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ORIGINAL RESEARCH

Sorafenib Attenuates Fibrotic Hepatic Injury Through Mediating Lysine Crotonylation

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Background: Liver fibrosis is an independent contributor of chronic liver diseases, and regressing liver fibrosis is considered a potential therapeutic target for chronic liver diseases. We aimed to explore the effects and mechanism of sorafenib in liver fibrosis.

Methods: Male Sprague Dawley (SD) rats were subjected to subcutaneous injection of carbon tetrachloride (CCl_4) for 8 weeks to induce liver fibrosis and then treated with sorafenib. The degree of liver fibrosis was analyzed by hematoxylin–eosin (H&E) staining, Masson staining, and Picrosirius red (PSR) staining. Serum biochemical indexes were detected by fully automatic biochemical analyzer or enzyme-linked immunosorbent assay (ELISA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to detect the expression of pro-fibrotic genes. Immunohistochemical staining and Western blotting were carried out to evaluate the levels of lysine crotonylation.

Results: Liver index was reduced with oral sorafenib in CCl_4-induced rats. Serum liver function (alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL)) and fibrosis indicators (type III procollagen (PC-III), hyaluronic acid (HA), and laminin (LN)) were attenuated with sorafenib treatment. Sorafenib improved the hepatic structure and fibrotic progression. The expression of fibrosis-related genes was remarkely reduced with sorafenib treatment. Meanwhile, sorafenib inhibited α-SMA and collagen I cumulation induced by CCl_4 injection. Besides, protein lysine crotonylation especially the crotonylated H2BK12 (H2BK12cr) and crotonylated H3K18 (H3K18cr) were reversed by sorafenib, which were decreased in response to CCl_4 treatment. Spearman correlation analysis shown lysine crotonylation expression was negatively correlated with serum fibrotic indicators. Conversely, crotonylation-regulated enzymes, which negatively regulate protein crotonylation, were increased in response to CCl_4 treatment, while sorafenib reduced their expression.

Conclusion: Sorafenib exerts significant anti-fibrotic effects through mediating crotonylation-regulated enzymes and protein crotonylation in fibrotic rats.

Keywords: liver fibrosis, sorafenib, crotonylation, epigenetic regulation

Introduction

Liver fibrosis is a wound healing reaction caused by viral hepatitis, alcoholic liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), etc.[1] The long term of hepatic fibrosis could develop into cirrhosis and even hepatocellular carcinoma (HCC), which threatens life span and life quality of people.[2] Chronic liver injury leads to the overdeposition of extracellular matrix (ECM) in the liver, the sign of liver fibrosis. The hepatic stellate cells (HSCs) are the primary cells producing extracellular matrix (ECM).[3] Various pathological stimuli activate quiescent hepatic stellate cells (HSCs) to generate superabundant ECM. Moreover, sustained inflammatory reaction and fibrotic-related signaling pathway are involved in the activation of liver fibrogenesis.[4] Therefore, interrupting associated targets to remove superfluous ECM is a promising strategy to regress liver fibrosis.[4] However, there are no effective anti-fibrotic drugs in clinical. More researches need to explore the underlying mechanism in liver fibrosis to enhance our understanding of liver fibrosis.

Lysine crotonylation is a novel posttranslational modification (PTM) of proteins, initially identified on histones and subsequently found also on non-histones.[5,6] Lysine crotonylation is widely involved in the pathophysiological processes such as cardiac remolding,[7,8] endoderm differentiation,[9] DNA repair,[10] and spermatogenesis.[11] Accumulating evidence has found crotonylation-regulated enzymes were directly or indirectly involved in regulating protein crotonylation.[12] For example, class I histone

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deacetylases (HDACs) including HDAC1 and HDAC3 are major histone decrotonylases to diminish protein crotonylation.[13,14] Besides, chromodomain Y-like protein (CDYL) serves as crotonyl-CoA hydratase to regulate the levels of crotonyl-CoA, the direct donor of protein crotonylation, to negatively modulate protein crotonylation.[10,11,15] Surprisingly, a great deal of crotonylated proteins is identified in normal mouse liver. These proteins are mainly distributed in the nucleus and cytoplasm participating in metabolism-related biological processes.[16] Moreover, the level of lysine crotonylation is lower in hepatocellular carcinoma and increasing lysine crotonylation is effective to prevent proliferation and migration of hepatocellular carcinoma cells.[17] Thus, lysine crotonylation might take part in the initiation and progression of liver fibrosis.

Sorafenib, a multikinase inhibitor, is approved for treating hepatocellular and renal cell carcinomas and reportedly possesses hepatoprotective and anti-fibrotic roles.[18] Sorafenib prevents liver fibrosis supported by inhibiting the activation of hepatic stellate cells (HSCs), repressing epithelial-mesenchymal transition (EMT), and suppressing transforming growth factor β1 (TGFβ1) signaling pathway.[19–21] Recently, evidences have demonstrated sorafenib could induce hepatic stellate cells (HSCs) ferroptosis to retard liver fibrosis.[22] In addition, epigenetic modifications are critically involved in the liver fibrosis. The well-studied protein acetylation has been demonstrated to participate in the modulating liver fibrosis.[23,24] Nevertheless, the roles of lysine crotonylation underlying the liver fibrosis and sorafenib treatment remain indistinct.

In the present study, we found sorafenib significantly prevented the progression of CCl_4-induced liver fibrosis. The lysine crotonylation levels were decreased in CCl_4-treated rats, especially the crotonylated H2BK12 (H2BK12cr) and crotonylated H3K18 (H3K18cr), while sorafenib promoted the abundance of lysine crotonylation. Meanwhile, protein lysine crotonylation expression was negatively correlated with serum fibrotic indicators. Collectively, these findings might uncover the mystery of lysine crotonylation in liver fibrosis.

Materials and Methods

Animals

Thirty-eight male Sprague Dawley (SD) rats (160–200g) were purchased from Chengdu Dossy Experimental Animals Co., Ltd. (Chengdu, China). The rats were housed in a standard 12 h light–dark cycle and were allowed for free access to water and food. All animal protocols were approved by the Committee on Laboratory Animal Care of Chengdu University of Traditional Chinese Medicine and the rat experiments were performed according to the guidelines of the National Institutes of Health (USA).

Animal Treatments

The rats were randomly divided into three groups: with 8 in the control group (Ctr); 14 in the CCl_4 group (CCl_4); 16 in the CCl_4 plus sorafenib group (CCl_4+S). Liver fibrosis was induced by subcutaneous injection of 50% CCl_4 olive oil twice a week for 15 weeks. Oral sorafenib (1mg/kg, dissolved in Cremophor EL) was performed every day begin at ninth week for 7 weeks.[25] Finally, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the serum samples were collected from the abdominal aortic blood of the rats by centrifuging at 3500 r/min, 4 °C for 10 min, and then stored at −80 °C for further analyses. Rats were then euthanized and the fresh liver was collected to dissect into pieces for further analyses.

Histopathological staining

Four percent paraformaldehyde was used to fix the rats liver. Paraffin was implemented to embed the liver and then embedded liver was dissected into 5μm section. Hematoxylin-eosin (H&E) was performed to analyze the structure of the liver. Masson staining and Picrosirius red (PSR) staining were carried out to detect the collagen deposition. Histopathological staining results were analyzed through Image-Pro Plus 6.0.

Serum Biochemistry Analysis

A fully automatic biochemical analyzer (Chemray 800, Rayto) was used to detect the level of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) in the blood according to the manufacturer's instructions. The concentration of serum hyaluronic acid (HA), laminin (LN), and type III procollagen (PC-III) were detected via enzyme-linked immunosorbent assay (ELISA) according to the instructions.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA of rat liver was collected through TRIzol (Invitrogen), then the first-strand cDNA Synthesis kit (New England Biolabs, #M0277) was used to synthesize the first-strand cDNA. The relative mRNA levels of indicated genes were performed with the SYBR Green Master Mix (TaKaRa, #R820A) by qRT-PCR.[26] Sequences for qRT-PCR primers are presented in Table 1.

Immunohistochemical Staining Analysis

Xylene was used to deparaffinize the paraffin-embedded liver section, then the sections were rehydrated with gradient ethanol. Three percent hydrogen peroxide (H_2O_2) was used to quench endogenous peroxidases for 30min. Antigen retrieval was performed with EDTA-Tris (pH 8.0) to boil the section for 3min. The section was blocked with goat serum and incubated with indicated primary antibodies H2BK12cr (PTM Biolabs, PTM-509), H3K18cr (PTM Biolabs, PTM-517), pan anti-Kcr (PTM Biolabs, PTM-501), α-SMA (Servicebio, GB111364) and collagen I (Servicebio, GB11022) overnight at 4°C. Eventually, diaminobenzidine (DAB) staining was enforced and counterstained with hematoxylin.

Western Blotting

RIPA lysis buffer (Beyotime, #P0013B) supplemented with protease inhibitor cocktail and phosphatase inhibitor was used to extract liver protein according to previous published paper.[26] Briefly, homogenized hepatic tissues were sonicated and centrifuged at 4 °C for 15 minutes to obtain the supernatant for Western blotting. Then, the BCA Protein Assay Reagent (Thermo, # 23227) was used to detect the content of each supernatant protein. The supernatant protein was mixed with SDS-PAGE Sample Loading Buffer (Beyotime Biotechnology, # P0015) and boiled in water to denature protein. Twenty micrograms of protein was subjected to SDS-PAGE and transferred to a PVDF membrane. Five percent non-fat milk was used to block the PVDF membrane. Then, the membrane was incubated with indicated primary antibodies overnight at 4 °C. Subsequently, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO; #ZB2301, #ZB2305), and protein expression was detected by Pierce ECL Western Blotting Substrate (Thermo, #32106). Antibodies to α-SMA (Servicebio, GB111364), Col1a1 (Servicebio, GB11022), H2BK12cr (PTM Biolabs, PTM-509), H3K18cr (PTM Biolabs, PTM-517), pan anti-Kcr (PTM Biolabs, PTM-501), H3 (Abcam, ab1791), HDAC1 (Proteintech, 10197-1-AP), HDAC3 (CST, 3949), CDYL (Proteintech, 17763-1-AP), GAPDH (Abcam, ab8245) were used in the present study.

Statistical Analysis

The data were typically shown as mean±SEM. One-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was implemented to analyze the significant differences between groups. P values <0.05 were considered statistically significant. All statistical analyses were performed with GraphPad Prism 8.0.

Results

Sorafenib Improved the Liver Index of CCl_4-Induced Rats

To investigate the effect of sorafenib on liver index in fibrotic rats, the body weight and liver weight of the rats were documented. As shown in Figure 1A, the body weights of CCl_4-treated rats were significantly decreased compared to the

Table 1 mRNA Primer Sequences Used in This Study

Gene Names	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)
Col1a1	GTACATCAGCCCAAACCCCA	CAGGATCGGAACCTTCGCTT	101
Acta2	CATCCGACCTTGCTAACGGA	GTCCAGCACAATACCAGTTGT	162
Timp1	GACACGCTAGAGCAGATACCA	CCAGGTCCGAGTTGCAGAAA	144
Timp2	CTCGGCCTCCTGCTGC	TGGCCCTGATCACTACGTCT	109
Timp3	AGCAGCTACCATGACTCCCT	TTGGCCCGGATCACGATG	144
Col4a1	GGCCCTTCATTAGCAGGTGT	GCTGGTGTGCATCACAAAGG	140
Gapdh	GTGATGGGTGTGAACCACGAG	GTCATGAGCCCTTCCACGATG	134

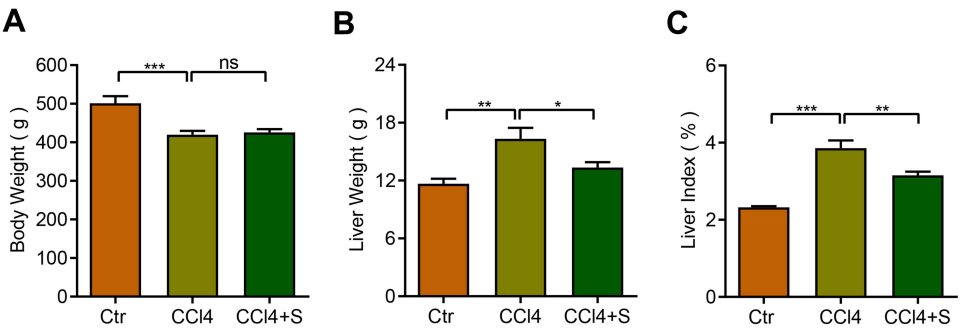

Figure 1 Body weight, liver weight, and liver index were analyzed between groups. (**A**) Body weight of rats in three groups. (**B**) Liver weight of rats in three groups. (**C**) Liver index = liver weight /body weight × 100%. *P<0.05, **P<0.01, ***P<0.001.
Abbreviations: Ctr, control group; CCl_4, CCl_4 group; CCl4 +S, CCl_4 plus sorafenib group; ns, not significant.

control group and the body weights of CCl_4 and CCl_4+S rats were comparable. Notably, CCl_4 increased the liver weight of rats, while sorafenib could reversed the increase of liver weight induced by CCl_4 (Figure 1B). Then, according to the body weight and liver weight of rats, we figured out the liver index of rats. CCl_4 treatment remarkably enhanced the liver index of rats, whereas sorafenib decreased the liver index of rats (Figure 1C). These data implicate that sorafenib could improve the liver index of fibrotic rats.

Sorafenib Relieved Liver Fibrosis in CCl_4-Induced Rats

To explore the effects of sorafenib on the liver fibrosis in rats treated by CCl_4. Hematoxylin–eosin (H&E) staining was used to analyze the histopathological changes of liver induced by CCl_4. The results indicated CCl_4 treatment promoted hepatocellular necrosis and inflammatory infiltration, while sorafenib improved these phenotypes (Figure 2A). As for the degree of liver fibrosis, Masson trichrome results indicated perivascular and interstitial fibrosis significantly increased in rats induced by CCl_4. Conversely, sorafenib treatment displayed notable decrease in perivascular and interstitial fibrosis in rats (Figure 2B and D). Consistently, Picrosirius red (PSR) staining assay also revealed that hepatocellular collagen accumulation was remarkably inhibited with sorafenib (Figure 2C and E). These results indicate sorafenib retards the progression of liver fibrosis.

Sorafenib Improved Liver Function and Liver Fibrosis Index of Fibrotic Rats

The concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) is common indicators to evaluate the liver function in clinical. Therefore, we evaluated the roles of sorafenib on these indicators. CCl_4 treatment increased the levels of serum ALT, AST, and TBIL, whereas sorafenib treatment dramatically reversed the serum ALT, AST, and TBIL levels (Figure 3A–C). These findings indicate that sorafenib could improve liver function of CCl_4-induced rats.

The non-invasive indicators for evaluating the liver fibrosis are the content of hyaluronic acid (HA), type III procollagen (PC III), laminin (LN). To analyze the effects of sorafenib on liver fibrosis indexes, we performed enzyme-linked immunosorbent assay (ELISA) and results showed the concentration of serum PC-III, HA, and LN were significantly up-regulated in CCl_4-induced rats. In contrast, these indexes were decreased in response to sorafenib (Figure 3D–F). These data demonstrate that sorafenib improves serum liver fibrosis indexes in CCl_4-induced rats.

Sorafenib Repressed HSC Activation and ECM Accumulation

During liver injury, persistent liver injury stimuli lead to transform the resting hepatic stellate cells into activated hepatic stellate cells, which would generate excess extracellular matrix (ECM).[27,28] To detect the roles of sorafenib on the activation of HSCs, we analyzed the expression of actin alpha 2, smooth muscle (*Acta2*, also called *α-SMA* in mice) in fibrotic livers, the indicator of HSCs activation. The qRT-PCR results demonstrated the expression of *Acta2* was markedly increased in response to CCl_4 and reduced by sorafenib treatment (Figure 4A). Moreover, the expression of collagen type I alpha 1 chain (*Col1a1*) was also up-regulated in CCl_4 group and down-regulated in the sorafenib group (Figure 4B). The expression tendency of another collagen-related gene called collagen type IV alpha 1 chain (*Col4a1*) was consistent to *Col1a1* (Figure 4C). Besides, TIMPs (tissue inhibitors of

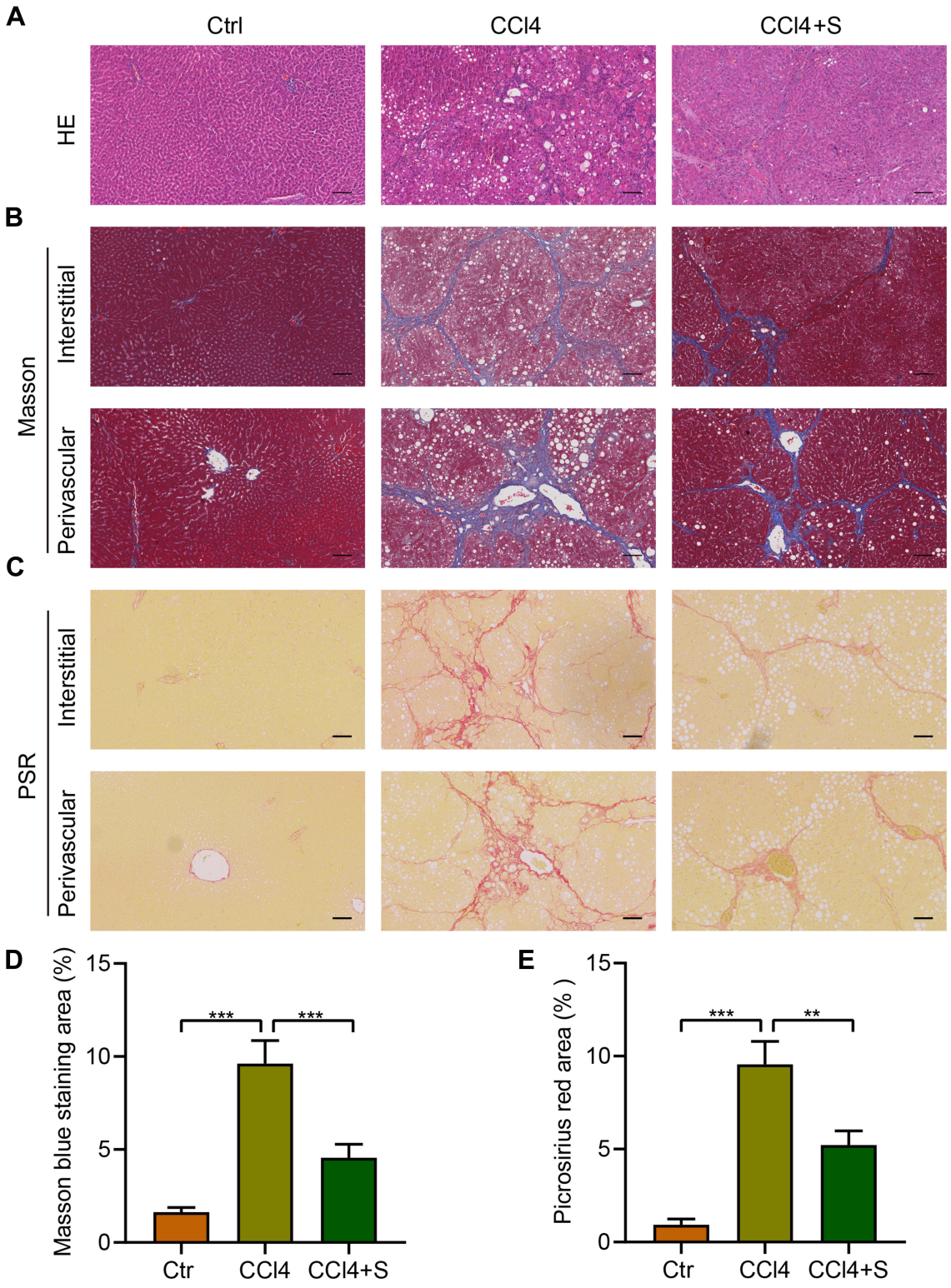

Figure 2 Sorafenib improved histopathological changes of liver induced by CCl4. (**A**) Hematoxylin–eosin (H&E, scale bar=100 μm) staining was used to analyze the hepatic structure of rats. (**B**) Masson trichrome (scale bar=100 μm) staining was used to analyze hepatic fibrosis. (**C**) Picrosirius red (PSR, scale bar=100 μm) staining was performed to determine hepatic fibrosis in rats. (**D**) Quantification of the fibrotic area of Masson trichrome staining in (**B**). (**E**) Quantification of the fibrotic area of Picrosirius red staining in (**C**). **P<0.01, ***P<0.001.

Abbreviations: Ctr, control group; CCl_4, CCl_4 group; CCl_4 +S, CCl4 plus sorafenib group.

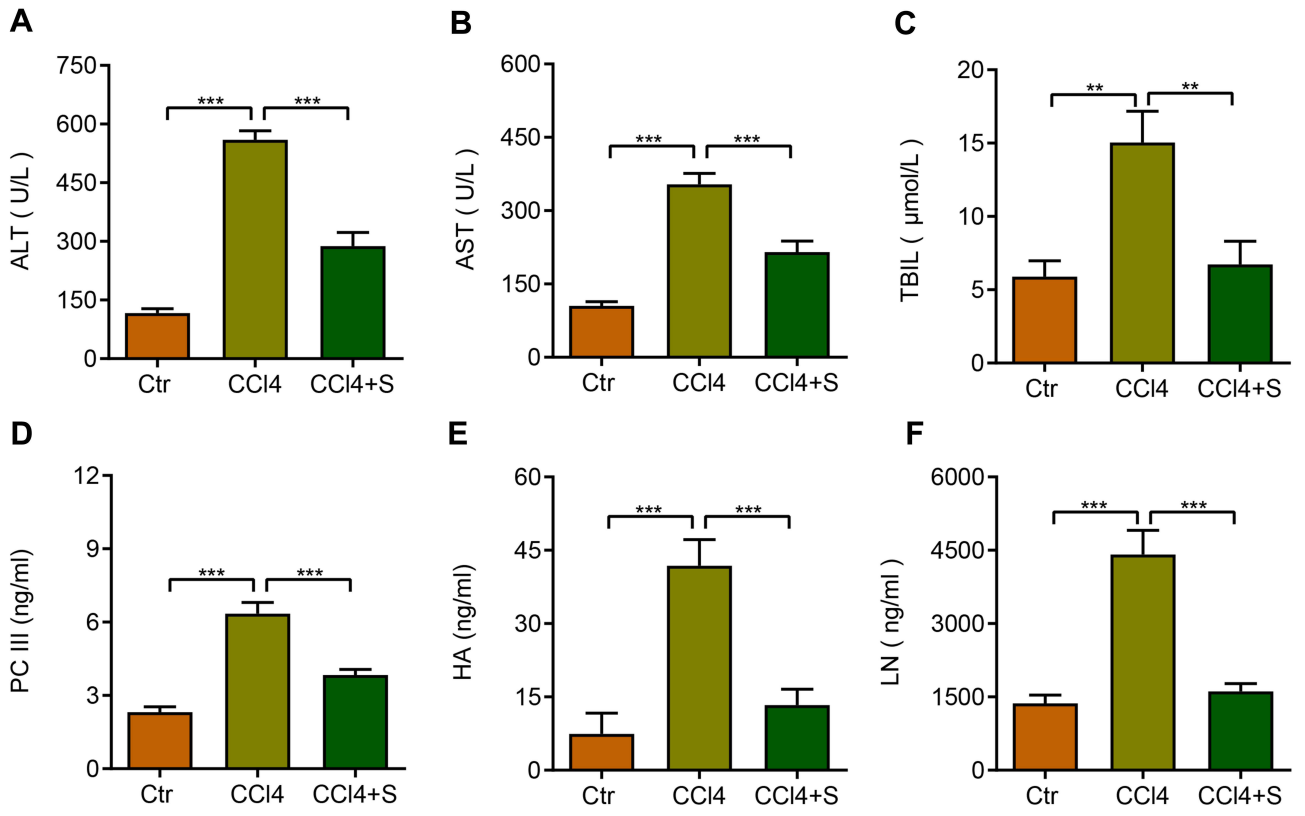

Figure 3 Serum biochemical indexes were determined in the control group (Ctr), CCl_4 group (CCl_4), and CCl_4 plus sorafenib group (CCl_4+S). (**A**) ALT: alanine aminotransferase. (**B**) AST: aspartate aminotransferase. (**C**) TBIL: total bilirubin. (**D**) PC III: type III procollagen. (**E**) HA: hyaluronic acid. (**F**) LN: laminin. **P<0.01, ***P<0.001.

metalloproteinases) play important role in maintaining homeostasis of ECM, which prevent metalloproteinases to inhibit the degradation of ECM.[29] Consistently, in our study, CCl_4 treatment promoted the expression of *Timp1, Timp2*, and *Timp3* and sorafenib reversed the high expression of *TIMPs* (Figure 4D–F). To further investigate the effects of sorafenib on HSC activation and ECM accumulation, immunohistochemical staining and Western blotting were performed to analyze the protein changes of α-SMA and collagen I. Similarly, α-SMA and collagen I accumulation induced by CCl_4 were improved upon sorafenib treatment (Figure 4G–K). These results indicate sorafenib could improve HSC activation and ECM accumulation.

Lysine Crotonylation Mediated Anti-Fibrotic Effect of Sorafenib

Lysine crotonylation is a novel post-translational modification of proteins widely participating in many biological processes.[5,30] Wan et al have uncovered lysine crotonylation expression was decreased in hepatocellular carcinoma and closely associated with hepatoma cell migration and proliferation.[17] However, the roles of lysine crotonylation in liver fibrosis remain indistinct. To explore the underlying mechanism of sorafenib in liver fibrosis, we analyzed the lysine crotonylation expression in our study. Surprisingly, compared to the control group, CCl_4 treatment led to a sharply decrease of total lysine crotonylation expression (Pan-Kcr) and sorafenib treatment increased the level of total lysine crotonylation (Figure 5A and D). Especially, the expression of crotonylated H2BK12 (H2BK12cr) and crotonylated H3K18 (H3K18cr) was significantly decreased after CCl_4 treatment and increased in response to sorafenib (Figure 5B, C, E and F). Western blotting results also demonstrated Pan-Kcr, H3K18cr and H2BK12cr levels were decreased in fibrotic liver. By contrast, sorafenib treatment increased histone crotonylation in liver (Figure 5G). These findings suggest that lysine crotonylation is important for antifibrotic effect of sorafenib.

To deeply explore the relationship between lysine crotonylation expression and liver fibrosis, we performed Spearman correlation analysis between lysine crotonylation expression and serum biochemical indicators. Heatmap Matrix showed that lysine crotonylation was negatively correlated with serum biochemical indicators (ALT, AST, TBIL, PCIII, HA, and

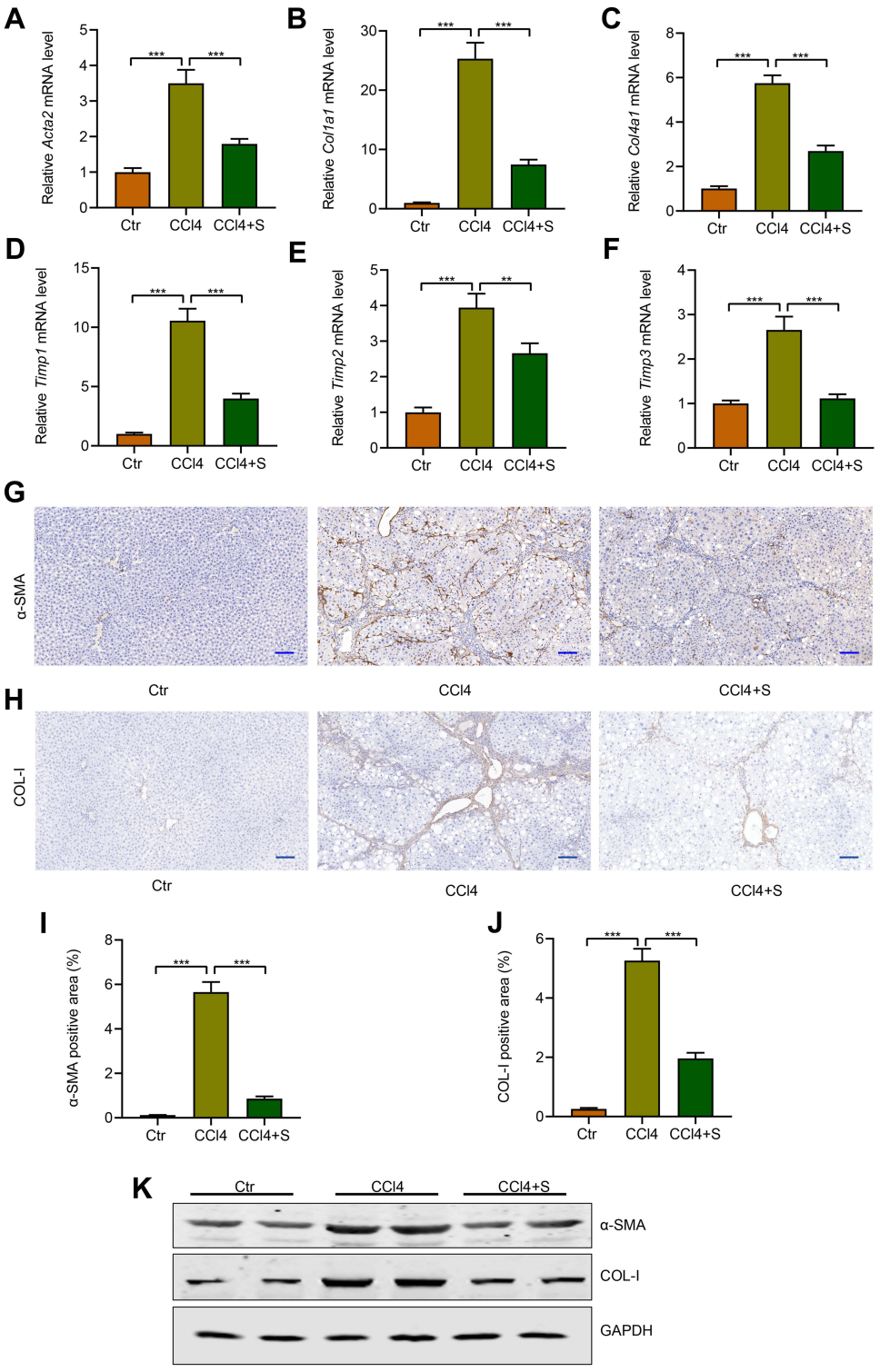

Figure 4 Sorafenib attenuated HSC activation and ECM accumulation. (**A**) *Acta2*: actin alpha 2, smooth muscle. (**B**) *Col1a1*: collagen type I alpha 1 chain. (**C**) *Col4a1*: collagen type IV alpha 1 chain. (**D**) *Timp1*: TIMP metallopeptidase inhibitor 1. (**E**) *Timp2*: TIMP metallopeptidase inhibitor 2. (**F**) *Timp3*: TIMP metallopeptidase inhibitor 3. (**G**) IHC staining was used to analyze α-SMA level. (**H**) IHC staining was used to analyze collagen I (COL-I) level. (**I**) Quantification of α-SMA IHC-positive staining area. (**J**) Quantification of collagen I (COL-I) IHC-positive staining area. (**K**) Western blotting was used to detect the expression of α-SMA and collagen I. **P<0.01, ***P<0.001.
Abbreviations: Ctr, control group; CCl_4, CCl_4 group; CCl_4 +S, CCl_4 plus sorafenib group.

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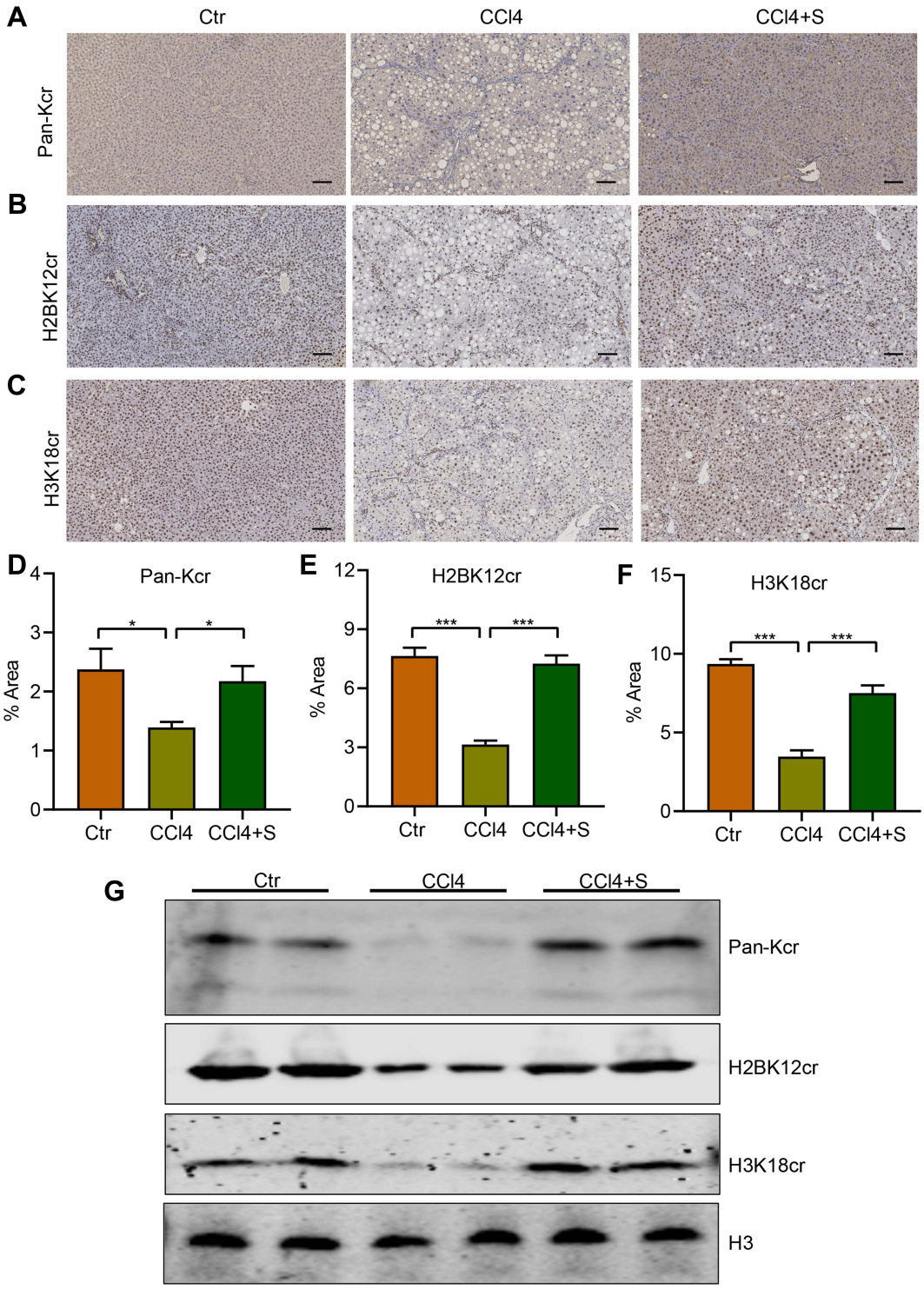

Figure 5 Sorafenib increased protein lysine crotonylation of fibrotic liver in CCl4-induced rats. (**A**) The levels of total lysine crotonylation (Pan-Kcr) in fibrotic livers. (**B**) The levels of crotonylated H2BK12 (H2BK12cr) in fibrotic livers. (**C**) The levels of crotonylated H3K18 (H3K18cr) in fibrotic livers. (**D**) Semi-quantitative analysis of Pan-Kcr IHC-positive staining areas. (**E**) Semi-quantitative analysis of H2BK12cr IHC-positive staining areas. (**F**) Semi-quantitative analysis of H3K18cr IHC-positive staining areas. (**G**) Western blotting was used to detect the expression of protein lysine crotonylation (Pan-Kcr, H2BK12cr, and H3K18cr). *P<0.05, ***P<0.001.
Abbreviations: Ctr, control group; CCl_4, CCl_4 group; CCl_4 +S, CCl_4 plus sorafenib group.

LN) (Figure 6A and Table 2). To further investigate how lysine crotonylation expression was disturbed, the levels of crotonylation-regulated enzymes (HDAC1, HDAC3, and CDYL) were detected by Western blotting. The results indicated the expression of HDAC1, HDAC3, and CDYL was increased in response to CCl_4 treatment, while sorafenib

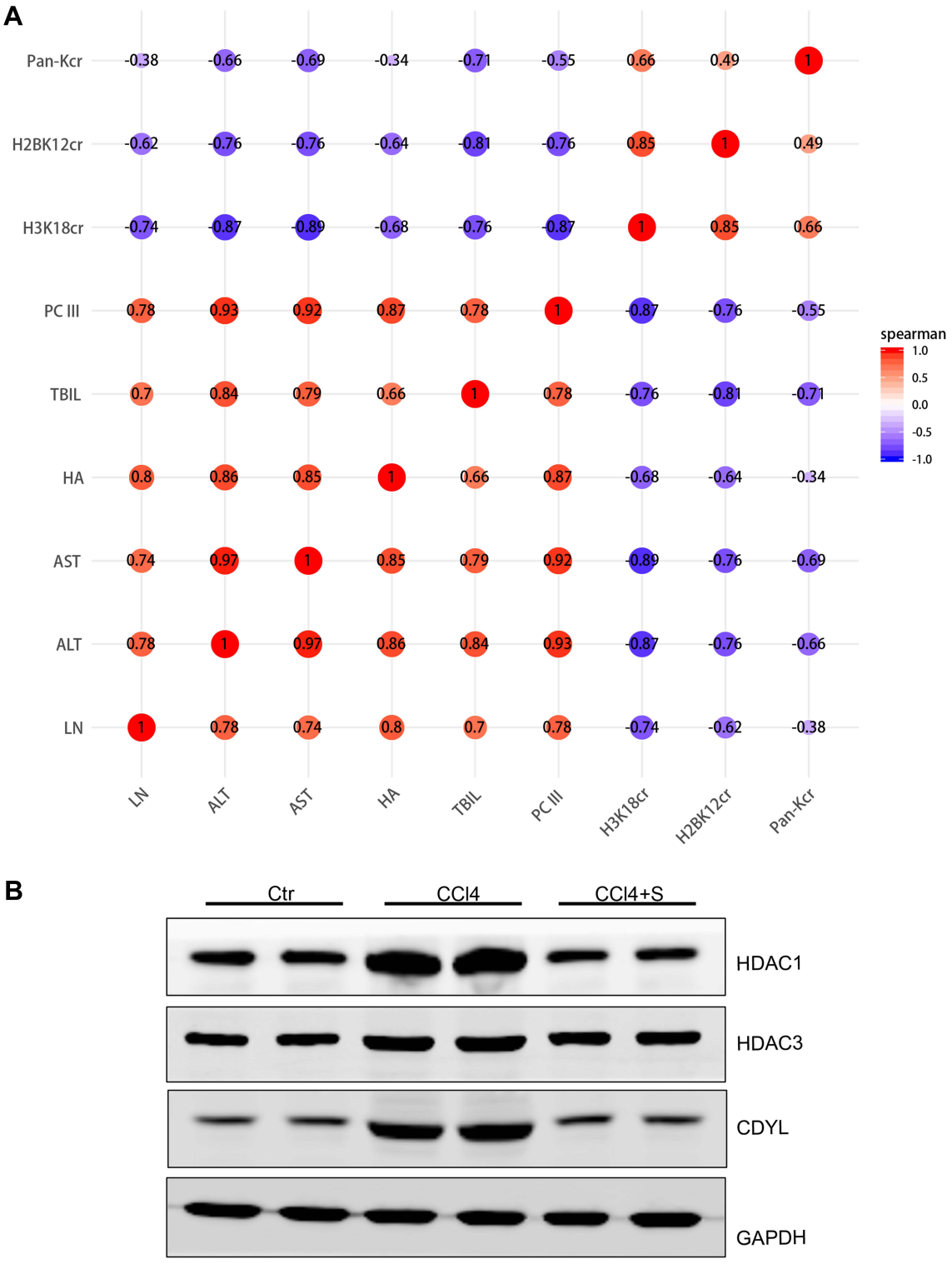

Figure 6 Analysis of the relationship between lysine crotonylation expression and each of the indicators of fibrosis and detection protein expression change in fibrotic liver. (**A**) Correlation analysis of lysine crotonylation and fibrotic serum biochemical indexes. The Heatmap Matrix was generated from https://cloud.oebiotech.cn/task/. (**B**) Western blotting was used to evaluate the expression of crotonylation-regulated enzymes.

Table 2 Correlation Analysis of Lysine Crotonylation and Fibrotic Serum Biochemical Indexes

Category 1	Category 2	Correlation	p-value	Type	Significance	AdjPvalue
LN	H3K18cr	−0.74126	0.008171	Group	**	0.009805
LN	H2BK12cr	−0.62238	0.0348	Group	*	0.0348
LN	Pan-Kcr	−0.37762	0.227443	Group		0.272932
ALT	H3K18cr	−0.87413	0.000309	Group	***	0.000618
ALT	H2BK12cr	−0.76224	0.005897	Group	**	0.009895
ALT	Pan-Kcr	−0.66434	0.022159	Group	*	0.044318
AST	H3K18cr	−0.88811	9.17E-05	Group	***	0.00055
AST	H2BK12cr	−0.75524	0.006597	Group	**	0.009895
AST	Pan-Kcr	−0.69231	0.015878	Group	*	0.044318
HA	H3K18cr	−0.67832	0.018825	Group	*	0.018825
HA	H2BK12cr	−0.63636	0.030114	Group	*	0.0348
HA	Pan-Kcr	−0.34266	0.276231	Group		0.276231
TBIL	H3K18cr	−0.75524	0.006597	Group	**	0.009805
TBIL	H2BK12cr	−0.81119	0.002369	Group	**	0.009895
TBIL	Pan-Kcr	−0.70629	0.013286	Group	*	0.044318
PC III	H3K18cr	−0.87413	0.000309	Group	***	0.000618
PC III	H2BK12cr	−0.76224	0.005897	Group	**	0.009895
PC III	Pan-Kcr	−0.54545	0.070679	Group		0.106018

Notes: The correlation table was generated from https://cloud.oebiotech.cn/task/. *P<0.05, **P<0.01, ***P<0.001.

reduced their expression (Figure 6B). These data indicated sorafenib attenuated liver fibrosis through regulating crotonylation-regulated enzymes to modulate the level of protein crotonylation.

Discussion

Liver fibrosis is a leading contributor of chronic liver diseases such as cirrhosis and hepatocellular carcinoma.[31] Sorafenib was approved to cure hepatocellular and renal cell carcinomas and had the potential to treat liver fibrosis.[18] However, the association between protein lysine acylation such as lysine crotonylation and liver fibrosis and whether sorafenib participated in remain unknown. In this study, we found CCl_4 treatment induced an increase in liver index, hepatocellular necrosis and inflammatory infiltration, serum liver function and fibrosis indexes, and expression of fibrosis-related genes and proteins in rats. The lysine crotonylation expression was negatively correlated with serum fibrotic indicators. Conversely, sorafenib improved these abnormities to inhibit liver fibrosis. Mechanistically, sorafenib reduced crotonylation-regulated enzymes expression and increased protein lysine crotonylation levels, which contributed to the anti-fibrotic effect of sorafenib.

Serum liver function (ALT, AST, and TBIL) and fibrosis indicators (PC-III, HA, and LN) were used to evaluate the degree of liver disease.[32] In our study, we found sorafenib dramatically inhibited the increase of these indicators in fibrotic rats. Besides, histopathological changes were also used to analyze the degree of liver fibrosis.[22] H&E, Masson trichrome and PSR were all demonstrated sorafenib repressed the hepatocellular necrosis, inflammatory infiltration and liver fibrosis. Moreover, the typical hallmark of fibrosis was the activation of hepatic stellate cells to induce *Acta2* expression.[33] We found sorafenib significantly reduced the expression of *Acta2* in CCl_4-induced rats. In addition, the expression of other fibrogenic genes such as *Col1a1, Col4a1, Timp1, Timp2, Timp3* were also inhibited by sorafenib. Collectively, these facts support the notion that sorafenib has the potential to treat liver fibrosis.

Histone short-chain lysine acylations, such as 2-hydroxyisobutyrylation, β-hydroxybutyrylation, glutarylation, and crotonylation, were reportedly to modulate gene expression and widely participate in the various pathophysiological process.[5,34–36] Histone lysine crotonylation was a novel short-chain lysine acylation and structurally and functionally distinctive from the well-studied lysine acetylation, which was mainly distributed in active promoters and latent enhancer.[5,8] It has been demonstrated lysine crotonylation was increased during acute kidney injury (AKI), which was protective for AKI.[37] Moreover, many researches have indicated lysine crotonylation also regulated spermatogenesis,[11]

endoderm differentiation,[9] cardiac homeostasis,[7] and tumorigenesis.[38] However, the effects of lysine crotonylation on the liver fibrosis remain to explore. The mouse liver lysine crotonylome has been globally profiled and proved crotonylated liver protein primarily influenced metabolism-related biological processes.[16] Besides, liver fibrosis would eventually lead to hepatocellular carcinoma, the high morbidity and mortality of malignancy. It has been proved the occurrence and development of hepatocellular carcinoma is accompanied by alteration of total lysine crotonylation.[17] Interestingly, we found total lysine crotonylation was decreased in fibrotic liver and reversed by oral sorafenib, especially H2BK12cr and H3K18cr levels. And the lysine crotonylation expression was negatively associated with fibrotic serum biochemical indexes. Besides, interruption of decrotonylases HDAC1 and HDAC3 to enhance the levels of total lysine crotonylation could suppress hepatoma cell proliferation, which suggested the potential role of lysine crotonylation on the liver fibrosis.[17] It has been reported that inhibiting HDAC activity could attenuate CCl_4-induced liver fibrosis.[39] Surprisingly, crotonylation-regulated enzymes (HDAC1, HDAC3, and CDYL), which negatively regulated protein crotonylation expression, were dramatically increased in fibrotic liver and decreased by sorafenib treatment. Therefore, deficiency of lysine crotonylation may be a mechanism that leads to liver fibrosis. However, we just discovered the expression changes and more attentions should be paid to uncover the potential mechanisms contributing to lysine crotonylation in liver fibrosis.

Conclusion

We demonstrate sorafenib is an effective drug for attenuating liver fibrosis. Sorafenib inhibits CCl_4-induced liver fibrosis through maintaining homeostasis of hepatic crotonylation-regulated enzymes and protein lysine crotonylation. These findings broaden our knowledge of anti-fibrotic effects of sorafenib and suggest lysine crotonylation may influence liver fibrosis progression.

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Disclosure

The authors report no conflicts of interest in this work.

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