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Original Article

CCM111 prevents hepatic fibrosis via cooperative inhibition of TGF- β , Wnt and STAT3 signaling pathways

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ABSTRACT

CCM111 is an aqueous extract of *Antrodia cinnamomea* (AC) that has exhibited anti-liver fibrosis functions. However, the detailed mechanisms of AC action against liver fibrosis have not been elucidated yet. The present research showed that CCM111 significantly lowered the levels of the hepatic enzyme markers glutamate oxaloacetate transaminase (GOT) and glutamic pyruvic transaminase (GPT), prevented liver damage and collagen deposition, and downregulated TGF- β /Smad signaling in a dose-dependent manner compared with CCl₄ treatment alone. CCM111 markedly inhibited TGF- β , Wnt and STAT3 signaling pathway-regulated downstream genes in the liver by next-generation sequencing. The antifibrotic mechanisms of CCM111 were further demonstrated in HSC-T6 cells. Our data demonstrated for the first time that CCM111 can protect against CCl₄-induced liver fibrosis by the cooperative inhibition of TGF- β , Wnt- and STAT3-dependent proinflammatory and profibrotic mediators, suggesting that CCM111 might be a candidate for preventing and treating chronic fibrotic liver diseases.

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1. Introduction

Liver fibrosis is a chronic liver disease caused by many stimulatory factors; the production of excessive extracellular matrix (ECM) proteins results in the formation of scar tissue that affects liver function and results in liver cirrhosis [1]. During the progression of liver fibrosis, the activation of hepatic stellate cells (HSCs) plays a critical role in the excessive production of ECM proteins, including type I collagen, type III collagen and α -smooth muscle actin (α -SMA); these proteins are recognized as fibrosis markers and are involved in a series of inflammatory and fibrotic processes [2]. Therefore, reducing the activation of HSCs, inducing the apoptosis of HSCs or decreasing the accumulation of ECM proteins are considered antifibrotic methods or methods that reverse liver fibrosis [3].

TGF- β is a key mediator in liver fibrosis through the activation of HSCs and fibroblasts to generate ECM proteins [3]. The TGF- β pathway regulates the activation of the MAPK pathway to promote the activation of HSCs [4]. In addition to TGF- β signaling, signal transducer and activator of transcription 3 (STAT3) also plays an important role in inflammatory responses through inducing a variety of proinflammatory cytokines and chemokines [5,6]. Furthermore, STAT3 inhibition ameliorates CCl₄-induced liver fibrosis *in vivo* [7]. Recently, Wnt signaling has been implicated in liver fibrosis progression since the TGF- β pathway regulates the activation of the Wnt pathway through downregulating the Wnt pathway antagonist Dkk-1 and activating HSCs [8]. Reducing the activation of the Wnt pathway attenuated liver fibrosis progression *in vitro* and *in vivo* [9].

Carbon tetrachloride (CCl₄), a hepatotoxin, is the most commonly used liver carcinogen in models that mimic human liver fibrosis and lesions in mice and rats [10]. Furthermore, CCl₄ induces the activation of Kupffer cells, to produce the proinflammatory cytokines that also promote the activation of HSCs [11].

Antrodia cinnamomea (also known as *Antrodia camphorata* and *Taiwanofungus camphorata*; AC) is a Polyporaceae fungus indigenous to Taiwan. AC is currently used as a food supplement for hepatic protection [12]. Previous studies have indicated that AC from different preparations reduced CCl₄- and TAA-induced liver fibrosis in rats [13–16]. Although a reduction in liver fibrosis markers affected by AC has been observed, the detailed mechanisms of AC action against liver fibrosis have not been elucidated yet. In our previous study, we analyzed the CCM111, an aqueous extract of AC, by HPLC-UV and LC/MS and found that CCM111 inhibited the inflammation response through reducing the activity of STAT3 and NF- κ B pathway [17]. Inflammation is one of the important factor in liver fibrosis progression [18]. In this study, we investigated the effects of CCM111 on the progression of liver fibrosis *in vitro* and *in vivo*.

Our results indicated that CCM111 reduced the progression of CCl₄-induced liver fibrosis through inhibition of the TGF- β 1/Smad signaling pathway and ECM protein expression. Furthermore, we revealed by the next-generation sequencing (NGS) analysis that CCM111 decreased the expression of genes downstream of Wnt, TGF- β , and STAT3, the levels of several proinflammatory cytokines and the production of ECM

proteins. CCM111 significantly inhibited the TGF- β 1-mediated expression phosphorylated of Smad2, Smad3, and STAT3, α -SMA, MMP2 proteins and the translocation of β -catenin in HSC-T6 cells. Therefore, CCM111 has hepatoprotective effects in CCl₄-induced liver fibrosis and has a potential application in clinical interventions.

2. Material and methods

2.1. Animals and treatments

Thirty-two male BltW:CD1 (ICR) mice, weighing 20–22 g, were purchased from BioLASCO (Taipei, Taiwan). The ICR mice were randomly divided into five groups: Group 1: Normal (corn oil only); Group 2: CCl₄ (CCl₄-treated only); Group 3: SIL (CCl₄ plus silymarin 200 mg/kg); Group 4: L-CCM111 (CCl₄ plus silymarin 20 mg/kg); and Group 5: H-CCM111 (CCl₄ plus CCM111 100 mg/kg). The CCl₄-treated animals were administered 40% CCl₄/corn oil (0.1 ml per 100 g body weight) via intraperitoneal (i.p.) injection twice a week for eight consecutive weeks. Animals in the normal group were given an equal volume of corn oil and ddH₂O. The SIL group was given CCl₄ plus silymarin at a dose of 200 mg/kg by oral gavage daily. Two of the CCl₄ groups were given either CCM111 at a dose of either 20 mg or 100 mg per kg body weight by oral gavage daily (Fig. S1).

2.2. Biochemical analysis of liver function

Serum was separated from the animal blood samples by centrifugation at 3000 rpm for 10 min. Serum levels of aspartate transaminase (GOT), alanine transaminase (GPT), triacylglycerol (TG), and total cholesterol (TCHO) were measured on Fujifilm Dri-Chem slides (Fujifilm, Kanagawa, Japan), and the level of each biochemical indicator was determined using a blood biochemistry analyzer (Fujifilm Dri-Chem 3500s; Fujifilm, Kanagawa, Japan). The liver sections (3 μ m) were stained with hematoxylin–eosin (H&E) stain and Sirius Red/Fast Green staining. All protocols for assessing liver function were conducted according to the manufacturer's instructions.

2.3. Chemicals

Carbon tetrachloride (CCl₄) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other chemicals used in this study were of the highest grade of purity and were purchased from Sigma, Inc. (St. Louis, MO, USA).

2.4. Preparation of the crude water extract

The *A. cinnamomea* mycelial culture broth was concentrated under vacuum and freeze-dried to a powder form. The preparation protocol, HPLC-UV and LC/MS analysis were performed in accordance with that of a previous study [17].

2.5. Cells and toxicity assay

HSC-T6 cells were cultured in Waymouth's medium with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-

glutamine in a humidified atmosphere at 5% CO₂ and 37 °C. The cells were plated in 96-well plates and treated with different concentrations of CCM111 (0, 5, 10, 30, 60, 120, and 160 µg/ml). After 24 h, the cell toxicity was determined by AlamarBlue assays (Invitrogen Life Technologies, NY, USA) according to the manufacturer's instructions. The fluorescence values were measured at excitation wavelengths of 530–560 nm and an emission wavelength of 590 nm. All values were measured using a Synergy HT microplate reader (BioTek, VT, USA).

2.6. Western blotting

Liver tissue from each rat was homogenized individually, and total protein was extracted from the tissue using ice-cold Gold lysis buffer. Protein on the gel was separated on the SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The α -SMA and matrix metalloproteinase 2 (MMP2) antibodies were obtained from Abcam (Cambridge, UK). The β -actin antibody was from Oncogene Science (Oncogene Science Inc., Uniondale, NJ). TGF- β 1 was purchased from Sigma, Inc. (Sigma-Aldrich, MO, USA). p-Smad2 (Ser465/467), p-Smad3 (Ser423/425), p-Stat3, Stat3, β -Catenin, Smad3, and TGF- β 1 antibodies were purchased from Cell Signaling Technology (Cell Signaling, MA, USA). α -SMA and MMP2 antibodies were purchased from Abcam (Abcam, MA, USA).

2.7. Hematoxylin–eosin staining and Sirius Red/Fast Green staining

For histological examination, mouse liver sections were fixed in 10% formalin for 48 h and embedded in paraffin. The sections (4 mm) were stained with hematoxylin–eosin stain (H&E) and Sirius Red/Fast Green stain and analyzed under a microscope.

2.8. Next-generation sequencing (NGS)

mRNA profiling was performed by NGS. Total RNA was isolated from the mouse liver sections with TRIzol reagent (Invitrogen, USA). The total RNA was sent to Genomics BioSci & Tech. (Genomics, New Taipei City, Taiwan) for RNA-seq analysis. The criteria for the Gene Ontology (GO) analysis were reads per kilobase per million mapped reads (RPKM) > 3.5 and coverage > 50% [19]. Read counts obtained from NGS were normalized as RPKM to compare the gene expression levels of mRNA across samples. The relative fold change of the gene expression was determined by the normalized reads counts, RPKM. The pathways analysis was performed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) [20].

2.9. Subcellular fractionation

The cytoplasmic and nuclear fractions were prepared by the Buffer A (1 mM DTT pH7.9, 0.1 mM EDTA, 10 mM KCL, 10 mM HEPES, and proteinase inhibitor), 9% NP-40, and Buffer B (400 mM NaCl pH7.9, 20 mM HEPES, 1 mM EDTA, 1 mM DTT, and proteinase inhibitor) as previous protocol [17]. The cytoplasmic and nuclear fractions concentration was determined

by the BCA assay kit (Pierce). After collection, the fractions were performed for western blotting.

2.10. Statistical analysis

Data are expressed as the mean $\pm$ standard deviation. All statistical analyses were generated using SigmaPlot 10.0 (SYSTAT, CA, USA). Image analysis was performed using ImageJ 1.47 (NIH, Bethesda, MD, USA).

3. Results

3.1. Effects of CCM111 on CCl₄-treated mice

This experiment was performed to investigate the effects of CCM111 on liver fibrosis progression. The body weights of the CCl₄-treated mice were lower than those of the control animals at the end of the study (8 weeks), but there was no significant difference in body weight between the CCl₄-treated and the control animals (Fig. S2). The kidney weights of the mice in the CCl₄-treated alone group were significantly higher than those of the control animals. The oral administration of silymarin (SIL) at 200 mg/kg was the antifibrosis control for this experiment. Treatment with CCl₄ plus SIL decreased the kidney and spleen weight compared with treatment with CCl₄ only. The groups receiving oral administration of CCM111 at 20 (L-CCM111) or 100 (H-CCM111) mg/kg exhibited a slightly higher kidney weight than the control group, but the difference was not significant. The L-CCM111 group showed a significantly lower spleen weight compared with the CCl₄-only group (Table 1). The GOT, GPT and TCHO levels in serum and the liver injury markers in the CCl₄-only group were higher than those in the control group, but no difference was observed in the serum TG levels. The CCM111 and SIL treatment groups demonstrated significantly reduced serum GOT and GPT levels, but there were no effects on the serum TCHO or TG levels (Table 2). These data suggested that CCM111 could reduce CCl₄-induced liver injury.

The effects of CCl₄-treated liver injury were evaluated by histopathological examination of the liver sections. The liver samples were stained with Sirius Red/Fast Green stain to examine the collagen deposition and with hematoxylin–eosin

Table 1 – Relative organ weight of CCl₄-treated rats with or without CCM111.

Groups	Relative organ weight (g/of bw)		
	Liver	Kidney	Spleen
Control	5.09 $\pm$ 0.32	1.84 $\pm$ 0.27	0.27 $\pm$ 0.06
CCl ₄	5.69 $\pm$ 0.76	2.10 $\pm$ 0.14 [#]	0.49 $\pm$ 0.12 [#]
SIL	5.53 $\pm$ 0.93	2.03 $\pm$ 0.24	0.39 $\pm$ 0.10
L-CCM111	5.57 $\pm$ 0.35	2.17 $\pm$ 0.43	0.33 $\pm$ 0.05 [*]
H-CCM111	5.49 $\pm$ 0.70	2.14 $\pm$ 0.35	0.38 $\pm$ 0.11

CCl₄ was intraperitoneally given at a dose of 40% twice per week for 8 weeks to each group except control group. The data represent the mean $\pm$ SD of six mice. [#]Significantly different from the control group, *P* < 0.05. ^{*}Significantly different from the group treated with CCl₄ alone, *P* < 0.05.

Table 2 – Effect of CCM111 on the serum parameters in the CCl₄-treated mice.

Groups	Activity			
	GOT (U/L)	GPT (U/L)	TG (mg/dl)	TCHO (mg/dl)
Control	89.33 ± 47.9	26.83 ± 8.52*	213.50 ± 46.37	199.17 ± 40.51
CCl ₄	323.83 ± 188.09 [#]	262.50 ± 199.21 [#]	232.17 ± 74.99	133.83 ± 26.12 [#]
SIL	70.60 ± 37.87*	54.60 ± 52.64*	202.50 ± 43.54	125.50 ± 32.46
L-CCM111	71.33 ± 9.93*	71.33 ± 9.93*	176.67 ± 57.69	109.83 ± 19.55
H-CCM111	53.00 ± 13.88*	53.00 ± 13.88*	191.00 ± 39.22	125.20 ± 28.60

CCl₄ was intraperitoneally given at a dose of 40% twice per week for 8 weeks to each group except control group. The data represent the mean ± SD of six mice. [#]Significantly different from the control group, *P* < 0.05. *Significantly different from the group treated with CCl₄ alone, *P* < 0.05.

(H&E) stain to examine the extent of tissue damage. Compared with the control group, the CCl₄-only group showed substantial inflammation and collagen deposition. CCM111 or SIL administration markedly reduced collagen deposition (Fig. 1A and B). These results suggested that CCM111 alleviated liver inflammation and fibrosis progression.

3.2. CCM111 represses the progression of liver fibrosis through reducing activation of the TGF- β pathway

To further examine the mechanism of CCM111 on CCl₄-induced liver fibrosis, we analyzed the expression of ECM proteins and proteins in several signaling pathways associated with liver fibrosis. The CCl₄-only group had a significantly increased expression of α -SMA and MMP2 compared with the

control group, whereas CCM111 treatment significantly reduced the expression of these proteins. These results indicated that CCM111 suppressed the expression of ECM proteins in a dose-dependent manner. CCl₄ significantly induced the levels of phosphorylated Smad2, Smad3, and TGF- β 1, whereas CCM111 significantly reduced these levels in a dose-dependent manner (Fig. 2). All these data indicated that CCM111 suppressed liver fibrosis progression through inhibiting the activity of the TGF- β pathway.

3.3. CCM111 inhibits the expression of inflammation- and fibrogenesis-related genes

To further investigate the mechanism of CCM111 in liver fibrosis, we analyzed liver sections (Normal, CCl₄, H-CCM111)

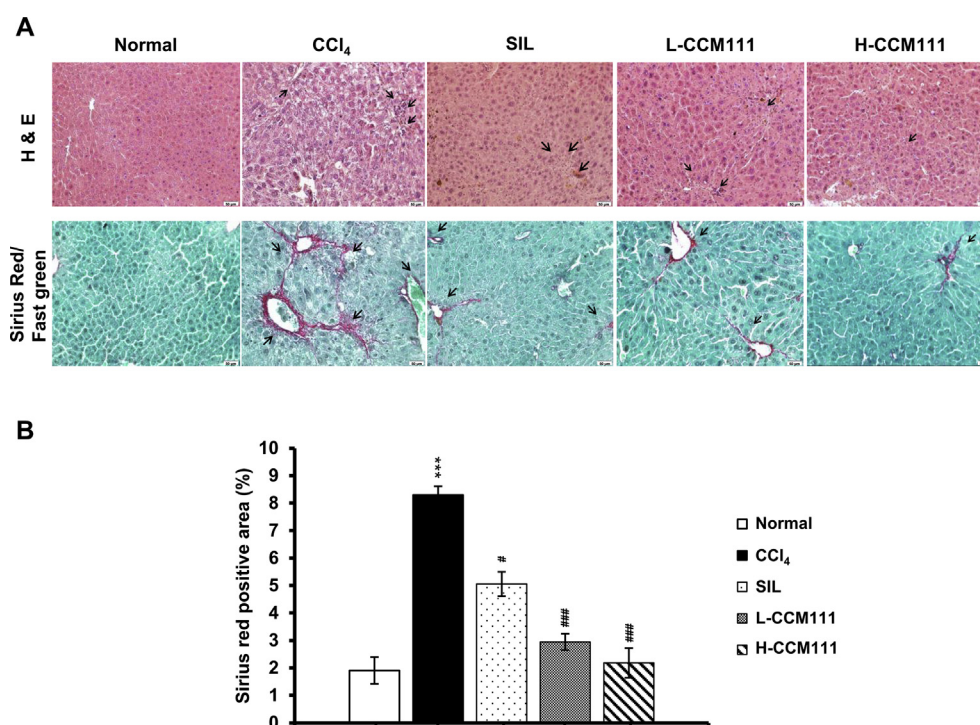


Fig. 1 – CCM111 suppressed the liver morphology in CCl₄-treated animals. (A) Images of liver tissue sections stained separately with H&E stain and Sirius Red/Fast Green stain. The sections were imaged at 200 $\times$ magnification. **(B)** Quantification of Sirius Red/Fast Green staining. All values are expressed as the mean $\pm$ SD (*n* = 6). Student's *t*-test: **P*-value < 0.05, ***P*-value < 0.01, ****P*-value < 0.001 for Normal vs. CCl₄. #*P*-value < 0.05, ##*P*-value < 0.01, ###*P*-value < 0.001, for CCl₄ vs. SIL, L-CCM111, or H-CCM111, respectively.

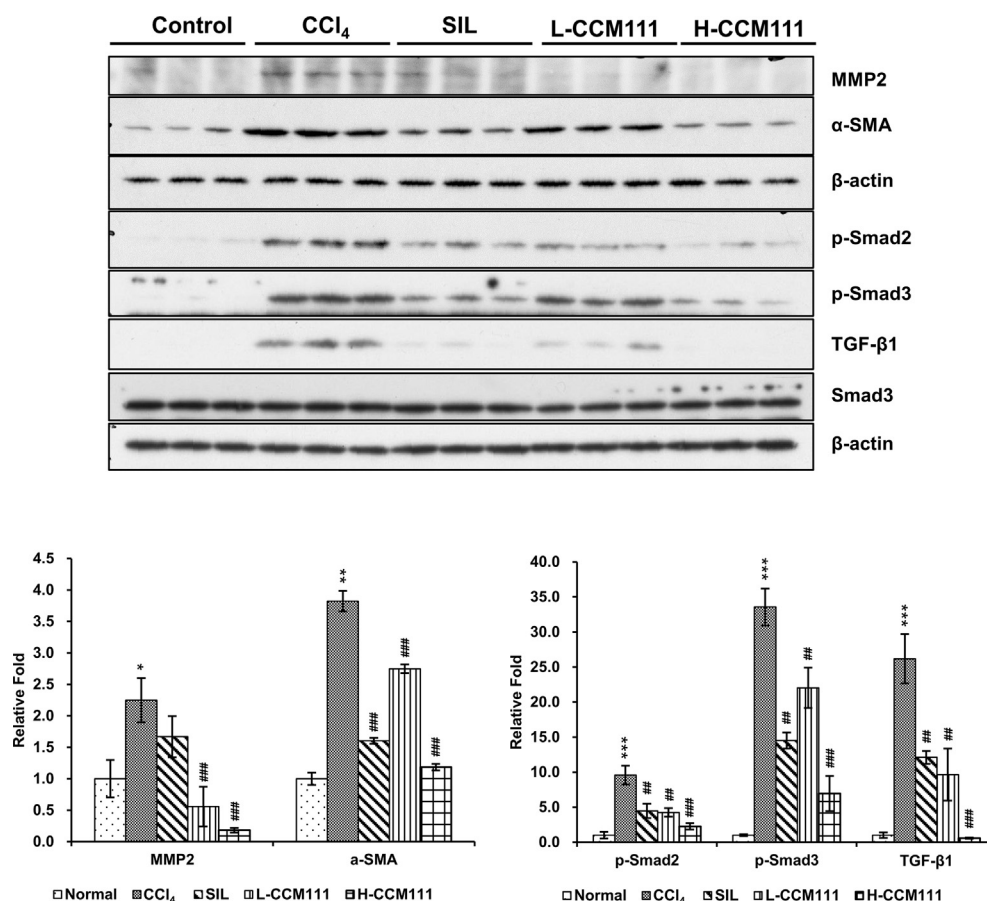


Fig. 2 – CCM111 reduced TGF-β1-induced expression of α-SMA and MMP2 and activation of the TGF-β signaling pathway in CCl₄-treated animals. Total liver cell lysates were analyzed for α-SMA, MMP2, p-Smad2, p-Smad3, TGF-β1, and Smad3 protein expression by western blot. β-Actin was the loading control. The quantification data are reported as the mean ± standard deviation of three independent experiments. Student's t-test: *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001 for Normal vs. CCl₄. #P-value < 0.05, ##P-value < 0.01, ###P-value < 0.001, for CCl₄ vs. SIL, L-CCM111, or H-CCM111, respectively.

by next-generation sequencing (NGS). We analyzed the functions of genes below the cutoff point (coverage: 50, RPKM: 3.5) according to GO terms (Fig. S3). We found that the metabolic and immune responses of the CCl₄ group were different from those of the normal group, which suggested that CCl₄ affected these functions. The response of the H-CCM111 group was similar to that of the normal group, which indicated that CCM111 may reverse the liver function in terms of gene expression levels (Fig. S4A and B).

We further analyzed the effects of CCM111 on the pathways important to liver fibrosis progression. We found that CCl₄ alone induced the some gene expressions of TGF-β, cytokines, and ECM proteins and that CCM111 reduced the expression of these genes (Fig. 3A–D). The Wnt pathway plays important roles in the development, regeneration and metabolism of the liver [21]. The Wnt pathway has also been reported to be involved in the activation of HSCs and the progression of fibrosis [22]. We also found that CCM111 reduced the some gene expressions of several downstream genes in the Wnt pathway (Fig. 3E). Previous reports indicated that the STAT3 pathway is involved in the inflammatory response and the progression of

liver fibrosis [23,24]. We also found that CCl₄ induced the expressions of several downstream genes of STAT3 pathway such as TIMP1, which is an important factor for the survival of HSCs and has a positive correlation with liver fibrosis (Fig. 3F). These results suggested that CCM111 repressed the progression of liver fibrosis and inflammatory responses through down-regulating the TGF-β, Wnt, and STAT3 pathways.

3.4. Effects of CCM111 on TGF-β1-induced MMP2, α-SMA and TGF-β pathway activation in HSC-T6

We used the HSC-T6 cell line to investigate the effects of CCM111 on the activation of HSCs. CCM111 did not induce toxicity in the HSC-T6 cells (Fig. 4A). We then tested the effects of CCM111 on the TGF-β1-induced TGF-β pathway and fibrosis markers α-SMA and MMP2. CCM111 significantly inhibited the TGF-β1-mediated expression of phosphorylated Smad2, Smad3, α-SMA, and MMP2 in a dose- and time-dependent manner (Fig. 4B and C).

To further investigate the candidate compounds of CCM111, we chromatographed CCM111 into 7 fractions (F1F7).

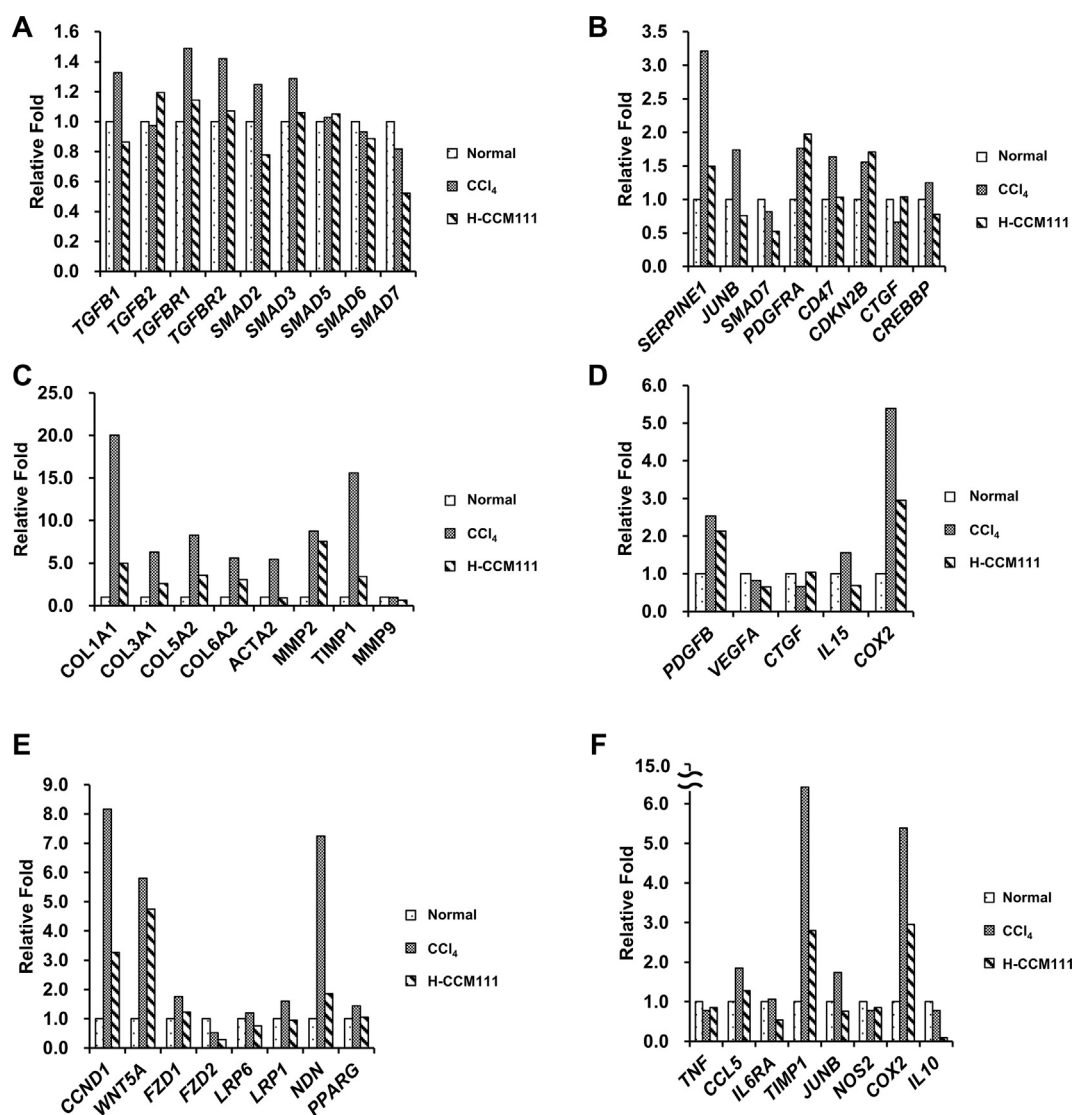


Fig. 3 – Assessment of genes differentially expressed in liver samples. Liver samples were analyzed by next-generation sequencing. The fold change was calculated by the RPKM of each group relative to the normal group. (A) TGF- β pathway genes. (B) Downstream genes of the TGF- β pathway. (C) ECM genes. (D) Cytokines. (E) Downstream genes of the Wnt pathway. (F) Downstream genes of the STAT3 pathway.

We examined the toxicity of these fractions by toxicity assay (Fig. S5A). We found that F4 and F5 fractions of CCM111 decreased the TGF- β 1-mediated the expression of α -SMA (Fig. S5B). We have analyzed the compounds of F4 by LC/MS in the previous study [17]. The results suggested that F4 and F5 are the two major fractions for alleviation of liver fibrosis.

3.5. Effects of CCM111 on TGF- β 1-induced STAT3 and Wnt pathways activation in HSC-T6

Our NGS analysis revealed that CCM111 reduced the expressions of some genes downstream of STAT3 and Wnt pathways (Fig. 3D and E). Therefore, we examined if CCM111 can affect TGF- β 1-mediated the activity of STAT3 and Wnt pathways by western blots. As consistent with previous results, we found that CCM111 reduced the TGF- β 1-mediated expression of

phosphorylated STAT3 and the translocation of β -catenin in HSC-T6 cells (Fig. 5A and B). The results suggested that CCM111 reduced liver fibrosis progression through repressing the activation of HSCs.

4. Discussion

CCM111 alleviated the liver damage in the CCl₄-only group by increasing the relative spleen and body weights, decreasing the levels of the liver damage markers GOT and GPT, and alleviating the inflammatory responses and collagen deposition in the liver sections (Tables 1 and 2, Fig. 1, and Fig. S2). We also investigated the CCl₄-treated liver sections and found that CCM111 also reversed the CCl₄-induced gene expression profile; these results imply that CCM111 may reverse the

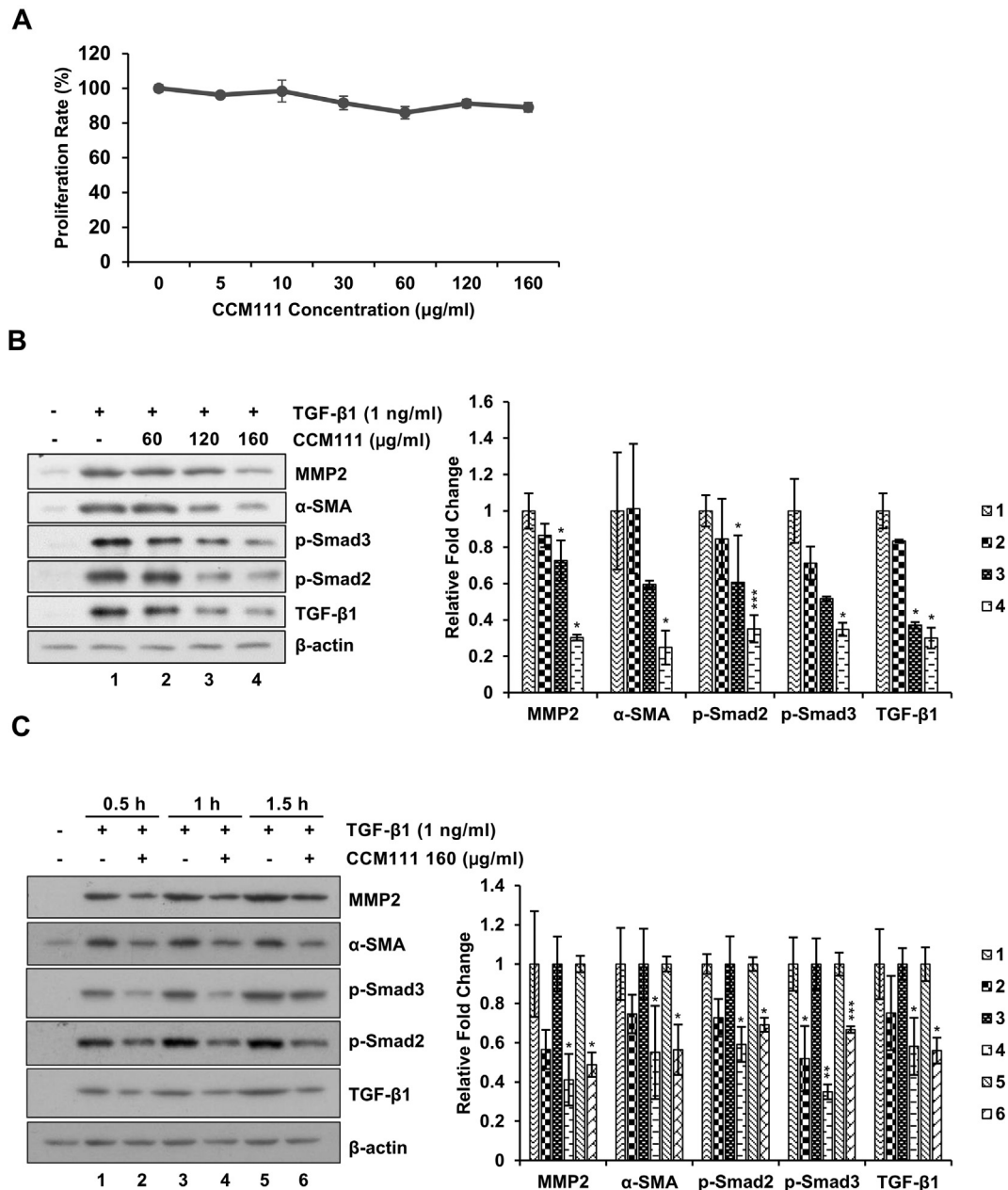


Fig. 4 – CCM111 reduced TGF-β1-induced expression of α-SMA and MMP2 and activation of the TGF-β signaling pathway in HSCs. (A) The cells were treated with CCM111 (0, 5, 10, 30, 60, 120 or 160 μg/ml) for 24 h. Proliferation was detected by the AlamarBlue assay. (B) The cells were starved for 24 h and treated with TGF-β1 (1 ng/ml) and CCM111 (0, 60, 120 or 160 μg/ml) for 1 h. (C) The cells were starved for 24 h and treated with TGF-β1 (1 ng/ml) and CCM111 (160 μg/ml) for 0.5, 1 and 1.5 h. Total cell lysates were prepared for western blot analysis to detect protein levels. β-actin was the loading control. The quantification data are reported as the mean ± standard deviation of three independent experiments. Student's t-test was performed to compare the CCM111 group with the TGF-β1-only group. *P-value < 0.05, **P-value < 0.01 and ***P-value < 0.001.

progression of fibrosis (Fig. S4). We analyzed genes important in fibrogenesis and found that CCM111 markedly down-regulated the expression of proinflammatory cytokines and chemokines and the expression of TGF-β, Wnt, and STAT3-regulated genes (Figs. 2 and 3). Furthermore, we performed the fractions of CCM111 by chromatography and found that F4 and F5 reduced the TGF-β expression of α-SMA (Fig. S5B). We

suggested that CCM111 has potential as an antifibrotic agent and has potential applications in clinical interventions.

HSCs play an important role in the progression of fibrogenesis. However, activated HSCs are a major source of ECM proteins [25]. The TGF-β/Smad signaling pathway, including the Smad and TGF-β1 families, is one of the major pathways that activate HSCs and induce expression and deposition of

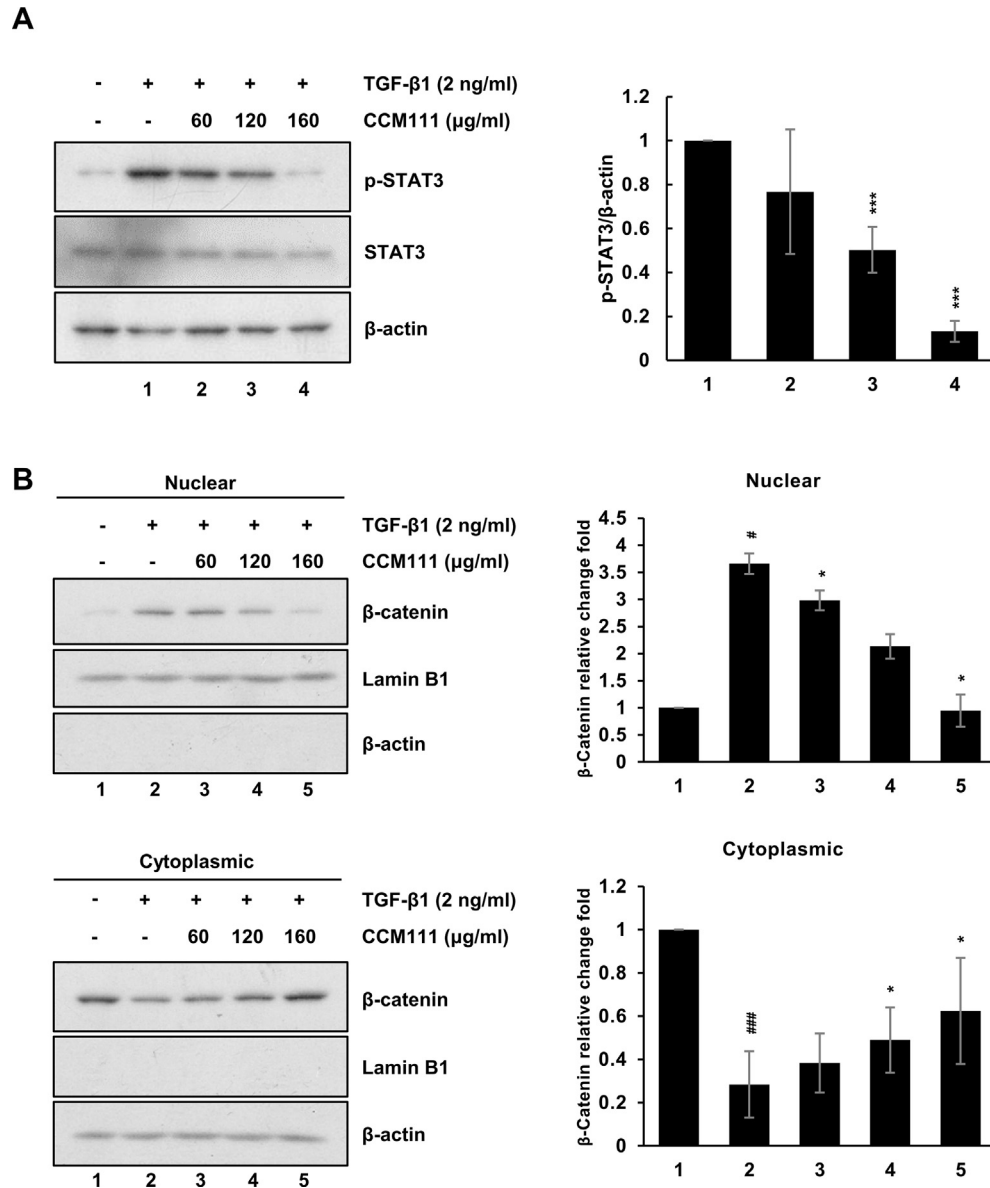


Fig. 5 – CCM111 reduced TGF-β1-induced the activation of the Wnt and STAT3 signaling pathways in HSC cells. The cells were starved for 24 h and treated with TGF-β1 (2 ng/ml) and CCM111 (0, 60, 120, 160 μg/ml) for 24 h. (A) The protein levels of p-STAT3 and STAT3 were detected. (B) The protein levels of β-catenin expression were detected. β-actin was used as the cytosol internal control, and laminB1 was used as the nuclear internal control. The quantification data are reported as the mean standard deviation of three independent experiments. Student's t-test compared the CCM111 treatment with the TGF-β1 only group. *P-value < 0.05, **P-value < 0.01 and ***P-value < 0.001. The control group vs. TGF-β1 only group. #P-value < 0.05, and ###P-value < 0.001.

collagen and the expression of fibrogenesis-related cytokines and chemokines [26]. We found that CCM111 reduced TGF-β pathway activity and the expression of genes downstream in the TGF-β pathway (Fig. 3B and C). However, CCM111 did not influence the expression of genes in the TGF-β pathway (Fig. 3A); rather, CCM111 specifically inhibits the expression of phosphorylated Smad proteins (Fig. 4B and C).

The major biological functions of matrix metalloproteinase (MMP) are degrading ECM proteins and promoting wound repair [27]. However, MMP-2 has been found to be involved in hepatic fibrogenesis [28]. Our study reported that CCM111 reduced the TGF-β1-induced expression of MMP2 and the

CCl₄-induced expression of MMP2 in ICR mice (Figs. 2 and 4B and C). Some potential antifibrotic targets may include the induction of HSCs apoptosis and the elimination of profibrogenic pathways; inducing apoptosis in activated HSCs appears to drive the regression of liver fibrosis. The inactivation of hepatic stellate cells to prevent further liver damage is a promising antifibrotic strategy.

Abnormal activation of the canonical Wnt pathway plays an important role in the activation of HSCs and the differentiation of myofibroblasts [29]. Treating with antagonists of Dickkopf1 (DKK1) or reducing the level of β-catenin can reduce the activation of HSCs [22]. In our study, we found that

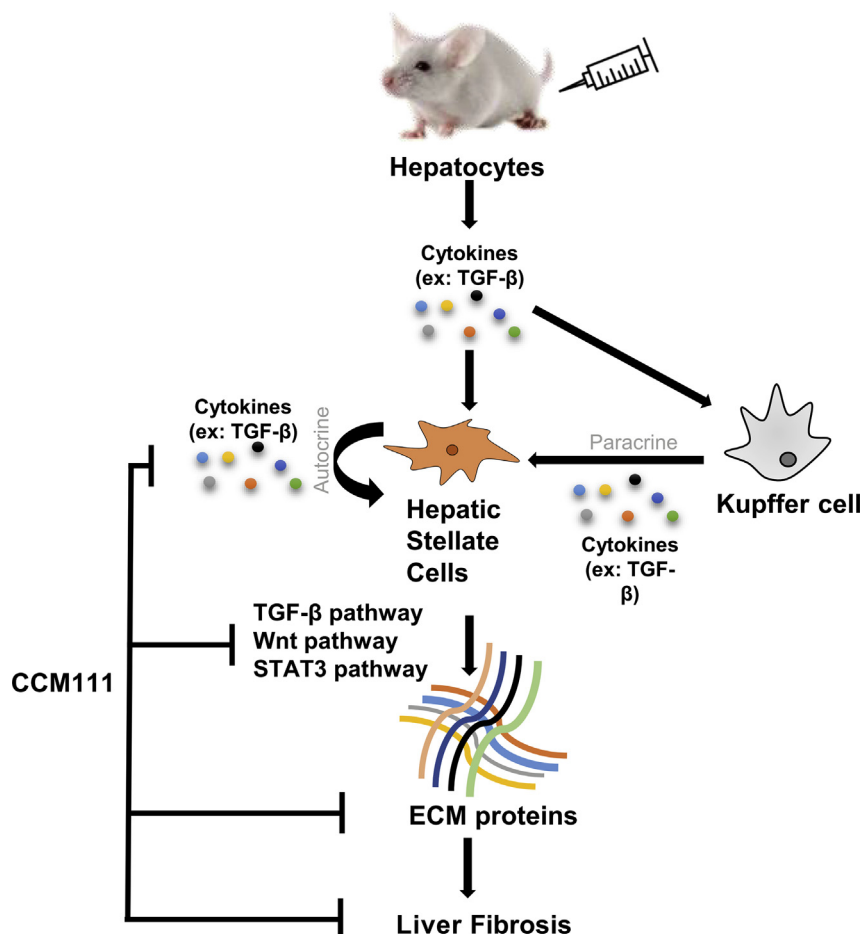


Fig. 6 – CCM111 remarkably attenuated the severity of CCL₄-induced liver fibrosis through the inhibition of the TGF-β1/Smad, Wnt, and STAT3 pathways and the expression of ECM proteins and cytokines. CCM111 exhibited hepatoprotective effects in CCL₄-induced experimental fibrosis, implying its potential application in clinical intervention.

CCM111 reduced the expression of genes downstream of the Wnt pathway and the translocation of β -catenin (Figs. 3E and 5B). This result implied that CCM111 represses the progression of liver fibrosis through reduced Wnt pathway activation.

Activation of the STAT3 pathway has been reported in a variety of diseases such as cancer, inflammation, liver injury and liver fibrosis [30]. The inhibition of inflammation alleviates liver injury [31]. However, some reports indicated that STAT3 pathway activation reduced fibrosis through preventing hepatocellular damage [32]. In our study, we found that the expression of genes downstream of STAT3 was lower in the H-CCM111 group than in the CCL₄ group, which implied that CCM111 may reduce the activation of the STAT3 pathway (Fig. 3F). We also found that CCM111 reduced the expression of phosphorylated STAT3 in HSC cells (Fig. 5A). This result was consistent with that of our previous study, in which we found that CCM111 reduced the activity of the STAT3 pathway in RAW264.7 macrophages [17].

F4 and F5 fractions of CCM111 could reduce the TGF- β -mediated expression of α -SMA (Fig. S5A and B). We perfused the LC/MS to analysis the compounds of F4 in our previous studies [17]. The components of F4, antroquinonol B, 14-deoxy-11,12-didehydroandrographolide, and antrocinna-nomin C, have been reported on the anti-inflammation

functions [33–35]. In addition, 14-deoxy-11,12-didehydroandrographolide also has been indicated the anti-fibrosis ability in murine renal mesangial cell lines [36]. These studies supported our finding that compounds in F4 and F5 of CCM111 may play an important role to alleviate liver fibrosis.

In summary, our study provided novel insight into the mechanism by which AC ameliorates liver fibrosis. Our results suggested that CCM111 reduces the activity of TGF- β signaling pathways through inhibiting the levels of phosphorylated Smad2 and Smad3 and reducing the expression of α -SMA, MMP2, and TGF- β 1. CCM111 also decreased the activity of STAT3 and Wnt pathways through reducing the expression of phosphorylated STAT3 and the translocation of β -catenin in HSC-T6. CCM111 decreases CCL₄-induced expression of ECM proteins by downregulating the activation of the TGF- β , Wnt, and STAT3 pathways and by reducing inflammatory responses (Fig. 6).

Conflicts of interest

The authors have declared no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data related to this article can be found online at <https://doi.org/10.1016/j.jfda.2018.09.008>.

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