rinsing with distilled water 3-4 times. The sections were then stained with haematoxylin for 5 minutes, rinsed with distilled water, stained with eosin for 5 minutes, rinsed with distilled water 3-4 times, dehydrated with ethanol, cleared with xylene, and finally sealed with neutral resin.

Immunohistochemistry

Tissue histology was assessed using immunohistochemistry (IHC) following previously des-

cribed methods [25]. Slides were incubated with primary antibodies against specific targets, including Wnt-1 (catalog No. ab189001; 1:60, Abcam, Cambridge, MA, US), β-catenin (catalog No. P35222; 1:100, Cell Signaling Technology, Inc., US), Axin-1 (catalog No. 015169; 1:50, Cell Signaling Technology, Inc., US), E-cadherin (catalog No. ab10982676; 1:10, Thermo Fisher Scientific, CN), N-cadherin (catalog No. 3608; 1:400, Affinity Biosciences., Ltd., China), and vimentin (catalog No. ab92547; 1:200, Abcam, Cambridge, MA, US). The slides were then washed and incubated with secondary antibodies according to the manufacturers' instructions, followed by incubation with diaminobenzidine chromogen. Images were captured using an Olympus BX51T-PHD-J11 microscope (Olympus, Tokyo, Japan).

Immunohistochemical staining was quantified using Image-Pro Plus 5.1 for Windows software (Media Cybernetics Inc., Rockville, MD, US) with the Measurement function. The number of positive cells and the color intensity of positive cells were used to calculate the immunohistochemistry score (IHS) as follows: A) grading of positive cell number: 0-1% = 0, 1-10% = 1, 10-50% = 2, 50-80% = 3, and 80-100% = 4; B) grading of color intensity of positive cells: grade 0 (negative), 1 (weak positive), 2 (positive), and 3 (strong positive). The IHS was calculated as A multiplied by B [26]. An IHS value greater than 100 was considered positive, while an IHS value of 100 or less was considered negative.

In human HCC tissues, β-catenin expression status was determined as previously described by Maruyama et al. [27]. Normal expression was defined as cell membrane staining in over 70% of cells; otherwise, expression was considered absent. Cytoplasmic and nuclear expression in >10% of cells was considered as ectopic expression. Lack of expression and ectopic expression were collectively referred to as abnormal positive expression [27].

Collection of clinical data related to liver function from HCC patients

From January 2021 to March 2022, data were collected from a total of 131 patients with unresectable advanced HCC. After their initial admission, liver function tests were conducted for each patient. One group received treatment with compound matrine intravenously during hospitalization, while the control group received other hepatoprotective drugs. Patients were hospitalized every 3-4 weeks, and liver function was re-evaluated after each hospitalization. More than two examinations were required before a patient was included in the evaluation group. Ultimately, a total of 69 patients were included in the study, with 41 in the matrine group and 28 in the control group. Due to a high number of patients lost to follow-up during the later stages, liver function data were collected only up to the sixth hospitalization. The study was approved by the ethics committee under the number 2021KY103.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software. The expression levels in each group were compared using t-tests or one-way ANOVA tests for measured data, while X^2 tests or Fisher tests were used for comparison of counted data. Survival analysis was conducted using Kaplan-Meier and Cox regression analyses. Multivariate linear regression analysis and logistic regression analysis was utilized to determine correlations of count data. Column charts were generated using Graph Pad Prism 6.01 software. A significance level of *P*<0.05 was considered significant.

Results

Abnormal activation of the Wnt-1/β-catenin signaling pathway in HCC tissues affected patient prognosis and promoted epithelial-mesenchymal transition (EMT)

The immunohistochemistry results revealed that Wnt-1 exhibited predominant expression in the cytoplasm, displaying a brown-red staining. The expression of Wnt-1 in HCC tissues was significantly higher compared to hepatic cirrhosis tissues (*P*<0.05). In paracancerous hepatic cirrhosis tissues, β-catenin was mainly expressed in the cell membrane, showing a linear brown staining pattern, while it was absent in the cytoplasm. Conversely, in HCC tissues, β-catenin expression was absent in the cell membrane but showed ectopic expression in the cytoplasm and nucleus, which was significantly higher compared to adjacent hepatic cirrhosis tissues (*P*<0.05). Axin-1 exhibited expression in both the cytoplasm and nucleus, with no significant difference between adjacent liver cirrhosis and HCC tissues (**Figure 1A**). These findings
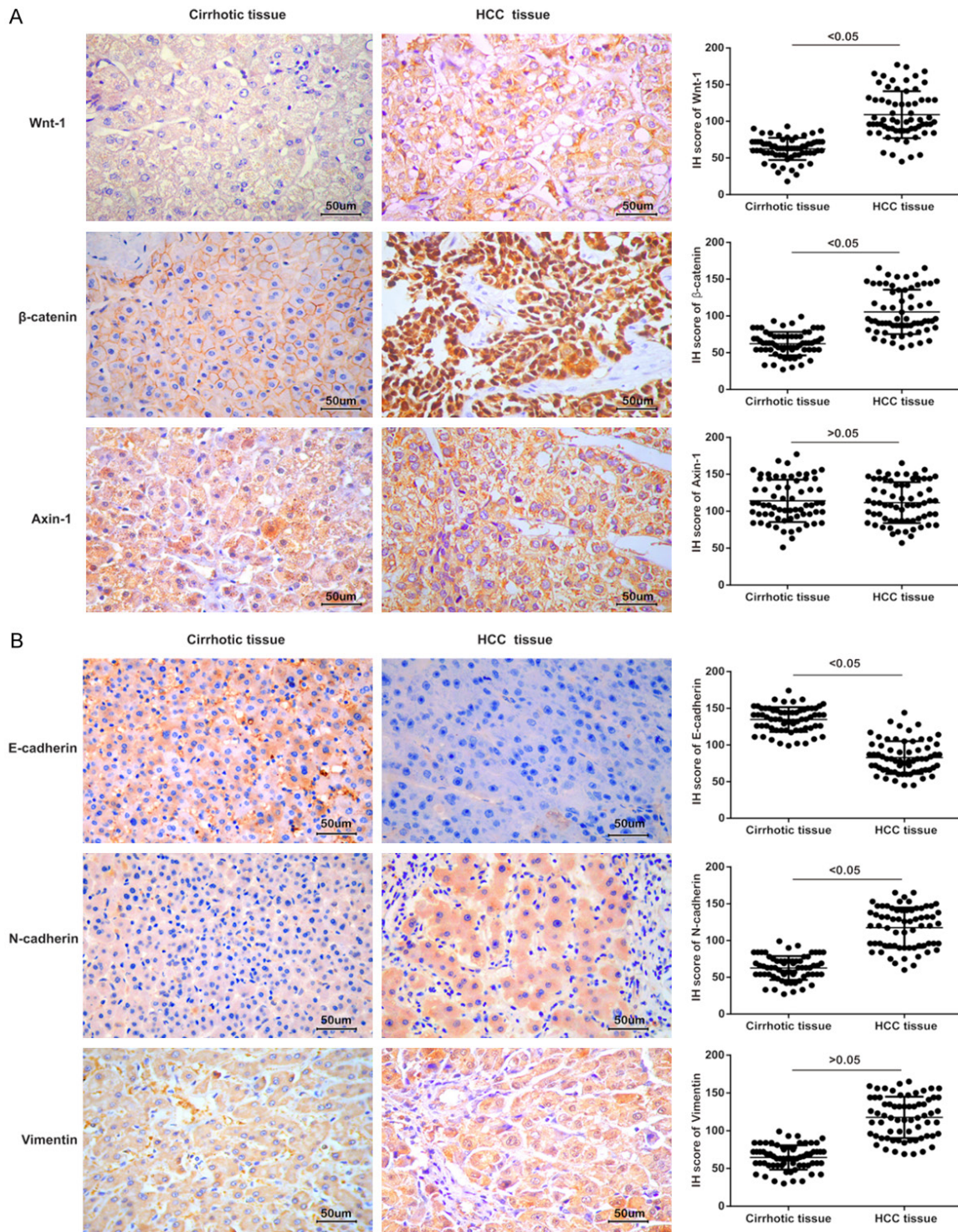
Figure 1. Expression of the Wnt-1/β-catenin signaling pathway and EMT-associated proteins in cirrhotic tissues and HCC tissues as assessed by immunohistochemistry. A. Orange signal indicates the presence of Wnt-1, β-catenin, and Axin-1. Wnt-1 and Axin-1 were mainly present in the cytoplasm. β-Catenin was mainly expressed on the cell membrane in cirrhotic tissues and in the cytoplasm and nucleus in HCC tissues (magnification, 400×), *P*<0.05. B. Orange signal indicates the presence of E-cadherin, N-cadherin, and vimentin, which were mainly present in the cytoplasm. HCC: Hepatocellular carcinoma (magnification, 400×), *P*<0.05.

indicated significant activation of Wnt-1 and β-catenin in HCC tissues.

The immunohistochemical results demonstrated that E-cadherin was primarily expressed in

the cytoplasm, exhibiting a brown-red staining pattern. The expression of E-cadherin was significantly reduced in HCC tissues compared to cirrhotic tissues ($P<0.05$). N-cadherin and vimentin were predominantly expressed in the cytoplasm, displaying a brown-red staining pattern. HCC tissues exhibited significantly higher expression levels of N-cadherin and vimentin compared to adjacent liver cirrhosis tissues ($P<0.05$) (**Figure 1B**). The expression of E-cadherin was lower in Wnt-1- and β-catenin-positive HCC tissues. Furthermore, there was a negative correlation between Wnt-1 and β-catenin expression. Additionally, Wnt-1- and β-catenin-positive HCC tissues showed a higher proportion of N-cadherin- and vimentin-positive cells, with a positive correlation between Wnt-1 and β-catenin expression and the expression of N-cadherin and vimentin (Supplementary Table 1; Supplementary Figure 1).

We conducted an analysis of the correlations between the expression of Wnt-1 and β-catenin with clinical data. The χ^2 test results revealed that Wnt-1-positive patients had larger tumors, a higher rate of portal vein tumor thrombus, more advanced BCLC stage, poorer tumor differentiation, and higher blood AFP levels. Multivariate correlation analysis demonstrated that Wnt-1 expression was an independent factor correlated with BCLC stage and tumor grade (Supplementary Table 2). Similarly, the χ^2 test results indicated that β-catenin-positive patients also had larger tumors, a higher rate of portal vein tumor thrombus, more advanced BCLC stage, poorer tumor differentiation, and higher blood AFP levels. Multivariate correlation analysis showed that β-catenin expression was independently correlated with BCLC stage and AFP level (Supplementary Table 3).

Kaplan-Meier analysis demonstrated that patients with larger tumor diameter, portal vein tumor thrombus, more advanced BCLC stage, poorer tumor differentiation, higher blood AFP level, HbeAb negativity, Wnt-1 positivity, and β-catenin positivity exhibited shorter survival times (**Figure 2A-H**; **Table 2**). Furthermore, decreased expression of E-cadherin was associated with a shortened survival time in HCC patients, while increased expression of N-cadherin and vimentin was linked to a worse prognosis (**Figure 2I-K**; **Table 2**). Cox regression analysis revealed that BCLC stage and β-catenin

positivity were independent risk factors that influenced the prognosis of HCC patients, with patients in more advanced BCLC stages and β-catenin positivity exhibiting a worse prognosis (**Table 2**).

Abnormal activation of the Wnt-1/β-catenin signaling pathway in HCC tissue affected patient hepatic functions

T-test results revealed that Wnt-1-positive patients had elevated levels of aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT), a prolonged prothrombin time (PT), and decreased A/G (albumin/globulin) ratio. Multiple curve regression analysis demonstrated a significant linear relationship between the Wnt-1 immunohistochemistry score (IHS) and AST and GGT levels (**Table 3**). Additionally, the T-test results indicated that β-catenin-positive patients exhibited higher AST levels but lower A/G ratio and albumin (ALB) levels. Multiple curve regression analysis indicated a significant linear correlation between the β-catenin IHS and AST and A/G levels (**Table 4**). The activation of Wnt-1/β-catenin signaling may contribute to impaired hepatic function, promote EMT, and ultimately result in disease progression and mortality in HCC patients.

Matrine inhibited accretion and metastasis of HCC, downregulated the Wnt-1/β-catenin pathway, and reversed EMT in HuH-7 cells

The CCK-8 assay was conducted to assess the growth of HuH-7 cells after treatment with varying concentrations of matrine for 48 hours and 72 hours. The results showed that matrine inhibited the viability of HCC cells in a time- and concentration-dependent manner (**Figure 3A**). Higher concentrations and longer exposure times led to more pronounced inhibition of HCC cell proliferation. The IC_{50} value of matrine over 48 hours was 1.13 mg/ml, an IC_{50} value could not be obtained after 72 hours of treatment. In the subsequent experiment, HCC cells were treated with matrine over 48 hours at a concentration of 1 mg/ml for the high-dose group and 0.5 mg/ml for the low-dose group.

The colony formation assay demonstrated that matrine weakened the proliferative ability of HuH-7 cells with the high-dose group exhibited more pronounced inhibition of HCC cell prolif-
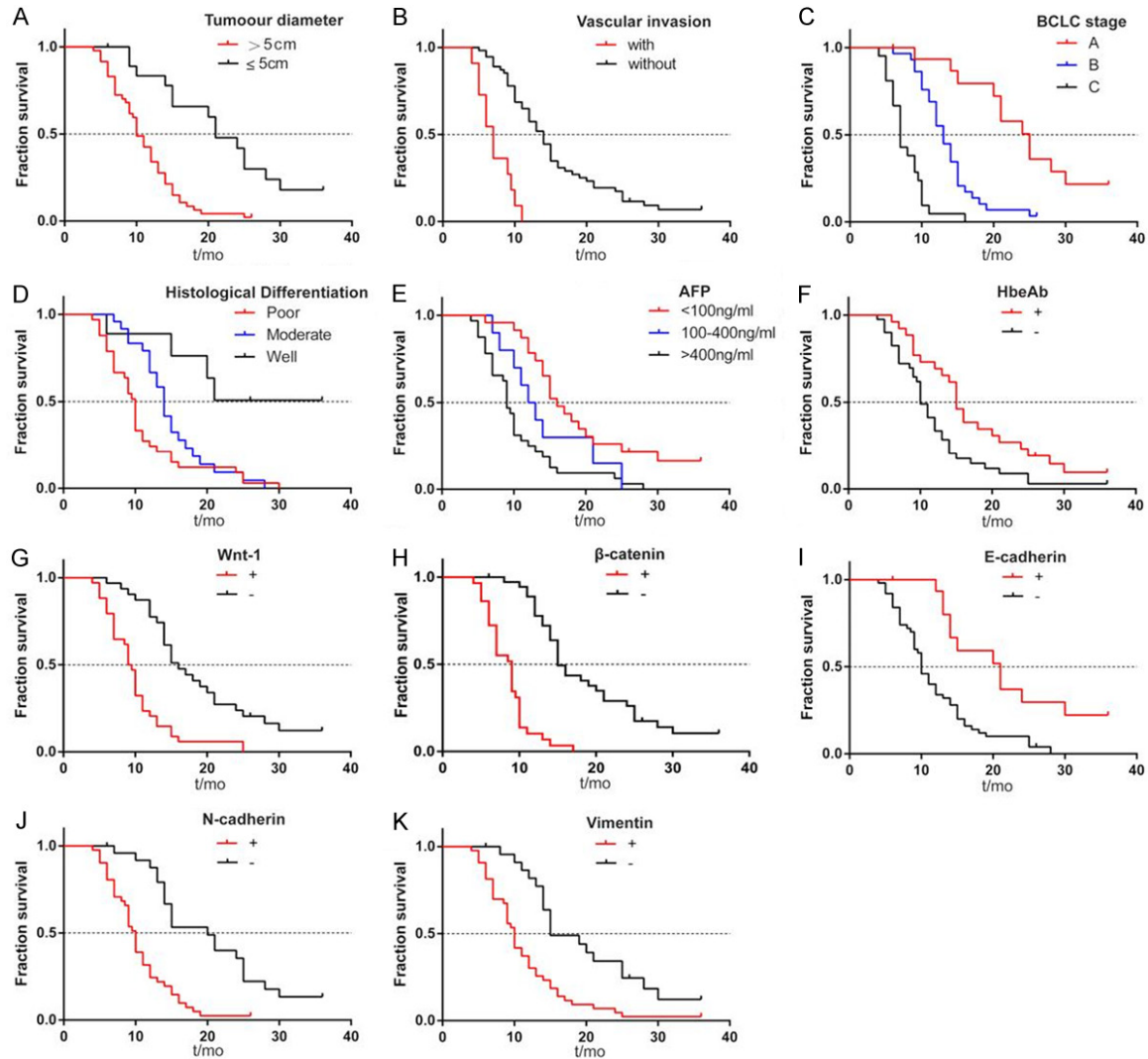
Figure 2. Kaplan-Meier assessment of the impact of clinicopathologic factors, Wnt-1/β-catenin signaling pathway, and EMT-related protein expression on survival time. The aforementioned variables were significantly correlated with the postoperative survival of HCC patients. A. Tumor diameter. B. Vascular invasion. C. BCLC stage: Barcelona clinical liver cancer stage. D. Histologic differentiation. E. AFP: Alpha Fetoprotein. F. HbeAb. G. Wnt-1. H. β-catenin. I. E-cadherin. J. N-cadherin. K. Vimentin.

eration ($P<0.05$) (**Figure 3B**). Furthermore, when examining the effect of matrine on HCC cell invasion after pre-incubation for 48 hours, it was observed that matrine reduced the number of migrating cells compared to the control group. The high-dose group showed a more significant inhibitory effect on HCC cell migration, demonstrating a dose-dependent response ($P<0.05$) (**Figure 3C**). Wound healing assay results revealed that HCC cells treated with matrine migrated at a slower rate compared to the control group, indicating a strong inhibitory effect on cell migration. The high-dose group exhibited a more pronounced effect than the

low-dose group in HuH-7 cells ($P<0.05$) (**Figure 3D**).

Western blotting analysis demonstrated that treatment with matrine led to significantly lower levels of Wnt-1 and β-catenin in HuH-7 cells compared to the control group, while Axin-1 expression increased. Moreover, the high-dose group exhibited a more pronounced effect than the low-dose group, showing a dose-dependent response. Additionally, the levels of N-cadherin and vimentin in HuH-7 cells were significantly lower in the matrine-treated group compared to the control group, while E-cadherin expression

Table 2. Kaplan-Meier analysis and cox regression analysis of prognostic factors for HCC patients

Variables	Kaplan-Meier analysis				Cox Regression analysis		
	No. of patients (%)	Median survival time (m)	Standard error	P	HR	95% CI	P
Gender							
Male	54 (81.8)	13	1.30	0.775			
Female	12 (18.2)	10	1.21				
Age							
<50 (y)	25 (37.9)	14	1.78	0.758			
≥50 (y)	41 (62.1)	12	0.89				
Tumour diameter (cm)							
>5 cm	47 (71.2)	10	0.64	<0.001			
≤5 cm	19 (28.8)	21	2.70				
Vascular invasion							
With	11 (16.7)	7	0.80	<0.001			
Without	55 (83.3)	14	1.04				
BCLC stage (A/B/C)							
A	16 (24.2)	25	2.37	<0.001			
B	29 (44.0)	13	0.89		1		
C	21 (31.8)	7	0.45		4.35	1.36-13.93	0.013
Histologic Differentiation							
Well	10 (15.2)	30	11.1	<0.001			
Moderate	24 (36.4)	14	0.60				
Poor	32 (48.4)	9	0.42				
Peritumor metastasis							
With	19 (28.8)	11	1.04	0.223			
Without	47 (71.2)	13	0.98				
AFP (ng/ml)							
>400	31 (47.0)	9	0.46	<0.001			
100-400	11 (16.7)	12	1.10				
≤100	24 (36.3)	16	1.44				
HbsAg							
Positive	52 (78.8)	11	1.05	0562			
Negative	14 (21.2)	14	1.00				
HbeAb							
Positive	26 (39.4)	15	0.85	0.007			
Negative	40 (60.6)	10	0.58				
Pres1							
Positive	40 (60.6)	10	1.22	0.848			
Negative	26 (39.4)	14	1.05				
ALBI grade							
1	32 (48.5)	12	1.31	0.068			
2	32 (48.5)	13	1.62				
3	2 (3)	6	1.50				
Wnt-1							
Positive	34 (51.5)	9	0.44	<0.001			
Negative	32 (48.5)	16	1.60				
β-catenin							
Positive	28 (42.4)	8.5	0.72	<0.001	17.12	2.45-20.75	<0.001
Negative	38 (57.5)	16	1.10				

E-cadherin				
Positive	16 (71.2)	21	3.50	<0.001
Negative	50 (28.8)	10	0.587	
N-cadherin				
Positive	41 (24.2)	10	0.43	<0.001
Negative	25 (75.8)	20	2.73	
Vimentin				
Positive	43 (65.2)	10	2.77	<0.001
Negative	23 (34.8)	15	0.54	

HCC: Hepatocellular carcinoma; BCLC stage: Barcelona clinical liver cancer stage; AFP: Alpha Fetoprotein; ALBI grade: Albumin-bilirubin grade.

Table 3. T-test, linear regression, and multiple linear regression analysis of the correlation between Wnt-1 expression and liver function in 66 patients with hepatic cirrhosis with HCC

Variable	T-test			Linear Regression			Multiple linear Regression		
	Wnt-1-	Wnt-1+	P	R	R^2	P	R	R^2	P
ALT (U/L)	68.88±13.04	99.8±16.63	0.151	0.239	0.057	0.053			
AST (U/L)	48.53±5.54	79.88±12.7	0.029	0.373	0.139	0.003	0.373	0.19	0.002
ALB (g/L)	38.20±0.84	37.49±0.89	0.565	0.146	0.021	0.241			
TBIL (μmol/L)	14.87±1.07	16.93±2.10	0.394	0.211	0.044	0.089			
ALP (U/L)	104.21±6.00	132.8±24.2	0.27	0.225	0.051	0.069			
GGT (U/L)	67.44±7.9	123.2±15.45	0.002	0.372	0.138	0.002	0.451	0.204	0.001
PT (S)	11.69±0.18	12.29±0.22	0.043	0.203	0.041	0.102			
PTA (%)	99.4±2.38	94.74±2.76	0.208	0.095	0.009	0.447			

HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; TBIL: Total bilirubin; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; PT: Prothrombin time; PTA: Prothrombin time activity.

Table 4. T-test, linear regression, and multiple linear regression analysis of the correlation between the β-catenin expression and liver function in 66 patients with hepatic cirrhosis with HCC

Variable	T-test			Linear Regression			Multiple linear Regression		
	β-catenin-	β-catenin+	P	R	R^2	P	R	R^2	P
AST (U/L)	51.59±5.83	81.38±14.4	0.043	0.426	0.182	<0.001	0.539	0.29	<0.001
ALB (g/L)	39.1±0.6	36.22±1.1	0.017	0.256	0.065	0.038			
A/G	1.32±0.032	1.09±0.057	<0.001	0.473	0.224	<0.001	0.473	0.224	<0.001
TBIL (μmol/L)	14.5±0.911	17.74±2.45	0.18	0.28	0.078	0.023			
ALP (U/L)	103.7±5.57	138.34±28.19	0.182	0.282	0.079	0.022			
GGT (U/L)	82.05±11.88	114.15±14.72	0.091	0.353	0.124	0.004			
PT (S)	11.88±0.18	12.15±0.249	0.38	0.117	0.014	0.349			
PTA (%)	97.49±2.37	96.37±2.595	0.765	0.012	<0.001	0.992			

HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; TBIL: Total bilirubin; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; PT: Prothrombin time; PTA: Prothrombin time activity.

increased. These results also showed a dose-dependent response, with a significant effect (P<0.05) exhibited in the high-dose group (**Figure 3E**).
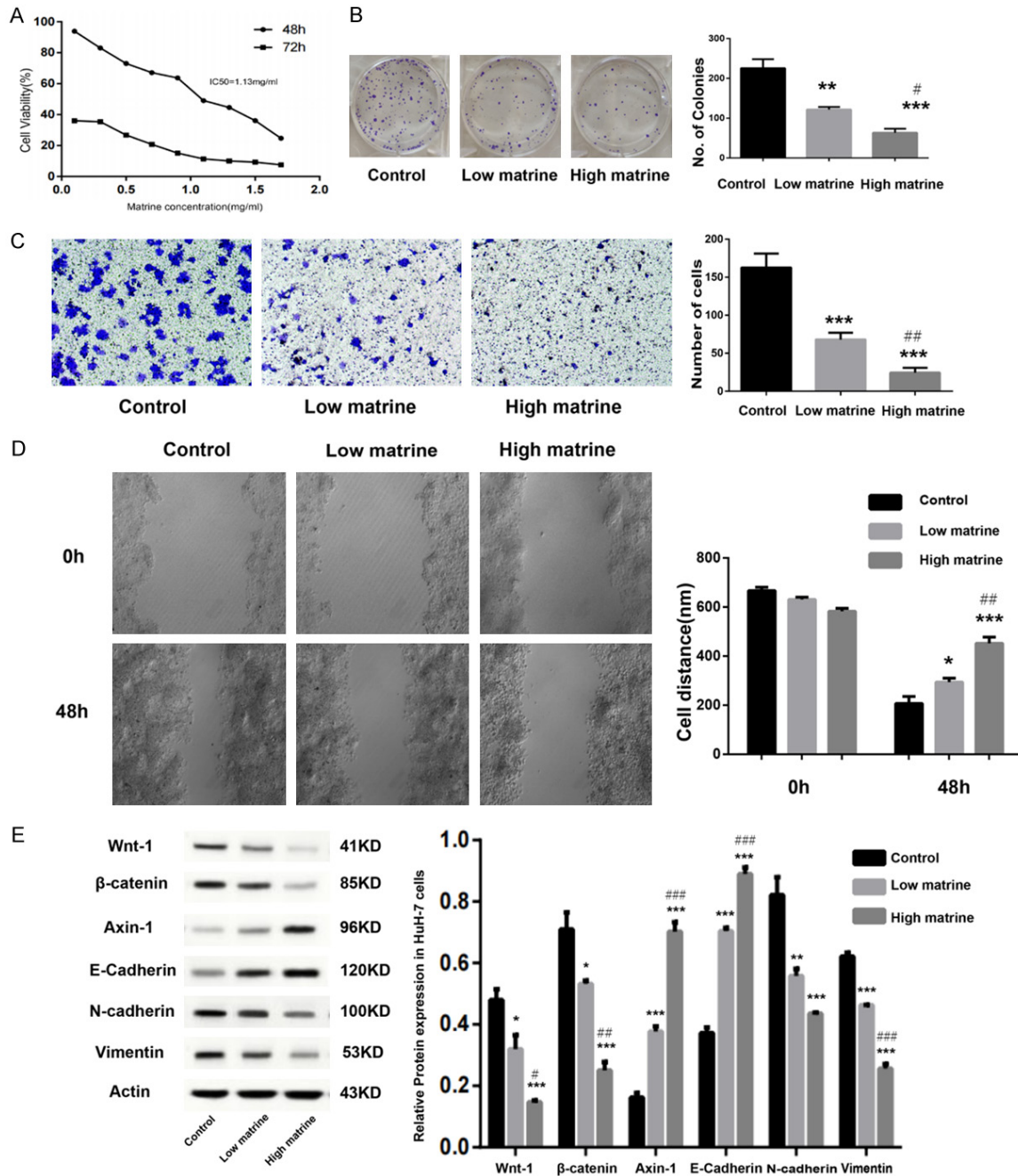
Matrine downregulates the Wnt-1/β-catenin pathway and reverses epithelial-mesenchymal transition (EMT) in HuH-7 cells. Collectively, these findings indicate that matrine has the potential to suppress HCC cell migration and invasion.

Matrine inhibited the Wnt-1/β-catenin pathway and improved liver function in a rat pre-hepatocellular carcinoma model

**Figure 4A** illustrates that in the sham-operated control group, the hepatic lobule structure,

Figure 3. HuH-7 cells were exposed to matrine. Matrine inhibited accretion and metastasis of HCC, downregulated the Wnt-1/β-catenin pathway and reversed EMT in HuH-7 cells. A. Proliferative viability of CCK-8. B. Colony formation assays of HuH-7 cells. C. Cellular migration of HuH-7 cells. D. Wound-healing assay for Huh-7 migration. E. Western blotting shows the expression of EMT makers in Wnt-1/β-catenin signalling pathway. Actin was used as the loading control. Control: control group, Low matrine: 0.5 mg/ml matrine, High matrine: 1 mg/ml matrine group. All data are expressed as the mean ± SEM. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs. the control group; #$P<0.05$, ##$P<0.01$ and ###$P<0.001$ vs. the low-matrine group.

hepatic cells, and hepatic cord structure remained normal at 2 weeks, 4 weeks, and 7 weeks after partial hepatectomy. No inflammatory cell exudation was observed in the portal area. In contrast, the operation model group displayed abnormal hepatic lobule structure and the absence of visible hepatocyte cords at 2 and 4 weeks post-operation. Additionally, sig-
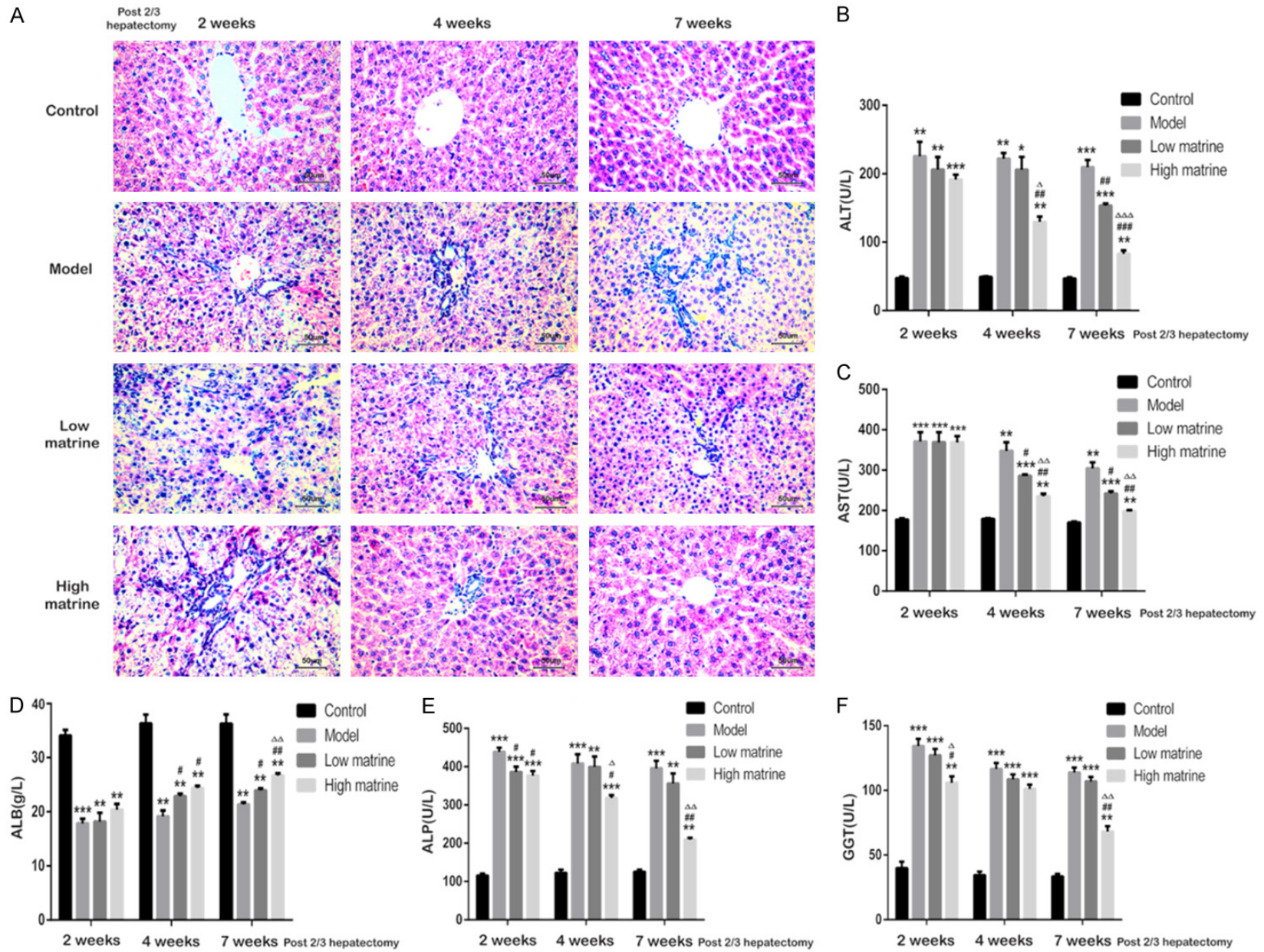
Figure 4. Matrine inhibited the Wnt-1/β-catenin pathway and improved liver function in a rat pre-hepatocellular carcinoma model. A. Haematoxylin and eosin staining (H&E staining) in rat liver. B-F. Expression levels of ALT, AST, ALB, GGT and ALP in rat serum. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: albumin; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; Control: saline group; model: model group; low matrine: low-matrine group; high matrine: high-matrine group. 2 weeks: 2 weeks after partial hepatectomy; 4 weeks: 4 weeks after partial hepatectomy; 7 weeks: 7 weeks after partial hepatectomy. All data are expressed as the mean ± SEM. $^*P<0.05$, $^{**}P<0.01$ vs. the control group; $^#P<0.05$, $^{##}P<0.01$ and $^{###}P<0.001$ vs. the model group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ and $^{\triangle\triangle\triangle}P<0.001$ vs. the low-matrine group. Three to 4 rats were examined in each group.

nificant damage to the hepatic cell membrane structure, hepatocyte edema, degeneration, and infiltration of inflammatory cells in the portal area were observed. At 7 weeks after the operation, hepatic lobules were still absent, and hepatocyte edema and necrosis were apparent. In the matrine group, 2 weeks after the operation, hepatic lobules were not present in the hepatic tissue. Moreover, hepatocyte edema and degeneration, along with notable infiltration of inflammatory cells in the portal area, were observed. Overall, the outcomes of the matrine groups were similar to those of the model group. However, starting from the 4th week after the operation, hepatic lobule injury and portal inflammation tended to decrease in the matrine groups compared to the model group. The high-dose matrine group exhibited a significant reduction in hepatocyte edema and portal inflammatory cell infiltration. At 7 weeks after the operation, the low-dose matrine group showed a significant reduction in hepatocyte edema and degeneration compared to the model group. In the high-dose matrine group, hepatic lobules were observed at the 7th week after the operation, showing a return to normal hepatic lobule structure, presence of normal hepatocyte cables, and mild hepatocyte edema-like degeneration.

The model group demonstrated significantly elevated levels of alanine aminotransferase (ALT), AST, GGT, and alkaline phosphatase (ALP) compared to the sham operation group. Additionally, a decrease in ALB levels was observed 2 weeks after the operation, indicating liver damage. However, in the low-dose matrine pretreatment group, AST levels were significantly decreased, and ALB levels were increased at 4 weeks after the operation compared to the model group. The low-dose matrine pretreatment group exhibited reduced levels of ALT and AST, and increased levels of ALB compared to the model group 7 weeks after hepatectomy. The high-dose matrine group showed significant reductions in ALT, AST, GGT, and ALP

levels, as well as increased ALB levels at weeks 4 and 7 after liver surgery, indicating a more pronounced hepatoprotective effect (**Figure 4B-F**; Supplementary Tables 4, 5, 6, 7, 8) provide further visualization and detailed data on the observed outcomes.

Immunohistochemistry was conducted to assess the expression of Wnt-1, β-catenin, and Axin-1 in rat liver tissues. In the control group, Wnt-1 exhibited weak expression at 2 weeks, 4 weeks, and 7 weeks after partial hepatectomy. In contrast, the model group displayed strong expression of Wnt-1 at the corresponding time points after the operation. Staining appeared reddish-brown, primarily located in the cytoplasm, and the intensity of Wnt-1 staining was greater in the model group compared to the control group. At 7 weeks after the operation, the expression of Wnt-1 in the low-dose matrine group was significantly lower than that of the model group, whereas the high-dose matrine group demonstrated weaker expression of Wnt-1 at 2 weeks, 4 weeks, and 7 weeks after the operation ($P<0.05$, **Figure 5A**).

The control group showed no expression of β-catenin at 2 weeks, 4 weeks, or 7 weeks after partial hepatectomy. In the model group, there was slightly stronger expression of β-catenin at the corresponding time points after the operation. The staining for β-catenin appeared reddish-brown and was mainly observed in the cell membrane, with a small amount of expression in the cytoplasm. The intensity of β-catenin staining was higher in the model group compared to the control group ($P<0.05$). In the low-dose matrine group, the expression of β-catenin was lower than that in the model group at 2 and 4 weeks after the operation ($P<0.05$). Similarly, the high-dose matrine group exhibited lower expression of β-catenin compared to the model group at 2 weeks, 4 weeks, and 7 weeks after the operation ($P<0.05$, **Figure 5B**).

Axin-1 demonstrated strong expression at 2 weeks, 4 weeks, and 7 weeks after partial hep-
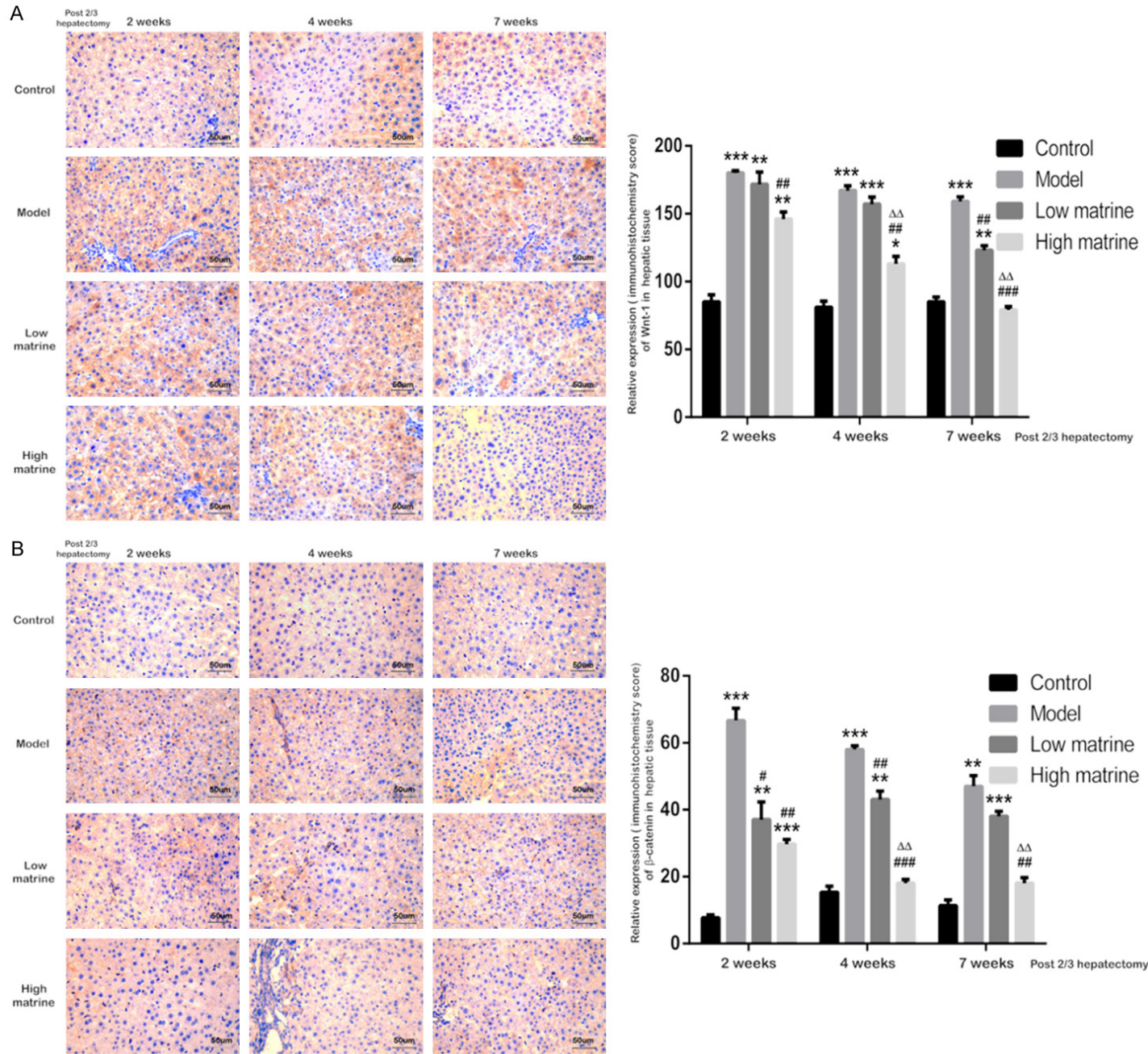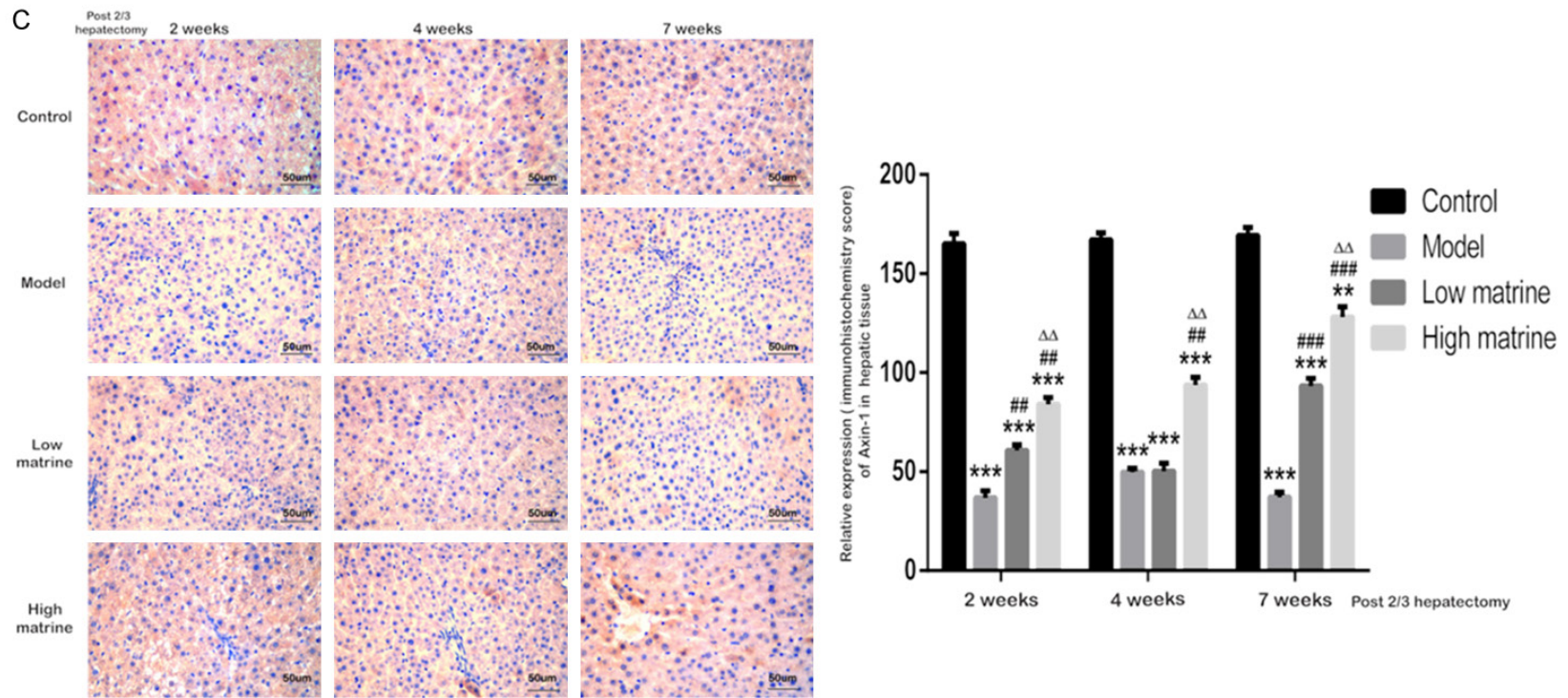
Figure 5. Immunohistochemical assessment of the expression of Wnt-1/β-catenin pathway components in a rat pre-hepatocellular carcinoma model (magnification, 400×). The orange-red signal indicates the presence of protein: A. Wnt-1; B. β-catenin; C. Axin-1. Control: saline group; model: model group; low matrine: low-matrine group; high matrine: high-matrine group. 2 weeks: 2 weeks after partial hepatectomy; 4 weeks: 4 weeks after partial hepatectomy; 7 weeks: 7 weeks after partial hepatectomy. All data are expressed as the mean ± SEM (n = 3 per group). $^{*}P<0.05$, $^{**}P<0.01$ vs. the control group; $^{#}P<0.05$, $^{##}P<0.01$ and $^{###}P<0.001$ vs. the model group; $^{Δ}P<0.05$, $^{ΔΔ}P<0.01$ and $^{ΔΔΔ}P<0.001$ vs. the low-matrine group. Three to 4 rats were examined in each group.

atectomy in the control group. The staining for Axin-1 appeared reddish-brown and was primarily located in the cytoplasm. In the model group, the expression of Axin-1 was significantly decreased at the corresponding time points after the operation, exhibiting a weaker intensity compared to the control group ($P<0.05$). At 2 and 7 weeks after the operation, the low-dose matrine group displayed stronger expression of Axin-1 compared to the model group. Additionally, significant differences were observed between the low-dose matrine group and the high-dose matrine group, with higher expression of Axin-1 in the high-dose matrine group compared to the low-dose matrine group ($P<0.05$, **Figure 5C**).

The activation of the Wnt-1/β-catenin signaling pathway was associated with significant liver damage in the precancer model. Matrine administration inhibited the activation of the Wnt-1/β-catenin signaling pathway, alleviating liver damage in a dose-dependent manner.

Matrine reversed EMT in a rat pre-hepatocellular carcinoma model

Immunohistochemistry analysis was performed to evaluate the expression of E-cadherin, N-cadherin, and vimentin, which are markers associated with epithelial-mesenchymal transition (EMT), in a rat pre-hepatocellular carcinoma model. In the control group, E-cadherin exhibited strong expression at 2 weeks, 4 weeks, and 7 weeks after partial hepatectomy. The staining appeared reddish-brown and was primarily located in the cytoplasm. In the model group, the expression of E-cadherin was significantly reduced at the corresponding time points after the operation, exhibiting a weaker intensity compared to the control group. At 2 weeks, 4 weeks, and 7 weeks after the operation, both the low-dose and high-dose matrine groups displayed higher expression of E-cadherin compared to the model group. Moreover, the high-dose matrine group exhibited significantly higher expression of E-cadherin compared to the low-dose matrine group ($P<0.05$, **Figure 6A**).

In the control group, N-cadherin was not expressed at 2 weeks, 4 weeks, or 7 weeks after partial hepatectomy. However, in the model group, N-cadherin demonstrated strong expression at the corresponding time points after the

operation. The staining appeared reddish-brown and was mainly observed in the portal area and cytoplasm surrounding central vessels. In contrast, both the low- and high-dose matrine groups exhibited weaker expression of N-cadherin compared to the model group at 2 weeks, 4 weeks, and 7 weeks after the operation. Furthermore, the high-dose matrine group displayed significantly lower expression of N-cadherin compared to the low-dose matrine group ($P<0.05$, **Figure 6B**).

Similarly, vimentin was not expressed in the control group at 2 weeks, 4 weeks, or 7 weeks after partial hepatectomy. However, in the model group, vimentin demonstrated strong expression at the corresponding time points after the operation. Staining appeared reddish-brown and was primarily located in the nucleus and cytoplasm of the portal area. In contrast, both the low- and high-dose matrine groups exhibited lower expression of vimentin compared to the model group at 2 weeks, 4 weeks, and 7 weeks after the operation. Furthermore, the high-dose matrine group displayed significantly lower expression of vimentin compared to the low-dose matrine group ($P<0.05$, **Figure 6C**).

In conclusion, EMT was observed in the precancer model. Matrine treatment reversed EMT, with a more pronounced effect observed in the high-dose matrine group.

Matrine may improve liver function in patients with unresectable HCC

ALT levels in patients with HCC during the second hospitalization, as well as the levels of AST, Total Bilirubin (TBIL), Direct Bilirubin (DBIL), and Indirect Bilirubin (IBIL) in patients during the second and third hospitalizations, were significantly reduced compared to those in control patients. However, after the fourth hospitalization, there was no significant difference in the levels of liver function markers between the two groups (**Figure 7A-E**). Furthermore, we examined the effects of matrine treatment in HCC patients with different BCLC stages. In patients with BCLC B stage disease, those who received matrine treatment had significantly lower DBIL levels during the second and third hospitalizations, as well as significantly lower IBIL levels during the fourth hospitalization, compared to patients without matrine treat-
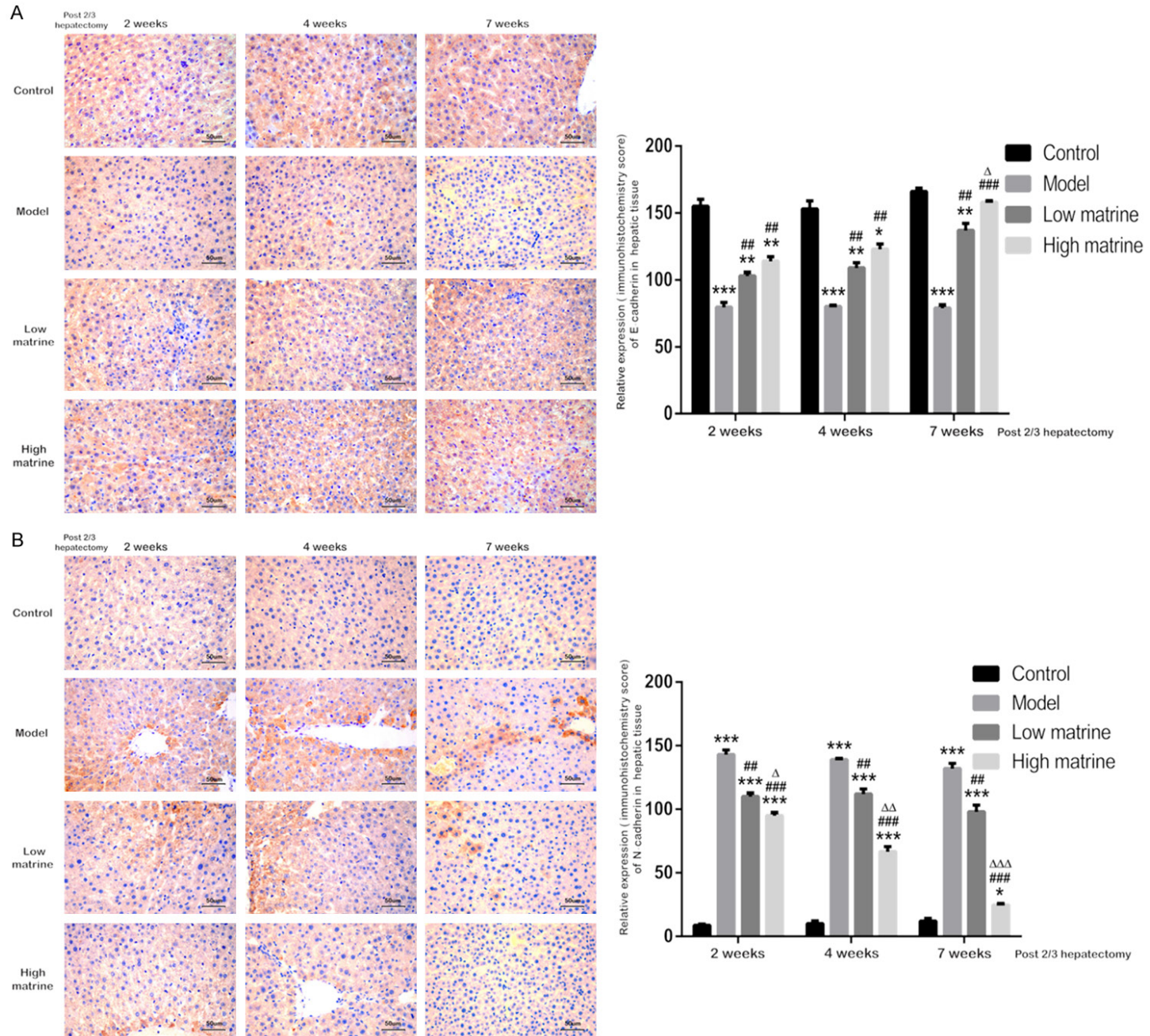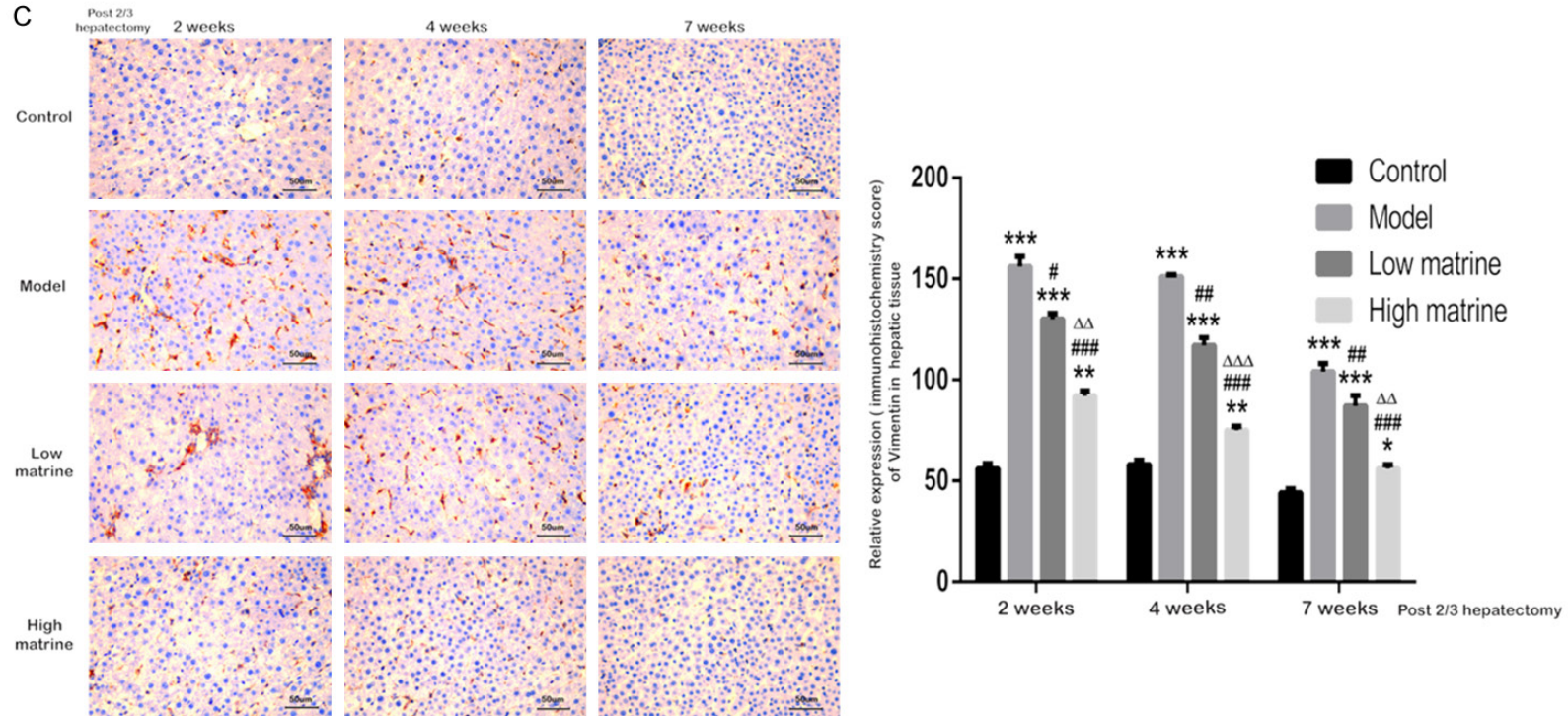
Figure 6. Assessment of the expression of the EMT markers E-cadherin, N-cadherin, and vimentin in a rat pre-hepatocellular carcinoma model (magnification, 400×). The orange-red signal indicates the presence of protein: A. E-cadherin; B. N-cadherin; C. Vimentin. Control: saline group; model: model group; low matrine: low-matrine group; high matrine: high-matrine group. 2 weeks: 2 weeks after partial hepatectomy; 4 weeks: 4 weeks after partial hepatectomy; 7 weeks: 7 weeks after partial hepatectomy. All data are expressed as the mean ± SEM (n = 3 per group). $^*P<0.05$, $^{**}P<0.01$ vs. the control group; $^\#P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ vs. the model group; $^\Delta P<0.05$, $^{\Delta\Delta}P<0.01$ and $^{\Delta\Delta\Delta}P<0.001$ vs. the low-matrine group. Three to 4 rats were examined in each group.
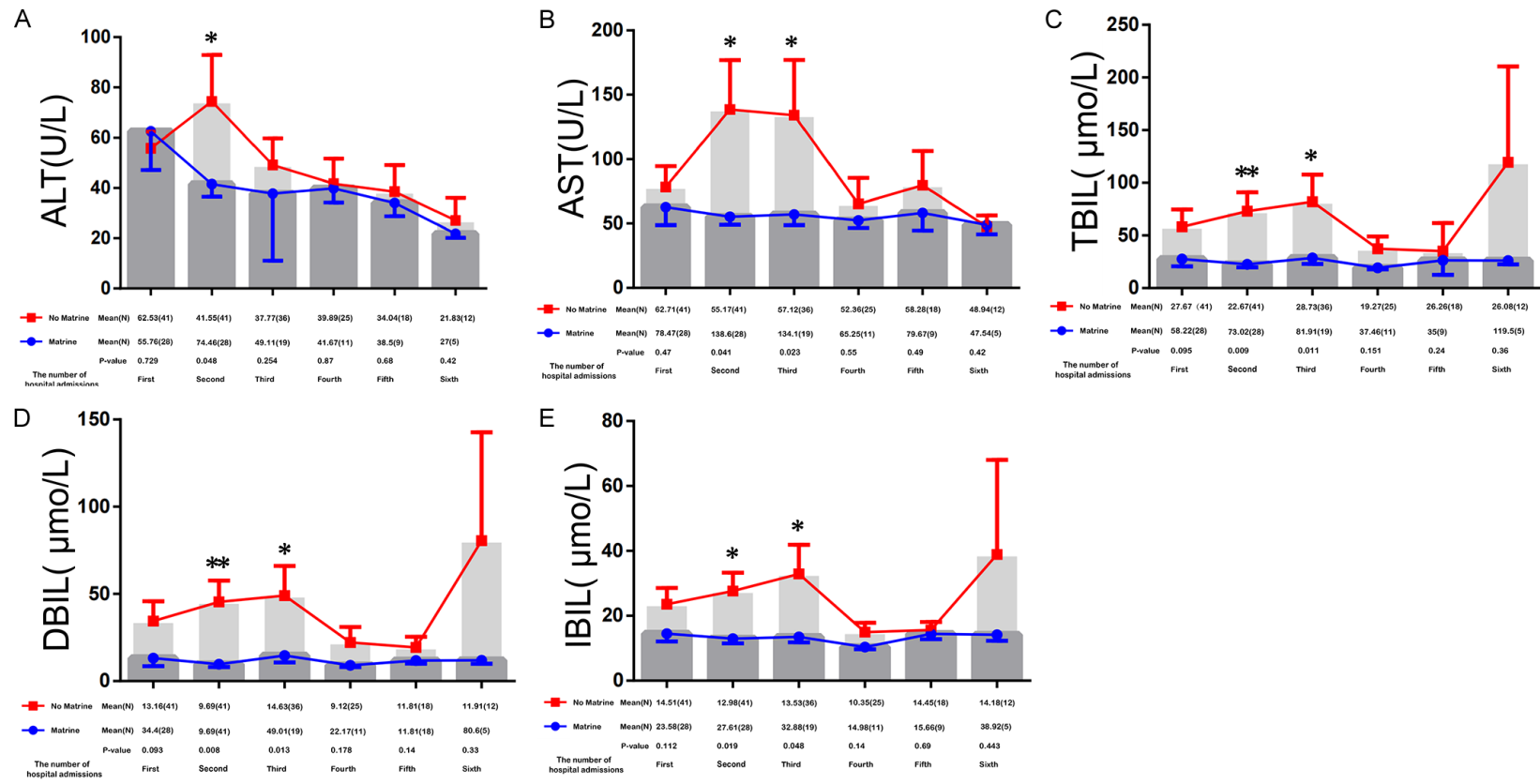
Figure 7. Hepatoprotective effect of matrine on patients with unresectable HCC. A-E: Differences in liver function between HCC patients treated with matrine and those who were not treated with matrine. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; TBIL: Total bilirubin; DBIL: Direct bilirubin; IBIL: Indirect bilirubin. All data are expressed as the mean ± SEM. $^*P<0.05$, $^{**}P<0.01$ matrine vs. no matrine. All data are expressed as the mean ± SEM.

ment. Similarly, in patients with BCLC C stage disease, matrine treatment led to significantly decreased levels of ALT, AST, and DBIL during the second hospitalization, as well as IBIL levels during the fourth hospitalization, compared to patients without matrine treatment. Matrine demonstrated a more pronounced effect in patients with BCLC C stage disease (Supplementary Figure 2A-D). Lastly, we compared the effects of matrine in patients with HCC of different Child-Pugh grades. In patients with Child-Pugh B grade disease, matrine administration significantly reduced AST levels during the fourth hospitalization compared to patients who did not receive matrine administration. Additionally, the level of IBIL was significantly reduced during the third and fourth hospitalizations. Patients with Child-Pugh grade A disease exhibited significantly lower DBIL levels during the second hospitalization compared to patients who were not treated with matrine. Matrine displayed a better hepatoprotective effect in patients with Child-Pugh B grade disease (Supplementary Figure 2E-G). These results indicate that matrine injection can partly improve liver function in patients with unresectable HCC in the early disease stage, with a more significant effect observed in patients with BCLC C stage and Child-Pugh B grade disease.

The above findings demonstrate a close association between abnormal activation of the Wnt-1/β-catenin signaling pathway and EMT, which can exacerbate inflammation. The hepatoprotective effect of matrine may be attributed to the suppression of the Wnt-1/β-catenin signaling pathway.

Discussion

HCC is a highly prevalent and lethal malignant tumor. Over the past decade, significant advancements have been made in HCC treatment. Targeted drugs, including small molecule TKIs such as sorafenib, lenvatinib, and regorafenib, as well as the macromolecular agent bevacizumab, have emerged as effective therapies. Immune checkpoint inhibitors like pembrolizumab and Opdivo have also shown promise. Local treatments such as transarterial chemoembolization (TACE) and hepatic arterial infusion chemotherapy (HAIC) have achieved favorable overall response rates (ORRs) and

improved prognoses, establishing them as first-line treatment options for HCC. However, the efficacy of these treatments is primarily limited to patients with Child-Pugh A-B7 disease [28-30]. In China, a majority of HCC cases are associated with significant cirrhosis. In a study of 69 patients with unresectable HCC, 57 patients (83%) had cirrhosis. Furthermore, many patients with complications such as abdominal effusion, jaundice, and upper gastrointestinal bleeding are unable to tolerate targeted drug therapy or immunotherapy. Even in patients with early-stage HCC following surgery, new lesions can reappear during immunological and targeted drug therapies. This is because HCC patients often have an inflammatory microenvironment, liver fibrosis, and liver cirrhosis, which contribute to the deterioration of the pre-hepatocellular microenvironment. This inflammatory microenvironment is characterized by abnormal pseudolobules, heterotypic cells, and depleted immune cells [31-33]. Consequently, the currently available targeted and immune therapies, as well as liver resection, cannot effectively reverse the deterioration of the microenvironment.

Abnormal activation of the Wnt-1/β-catenin signaling pathway plays a crucial role in the deterioration of the intrahepatic microenvironment. This pathway's activation can stimulate hepatic stellate cells, exacerbate intrahepatic inflammation, and contribute to liver fibrosis and cirrhosis [8, 9]. However, there have been limited studies on the correlation between inflammatory markers and the Wnt-1/β-catenin signaling pathway in HCC patients. In this study, the expression of Wnt-1, a member of the Wnt/β-catenin signaling pathway, as well as β-catenin and Axin-1, core proteins in the β-catenin destruction complex, were examined in human HCC tissues. The findings demonstrated significant activation of the Wnt-1/β-catenin signaling pathway and EMT in HCC patients. Activation of the Wnt-1/β-catenin signaling pathway was associated with more advanced stages, poorer tumor differentiation, and worse prognosis in HCC. This association is supported by the finding thatactivators of the Wnt-1/β-catenin signaling pathway can exacerbate inflammation in the hepatic microenvironment.

In this study, it was observed that patients with activation of the Wnt-1/β-catenin signaling

pathway exhibited higher levels of AST and GGT, prolonged PT, and lower levels of A/G ratio and ALB. These findings suggest that abnormal activation of the Wnt-1/β-catenin signaling pathway promotes the development of intrahepatic inflammation. To further validate this result, a precancerous rat hepatocellular lesion model was generated. In this model, specific time points showed increased levels of ALT, AST, GGT, and ALP, while the ALB level was decreased. Histologic examination of the liver tissue revealed severe necrosis of liver lobules and aggravated inflammation. Simultaneously, abnormal activation of the Wnt-1/β-catenin signaling pathway was observed, revealing that Wnt-1/β-catenin signaling promotes intrahepatic inflammation, influencing prognosis.

These findings highlight the significance of the Wnt-1/β-catenin signaling on inflammation within the liver and its impact on the prognosis of HCC patients. Understanding the role of this pathway may contribute to the development of novel therapeutic strategies targeting intrahepatic inflammation and improving patient outcomes.

Abnormal activation of the Wnt-1/β-catenin signaling pathway promotes the development of intrahepatic inflammation and accelerates tumor progression in HCC. Inhibition of this signaling pathway may lead to improved intrahepatic inflammatory microenvironment and inhibition of tumor progression. For example, in a mouse model of porphyria induced by feeding a diet containing 3,5-dimethoxycarbonyl-1,4-dihydrocollidine (DDC), which results in the accumulation of porphyrin intermediates and hepatobiliary injury, the formation of liver injury and porphyria is significantly inhibited by in mice deficient in β-catenin or transfected with β-catenin dsiRNA [34]. In a liver injury model induced by carbon tetrachloride (CCL4), Li et al. used the oligopeptide ICF-6 to inhibit the activation of hepatic stellate cells (HSCs). They observed that liver inflammation and fibrosis were alleviated, possibly due to the downregulation of the Wnt/β-catenin signaling pathway [35].

Matrine, a compound with anti-hepatitis B virus effects, anti-liver fibrosis properties, and anti-HCC effects, has been investigated in cell and animal experiments. In cell experiments, matrine was found to suppress HCC cell prolifera-
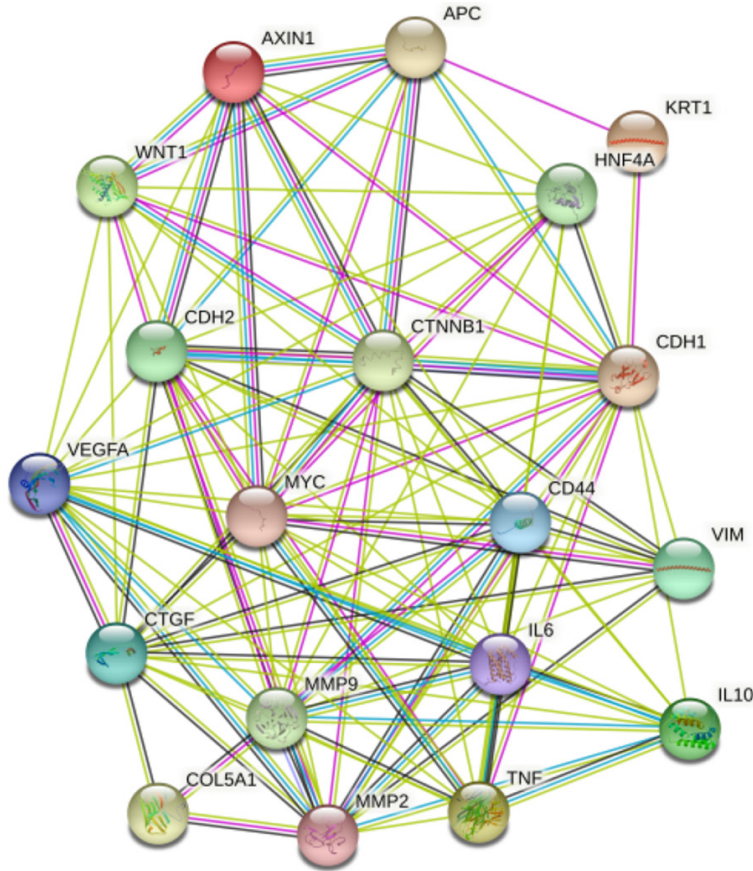
tion, migration, and invasion, as well as down-regulate the Wnt-1/β-catenin pathway. In animal experiments, matrine demonstrated a dose-dependent suppression of the Wnt-1/β-catenin signaling pathway, resulting in reduced levels of liver enzymes such as ALT, AST, GGT, and ALP, and increased levels of ALB. Histologic observations showed that matrine alleviated inflammatory cell infiltration, edema, and degeneration in a pre-hepatocellular carcinoma model, improving overall liver function.

In clinical practice, matrine is used for liver protection in patients with unresectable HCC. We found that matrine administration significantly reduced ALT, AST, TBIL, DBIL, and IBIL levels in patients following initial hospitalization, while patients with BCLC stage C and Child-Pugh stage B disease had better liver protection. However, the improvement in liver function after matrine administration was not as evident after repeated hospitalization, which may be attributed to the loss of many patients in the later stages of the study.

These findings highlight the potential of targeting the Wnt-1/β-catenin signaling pathway, including using compounds like matrine, to improve the intrahepatic inflammatory microenvironment and provide liver protection in HCC. Further research is needed to explore the precise mechanisms and optimize treatment strategies in order to improve the outcomes for HCC patients.

EMT is a process that facilitates development of carcinogenesis within the intrahepatic microenvironment [36]. Abnormal activation of the Wnt/β-catenin signaling pathway in HCC can promote EMT, carcinogenesis, cancer invasion, metastasis, and aggravate liver damage. In experiments using HuH-7 cells, matrine was shown to downregulate the Wnt-1/β-catenin pathway, reverse EMT, and suppress the migration and invasion of HCC. In our pre-hepatocellular carcinoma rat model, activation of the Wnt-1/β-catenin signaling pathway along with a decrease in E-cadherin expression and an increase in N-cadherin and vimentin expression was observed, indicating significant EMT. Matrine significantly inhibited the Wnt/β-catenin signaling pathway, increased the expression of E-cadherin, inhibited expression of N-cadherin and vimentin, reversed EMT, and improved the intrahepatic microenvironment. Dai et al. dis-

Figure 8. Relationships between the Wnt-1/β-catenin signaling pathway, EMT, liver fibrosis, and hepatitis were assessed using STRING software.

E-cadherin, N-cadherin, and vimentin. Moreover, it was found that β-catenin signals through its downstream genes, such as C-myc, CD44, MMP2, and MMP9, to activate inflammatory factors like CTGF-B1, TNF-A, VEGF, IL-6, and IL-10, which further contribute to liver damage (**Figure 8**). Matrine, on the other hand, was able to inhibit the activation of the Wnt-1/β-catenin signaling pathway and alleviate liver inflammation.

However, it is important to acknowledge several limitations of this study. First, while patients with unresectable HCC were enrolled, cancerous liver tissue was not obtained, which could have provided further insights. Additionally, there was a large patient dropout during the later stages of the study, leading to no observed significant difference in the improvement of liver function between the matrine treatment group and the non-treatment group. Secondly, in the rat model, β-catenin exhibited a different staining pattern compared to the strong expression and nuclear localization seen in HCC tissues. This difference could be attributed to the failure to induce typical HCC nodules in the rat model, possibly due to inadequate dosage of the inducer and insufficient treatment duration in the rats. Nonetheless, the study successfully established a model of precancerous overt hepatitis and demonstrated the potential of matrine in alleviating liver damage.

Conclusions

The poor prognosis of HCC patients, in our study, was closely related to the deterioration of the microenvironment in the liver. Furthermore, the Wnt/β-catenin signaling pathway was identified as promoting hepatic microenvironment deterioration while administration of matrine effectively inhibited the Wnt/β-catenin signaling pathway, improving the inflammatory

covered that matrine could effectively inhibit the migration and invasion of HCC and reverse EMT under hypoxic conditions [37]. Similarly, Li found that matrine analogues could inhibit EMT by regulating the AKT/GSK-3β/β-catenin and PI3K/AKT signaling pathways, thereby preventing the occurrence and development of HCC [38].

These findings suggest that matrine can inhibit EMT, suppress cancer cell migration and invasion, and improve the intrahepatic microenvironment by targeting the Wnt/β-catenin signaling pathway. Further research and exploration of the underlying mechanisms will be important in developing effective strategies for the prevention and treatment of HCC.

To further validate the role of the Wnt/β-catenin signaling pathway in promoting EMT and exacerbating liver damage, the STRING tool was utilized to confirm the close relationship between β-catenin expression and the expression of

microenvironment within the liver of HCC patients.

Disclosure of conflict of interest

None.

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Supplementary Table 1. Correlation between the Wnt-1/β-catenin signalling pathway and EMT-related proteins

	E-cadherin		N-cadherin		Vimentin	
	R	P	R	P	R	P
Wnt-1	-0.524	<0.001	0.527	<0.001	0.598	<0.001
β-catenin	-0.409	0.001	0.641	<0.001	0.628	<0.001
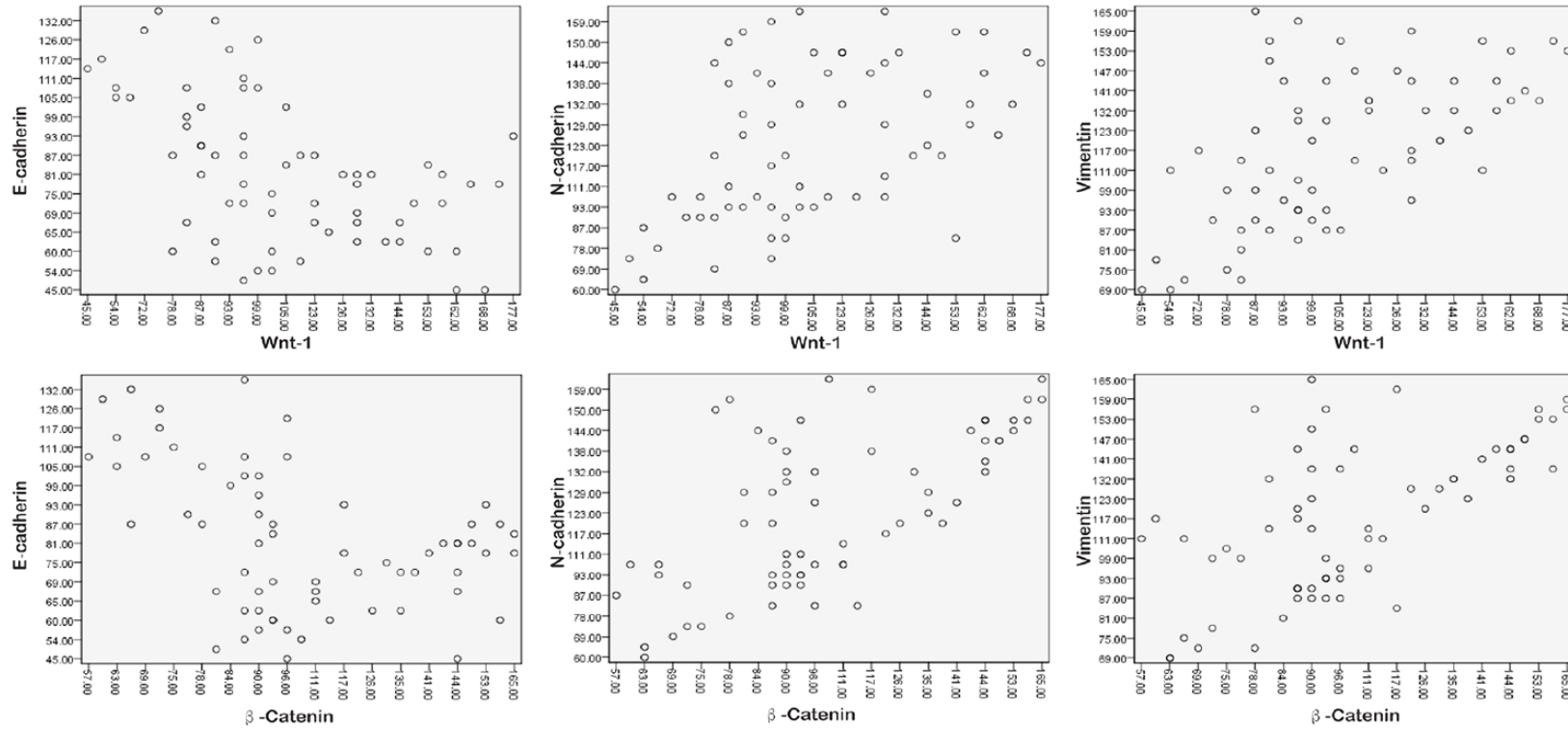
Supplementary Figure 1. Spearman's test was used to analyse the correlations between the expression of Wnt-1/β-catenin signalling pathway components and EMT protein expression.

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Supplementary Table 2. Univariate and multivariate logistic regression analysis of the correlation between Wnt-1 expression and clinical characteristics in 66 patients with hepatic cirrhosis with HCC

Variables	Univariate analysis			Multivariate analysis		
	Wnt-1 - n (%)	Wnt-1 + n (%)	P	OR	95% CI	P
Gender						
Male	25 (78.1)	29 (85.3)	0.45			
Female	7 (21.9)	5 (14.7)				
Age						
<50 (y)	10 (31.3)	15 (44.1)	0.281			
≥50 (y)	22 (68.7)	19 (55.9)				
Tumour diameter (cm)						
>5 cm	17 (53.1)	30 (88.2)	0.004			
≤5 cm	15 (46.9)	4 (11.8)				
Vascular invasion						
With	1 (3.1)	10 (29.4)	0.011			
Without	31 (96.9)	24 (70.6)				
BCLC stage (A/B/C)						
A	15 (46.9)	1 (2.9)	<0.001			
B	15 (46.9)	14 (41.2)		1		
C	2 (6.3)	19 (55.9)		6.365	1.461-27.72	0.014
Histological Differentiation						
Well	9 (28.1)	0 (0)	<0.001			
Moderate	19 (59.4)	5 (14.7)		1		
Poor	4 (12.5)	29 (85.3)		27.48	5.095-148.3	<0.001
Peritumor metastasis						
With	8 (25)	11 (32.4)	0.51			
Without	24 (75)	23 (67.6)				
AFP (ng/ml)						
>400	6 (18.8)	26 (76.5)	<0.001			
100-400	6 (18.8)	4 (11.8)				
≤100	20 (62.5)	4 (11.8)				
HbsAg						
Positive	23 (71.9)	29 (85.3)	0.183			
Negative	9 (28.1)	5 (14.7)				
HbeAb						
Positive	17 (53.1)	9 (26.5)	0.027			
Negative	15 (46.9)	25 (73.5)				
Pres1						
Positive	14 (50)	24 (63.2)	0.268			
Negative	14 (50)	14 (36.8)				
ALBI grade						
1	14 (43.8)	18 (52.9)				
2	17 (53.1)	15 (44.1)	0.754			
3	1 (3.1)	1 (2.9)				

HCC: Hepatocellular carcinoma; BCLC stage: Barcelona clinical liver cancer stage; AFP: Alpha Fetoprotein; ALBI grade: Albumin-bilirubin grade.

Supplementary Table 3. Univariate and multivariate logistic regression analysis of the correlation between β-catenin expression and clinical characteristics in 66 patients with hepatic cirrhosis with HCC

Variables	Univariate analysis			Multivariate analysis		
	β-catenin - n (%)	β-catenin + n (%)	P	OR	95% CI	P
Gender						
Male	30 (81.1)	24 (82.5)	0.861			
Female	7 (18.9)	5 (17.2)				
Age						
<50 (y)	13 (35.1)	12 (41.4)	0.604			
≥50 (y)	24 (64.9)	17 (58.6)				
Tumour diameter (cm)						
>5 cm	21 (56.8)	26 (89.7)	0.008			
≤5 cm	16 (43.2)	3 (29)				
Vascular invasion						
With	1 (2.7)	10 (34.5)	0.002			
Without	36 (97.3)	19 (65.5)				
BCLC stage (A/B/C)						
A	15 (40.5)	1 (3.4)	<0.001	1		
B	19 (51.4)	10 (34.5)		8.56	1.67-43.98	0.01
C	3 (8.1)	18 (62.1)				
Histological Differentiation						
Well	8 (21.6)	1 (3.4)	<0.001			
Moderate	19 (51.4)	5 (17.2)				
Poor	10 (27)	23 (79.3)				
Peritumor metastasis						
With	10 (27)	9 (31)	0.721			
Without	27 (73)	20 (69)				
AFP (ng/ml)						
>400	8 (21.6)	24 (82.8)	<0.001	1		
100-400	8 (21.6)	2 (6.9)		8.24	2.06-32.89	0.003
≤100	21 (56.8)	3 (10.3)				
HbsAg						
Positive	27 (73)	25 (86.2)	0.316			
Negative	10 (27)	4 (13.8)				
HbeAb						
Positive	20 (54.1)	6 (20.7)	0.006			
Negative	17 (45.9)	23 (79.3)				
HBV-Pres1						
Positive	19 (51.4)	21 (72.4)	0.082			
Negative	18 (48.6)	8 (27.6)				
ALBI grade						
1	20 (54.1)	12 (41.4)		1		
2	17 (45.9)	15 (51.7)	0.139	16.38	1.86-144.4	0.012
3	0 (0)	2 (6.9)				

HCC: Hepatocellular carcinoma; BCLC stage: Barcelona clinical liver cancer stage; AFP: Alpha Fetoprotein; ALBI grade: Albumin-bilirubin grade.

Supplementary Table 4. Alanine aminotransferase (ALT) level in rat serum

ALT	Ph 2 week					Ph 4 week					Ph 7 week				
Group (A-D)	Mean	SE	VS			Mean	SE	VS			Mean	SE	VS		
Control (A)	47.34	2.3	B	8.456	0.001	49.04	1.02	B	-21.13	0.001	46.77	1.97	B	-15.11	<0.001
			C	-8.614	0.001			C	-8.39	0.014			C	27.75	<0.001
			D	-19.56	<0.001			D	-10.22	0.001			D	-6.61	0.003
Model (B)	225.63	20.96				222.24	8.13	D	8.219	0.001	209.57	10.58	C	5.06	0.007
													D	10.79	<0.001
Low matrine (C)	206.14	18.29				205.96	18.66	D	3.776	0.02	153.43	3.3	D	11.64	<0.001
High matrine (D)	191.66	22.13				129.56	7.81				82.85	5.09			

Control: saline group, Model: Model group, Low matrine: Low-matrine group, High matrine: High-matrine group. 2 weeks: 2 weeks after partial hepatectomy, 4 weeks: 4 weeks after partial hepatectomy, 7 weeks: 7 weeks after partial hepatectomy.

Supplementary Table 5. Aspartate aminotransferase (AST) level in rat serum

AST	Ph 2 week					Ph 4 week					Ph 7 week				
Group (A-D)	Mean	SE	VS			Mean	SE	VS			Mean	SE	VS		
Control (A)	177.49	3.31	B	-12.62	<0.001	179.1	1.96	B	-7.71	0.002	170.3	2.48	B	-8.47	0.001
			C	-11.57	<0.001			C	-25.2	<0.001			C	-11.34	<0.001
			D	-18.79	<0.001			D	-8.46	0.001			D	-7.256	0.002
Model (B)	371.13	23.03				347.65	21.77	C	2.817	0.048	304.2	15.61	C	3.74	0.02
								D	4.956	0.008			D	6.667	0.003
Low matrine (C)	369.14	25				285.43	3.73	D	6.817	0.002	241.9	5.81	D	6.694	0.003
High matrine (D)	369.11	15.16				235.27	6.34				198.27	2.95			

Control: saline group, Model: Model group, Low matrine: Low-matrine group, High matrine: High-matrine group. 2 weeks: 2 weeks after partial hepatectomy, 4 weeks: 4 weeks after partial hepatectomy, 7 weeks: 7 weeks after partial hepatectomy.

Supplementary Table 6. Albumin (ALB) level in rat serum

ALB	Ph 2 week					Ph 4 week					Ph 7 week				
Group (A-D)	Mean	SE	VS			Mean	SE	VS			Mean	SE	VS		
Control (A)	34.1	1.03	B	-12.15	<0.001	36.32	1.62	B	8.513	0.001	36.3	1.67	B	8.709	0.001
			C	8.232	0.001			C	7.909	0.001			C	7.207	0.002
			D	9.286	0.001			D	7.103	0.002			D	5.591	0.005
Model (B)	17.85	0.86				19.62	1.1	C	-2.821	0.048	21.35	0.4	C	-4.603	0.01
								D	-4.004	0.016			D	-9.098	0.001
Low matrine (C)	18.17	1.64				22.99	0.66				23.94	0.394	D	-4.709	0.009
High matrine (D)	20.38	1.06				24.38	0.44				26.67	0.425			

Control: saline group, Model: Model group, Low matrine: Low-matrine group, High matrine: High-matrine group. 2 weeks: 2 weeks after partial hepatectomy, 4 weeks: 4 weeks after partial hepatectomy, 7 weeks: 7 weeks after partial hepatectomy.

Supplementary Table 7. Alkaline phosphatase (ALP) level in rat serum

ALP	Ph 2 week					Ph 4 week					Ph 7 week				
Group (A-D)	Mean	SE	VS			Mean	SE	VS			Mean	SE	VS		
Control (A)	115.6	15.46	B	-26.5	<0.001	122.5	8.66	B	-11.13	<0.001	125	5.14	B	-13.32	<0.001
			C	-18.1	<0.001			C	-9.843	0.001			C	-8.544	0.001
			D	-20.59	<0.001			D	-16.83	<0.001			D	-11.58	<0.001
Model (B)	438.5	10.89	C	2.977	0.041	408	24.15	D	3.552	0.024	395.57	19.6	D	9.193	0.001
			D	3.892	0.018										
Low matrine (C)	385.94	13.9				399.57	26.79	D	2.928	0.043	355.87	26.43	D	5.439	0.006
High matrine (D)	376.96	11.46				317.93	0.44				209.47	5.08			

Control: saline group, Model: Model group, Low matrine: Low-matrine group, High matrine: High-matrine group. 2 weeks: 2 weeks after partial hepatectomy, 4 weeks: 4 weeks after partial hepatectomy, 7 weeks: 7 weeks after partial hepatectomy.

Supplementary Table 8. γ-glutamyl transpeptidase (GGT) level in rat serum

GGT	Ph 2 week					Ph 4 week					Ph 7 week				
Group (A-D)	Mean	SE	VS			Mean	SE	VS			Mean	SE	VS		
Control (A)	39.97	4.87	B	-12.89	<0.001	34.46	2.73	B	-15.38	<0.001	33.48	2	B	-19.14	<0.001
			C	-12.4	<0.001			C	-15.98	<0.001			C	-18.42	<0.001
			D	-9.542	0.001			D	-15.74	<0.001			D	-7.668	0.002
Model (B)	134.35	5.47	D	3.887	0.018	116.6	4.6				113.84	3.96	D	8.296	0.001
Low matrine (C)	127.02	5.05	D	3.004	0.04	108.53	3.75				106.93	3.45	D	7.248	0.002
High matrine (D)	105.89	4.9				101.24	3.25				68.24	4.07			

Control: Saline group, Model: Model group, Low matrine: Low-matrine group, High matrine: High-matrine group. 2 weeks: 2 weeks after partial hepatectomy, 4 weeks: 4 weeks after partial hepatectomy, 7 weeks: 7 weeks after partial hepatectomy.

Supplementary Figure 2. Hepatoprotective effect of matrine on patients with unresectable HCC. A-D. Differences in liver function between patients with different BCLC stage disease who did and did not receive matrine. All data are expressed as the mean ± SEM. ▲P<0.05 matrine vs. no matrine in BCLC stage C disease. E-G. Differences in liver function between patients with different Child-Pugh grade disease who did and did not receive matrine. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; DBIL: Direct bilirubin; IBIL: Indirect bilirubin. All data are expressed as the mean ± SEM. *P<0.05 matrine vs. no matrine in Child-Pugh grade A disease. ▲P<0.05 matrine vs. no matrine in Child-Pugh grade B disease.