The NF-κB signalling pathway and TM7SF3 contribute to liver fibrosis caused by secreted phospholipase A2 of *Clonorchis sinensis*

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**Abstract**

**Background:** The NF-κB signalling pathway has been reported to be related to liver fibrosis, and we investigated whether the NF-κB signalling pathway is involved in liver fibrosis caused by secreted phospholipase A2 of *Clonorchis sinensis* (*CssPLA2*). Furthermore, expression of the receptor of *CssPLA2* on the cell surface of hepatic stellate cells (HSCs) may greatly contribute to liver fibrosis.

**Methods:** *CssPLA2* was administered to BALB/c mice by abdominal injection. The levels of markers of NF-κB signalling pathway activation in mouse liver tissue were measured by quantitative RT-PCR, ELISA and western blot. Additionally, HSCs were incubated with *CssPLA2*, and an NF-κB signalling inhibitor (BAY 11-7082) was applied to test whether the NF-κB signalling pathway plays a role in the effect of *CssPLA2*. Then, the interaction between *CssPLA2* and its receptor transmembrane 7 superfamily member 3 (TM7SF3) was confirmed by co-immunoprecipitation (co-IP) and GST pull-down. To determine how TM7SF3 influences the ability of *CssPLA2* to cause liver fibrosis, a TM7SF3 antibody was used to block TM7SF3.

**Results:** The levels of the NF-κB signalling pathway activation markers TNF-α, IL-1β and phospho-p65 were increased by *CssPLA2* in the context of liver fibrosis. In addition, the interaction between TM7SF3 and *CssPLA2* was confirmed by co-IP and GST pull-down. When TM7SF3 was blocked by an antibody targeting 1–295 amino acids of TM7SF3, activation of HSCs caused by *CssPLA2* was alleviated.

**Conclusions:** The NF-κB signalling pathway is involved in the activation of HSCs by *CssPLA2*. TM7SF3, the receptor of *CssPLA2*, plays important roles in liver fibrosis caused by *CssPLA2*.

**Keywords:** Pathogenesis, *Clonorchis sinensis*, Liver fibrosis, Phospholipase A2, Receptor

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**Background**

*Clonorchis sinensis* is a common parasite present mainly in East and South Asian countries, such as China, Korea and Vietnam [1, 2]. Infection of *Clonorchis sinensis* can cause cholangitis, cholecystitis and cholelithiasis and, in some serious cases, liver fibrosis or even cholangiocarcinoma [3]. Deposition of a large amount of collagen caused by the activation of hepatic stellate cells (HSCs) is key to liver fibrosis caused by infection with *Clonorchis sinensis* [4]. However, the mechanism by which HSCs are activated is still unclear. In our previous research, it was proven that *CssPLA2* can activate HSCs and result in liver fibrosis through the activation of the c-Jun N-terminal kinase (JNK) signalling pathway [5]. However, it is still unknown whether *CssPLA2* can activate HSCs through other cell signalling pathways. Cytokines such as TNF-α and IL-1β have
been found to be associated with the activation of HSCs in many reports [6]. The cytokines are related to the NF-κB signalling pathway and are released when this pathway is activated [7]. It is possible that CsssPLA2, an enzyme itself and member of the group III phospholipase family, can activate HSCs through the NF-κB signalling pathway. However, the mechanism by which CsssPLA2 causes liver fibrosis is associated with cell signalling pathways rather than its enzymatic activity [5]. Activation of cell signalling pathways is thought to be caused by the binding of a protein to its receptor on the surface of the cell [8–10]. In our previous research, TM7SF3, a transmembrane protein [11–13] and receptor of CsssPLA2, was screened by a yeast two-hybrid system. TM7SF3 contains seven putative transmembrane domains, and its open reading frame encodes a protein of 570 amino acids. In this research, the ability of CsssPLA2 to bind TM7SF3 is tested, and we investigate how TM7SF3 influences the ability of CsssPLA2 to cause liver fibrosis.

The excretory secretory products (ESPs) of parasites play important roles in host–parasite interactions and the pathogenesis of diseases, including immunosuppression, fibrosis and carcinoma [14]. The ESPs of Clonorchis sinensis, which include 110 proteins, such as glycometabolic enzymes, detoxification enzymes and a number of RAB family proteins, are closely related to biliary diseases and liver fibrosis [15].

CsssPLA2 is one of these ESPs. The gene that encodes CsssPLA2 contains 828 bp. CsssPLA2 is a 34-kDa protein, and we previously purified a recombinant MBP-CsssPLA2 protein with a molecular weight of 76 kDa. CsssPLA2 is a group III PLA2 and has been determined to cause liver fibrosis [16, 17]. It is being considered as a potential drug target [16]. It has been proven that this protein is able to bind to the HSC membrane and cause upregulation of collagen expression, which can result in liver fibrosis [16]. TM7SF3, the receptor of CsssPLA2, is expressed on the membranes of HSCs and may play important roles in liver fibrosis caused by CsssPLA2. The NF-κB signalling pathway has been reported to be associated with liver fibrosis [18]; therefore, it is necessary to investigate the relationship between liver fibrosis caused by CsssPLA2 and this signalling pathway.

**Methods**

**Expression and purification of MBP-CsssPLA2 protein**

The recombinant plasmids (pMAL-c2x-CsssPLA2) were transformed to *E. coli* BL21 (DE3). Overnight cultures carrying the recombinant plasmid pMAL-c2x-CsssPLA2 were inoculated in 2 L of Luria–Bertani broth medium containing 50 μg/ml of ampicillin. Cells were grown at 37°C until the A600 had reached 0.6. The expression of protein was induced by adding IPTG at a final concentration of 0.5 mM, followed by incubation at 37°C for 4 h with vigorous shaking at 250 rpm. MBP-CsssPLA2 protein was purified by a single step of amylose resin under native conditions (NEB, USA), following the manufacturer’s protocol. The endotoxin in the recombinant protein was removed by Detoxi-Gel™ Endotoxin Removing Gel (Thermo, USA), and then detected by limulus test, which confirmed that no endotoxin was found in the recombinant protein. The recombinant protein was stored at −80°C for later use.

**Measurement of the levels of NF-κB signalling pathway activation markers in mouse liver tissues**

Male BALB/c mice aged 8 weeks and weighing approximately 19–20 g were raised in a specific pathogen-free (SPF) environment. They were divided into three groups with three mice in each group. The three groups of Balb/c mice were given an abdominal injection of 100 μg PBS, 100 μg maltose-binding protein (MBP), or 100 μg MBP-CsssPLA2, twice a week for 4 weeks. The mice were sacrificed after 4 weeks.

Total RNA was extracted from mouse liver tissue by TRIzol (Invitrogen, USA). cDNA was obtained by reverse transcription from one microgram of total RNA with a reverse transcriptase kit (Thermo, Lithuania). SYBR Premix Ex Taq (Takara, China) was used for PCR amplification, and the transcribed cDNA was used as a template. The sequences of the primers for mouse TNF-α were 5′-CAT CCT CTC AAA ATT CGA GTGACA-3′ (forward primer) and 5′-TGG GAG TAG ACA AGG TAC AAC CC-3′ (reverse primer). The sequences of the primers for mouse IL-1β were 5′-AAA TGC CAC CTT TTG ACA GTG ATG-3′ (forward primer) and 5′-GCT CTG TGT GAT GTG CTT C-3′ (reverse primer). The sequences of the primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5′-CAA AAT GTG GAA GGT CGG TGT G-3′ (forward primer) and 5′-TGA TGT TAG TGG GGT CTG GT TCT C-3′ (reverse primer). Mouse GAPDH was used as the internal standard for normalization. The PCR conditions were as follows: 95°C for 30 s followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s, with an incremental increase of 0.5°C for 5 s from 60 to 95°C.

ELISA was used to measure the levels of TNF-α and IL-1β in mouse liver tissue. Total protein was extracted from liver tissue with a total protein extraction kit (Invitrogen, USA). TNF-α (1:2000 dilutions, CST, USA) and IL-1β antibodies (1:2000 dilutions, RD, USA) were then used as the primary antibodies, and HRP-conjugated goat anti-rabbit IgG (1:10,000 dilutions, CST, USA) was used as the secondary antibody. Tetramethylbenzidine (TMB) was added to each well, and 2 M H2SO4 was used
to stop the reaction. The absorbance of each well was measured at 450 nm.

Western blot analysis was used to determine the effect of the NF-κB signalling pathway on liver fibrosis caused by CssPLA2. Total protein was extracted from liver tissue with a total protein extraction kit (Invitrogen, USA). A BCA protein assay kit (Transgen, China) was used to measure the protein concentration. Mouse β-actin was used as an internal standard for normalization. SDS-PAGE (12% polyacrylamide gel) was used to separate the proteins, and the proteins were immobilized on a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk at room temperature for 2 h before being incubated with primary antibodies (p65 antibody and phospho-p65 antibodies, 1:1000 dilution in 1% BSA, CST, USA) overnight at 4 °C. Afterwards, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000 dilution, CST, USA) for 1 h at room temperature. Finally, Pierce™ ECL Plus Western Blotting Substrate (Thermo, USA) was used to visualize the bands.

**Analysis of the inhibitory effect of an NF-κB signalling pathway inhibitor (BAY 11-7082) on liver fibrosis**

Fatty-acid-binding protein (FABP) was another ESP of *Clonorchis sinensis*, and here, FABP was used as a negative control. To evaluate the effect of an NF-κB signalling pathway inhibitor (BAY 11-7082) on the liver fibrosis caused by MBP-CssPLA2, human HSC cells LX-2 were divided into four groups, and incubated with 25 μg/ml MBP, 25 μg/ml MBP-CssPLA2, 25 μg/ml FABP or 25 μg/ml MBP-CssPLA2 + 10 μM NF-κB inhibitor for 24 h.

The levels of markers of the NF-κB signalling pathway and HSC activation (TNF-α and collagen III, respectively) were measured by quantitative RT-PCR. The sequences of primers for human TNF-α were 5′-CCC AGG CAG TCA GAT CAT CTT CT-3′ (forward primer) and 5′-ATG AGG TAC AGG CCC TCT GAT-3′ (reverse primer). The sequences of primers for human collagen III were 5′-GGT CCT CCT GGA ACT GCC GGA-3′ (forward primer) and 5′-GAG GAC CTT GAG CAC CAG GT-3′ (reverse primer). The sequences of primers for human β-actin were 5′-GTC CAC CGC AAA TGC TTC TA-3′ (forward primer) and 5′-TGC TGT CAC CTT CAC CGT TC-3′ (reverse primer). Human β-actin was used as the internal standard for normalization.

The supernatant of cells was collected to measure the levels of markers of the NF-κB signalling pathway and HSC activation (TNF-α and collagen III, respectively) by ELISA. TNF-α (1:2000 dilutions, CST, USA) and collagen III antibodies (1:2000 dilutions, Abcam, UK) were then applied as the primary antibodies, and HRP-conjugated goat anti-rabbit IgG was applied as the secondary antibody (1:10,000 dilutions, CST, USA).

**Co-immunoprecipitation assay**

We transiently over-expressed GFP-tagged CssPLA2 and Myc-tagged TM7SF3 in 293T cells for 24 h. Transfected 293T cells were lysed using RIPA lysis buffer (Gibco, USA) and incubated with an anti-PLA2 antibody and protein A-agarose beads (Pierce, USA) overnight at 4 °C. After washing five times with RIPA lysis buffer (Gibco, USA), the beads were eluted with 2 × SDS sample buffer and boiled for 8 min at 100 °C. The samples were then analysed by western blot using anti-Myc antibodies (ProteinTech, USA).

**GST pull-down assay**

The recombinant proteins MBP-CssPLA2 (1.26 mg) and GST-TM7SF3 (0.5 mg) were incubated with 1 mg glutathione-conjugated resin for 2 h at room temperature. GST and MBP-CssPLA2, or MBP and GST-TM7SF3, were incubated with glutathione-conjugated resin under the same conditions as negative controls. After incubation, mixtures of two proteins were purified by a single-step glutathione-conjugated resin chromatography (GE, USA) under native conditions following the manufacturer’s protocol. The samples were then analysed by western blot using anti-PLA2 antibody.

**Inhibition of HSCs incubated with MBP-CssPLA2 in the presence of a TM7SF3 antibody by RT-PCR**

To assess whether HSCs incubated with MBP-CssPLA2 are inhibited when TM7SF3 is blocked, LX-2 cells were divided into three groups and incubated with either 25 μg/ml MBP-CssPLA2 + anti-TM7SF3 antibody (1:200 dilution), 25 μg/ml MBP-CssPLA2 or PBS. Quantitative RT-PCR was performed to measure the mRNA levels of collagen I and collagen III, markers of HSC activation. The sequences of primers for human collagen I were 5′-CTT CAC CTA CAG CTG TG-3′ (forward primer) and 5′-GGA TGG AGG GAG TTT ACA GG-3′ (reverse primer). The sequences of primers for human collagen III were 5′-GGT CCT CCT GGA ACT GCC GGA-3′ (forward primer) and 5′-GGA TGG AGG GAG TTT ACA GG-3′ (reverse primer). Human β-actin was used as the internal standard for normalization.

**Statistical analysis**

GraphPad Prism 6 software was used to analyse the results, and the differences between the control group and the experimental group were analysed by t tests at a significance level of P < 0.05.
Results
The NF-κB signalling pathway was associated with liver fibrosis in liver tissue of mice injected with MBP-CsPLA2. The level of markers of NF-κB signalling pathway activation in mouse liver tissue were measured by quantitative RT-PCR analysis, and the mRNA of TNF-α and IL-1β were determined. Unpaired \( t \) tests were performed, which showed that the differences in levels between the group of mice injected with 100 μg MBP and the group injected with 100 μg MBP-CsPLA2 were significant: \( t_{(4)} = 3.905, P = 0.0175 \) (Fig. 1a) and \( t_{(4)} = 4.095, P = 0.0149 \) (Fig. 1b).

The levels of TNF-α and IL-1β in mouse liver tissue were also measured by ELISA. Both TNF-α and IL-1β were increased in liver tissue of mouse injected with MBP-CsPLA2. Unpaired \( t \) tests were performed and showed that the differences in levels between the group injected with 100 μg MBP and the group injected with 100 μg MBP-CsPLA2 were significant: \( t_{(4)} = 7.873, P = 0.0014 \) (Fig. 1c) and \( t_{(4)} = 7.052, P = 0.0021 \) (Fig. 1d).

The levels of markers of NF-κB signalling pathway activation in mouse liver tissue were measured by western blot. p65 and phospho-p65 were measured in mouse liver tissue. Western blot of the liver tissues of mice injected with 100 μg MBP; lane 2: liver tissue of a mouse injected with 100 μg MBP-CsPLA2; lane 3: liver tissue of a mouse injected with PBS. The differences in levels between the control group and the experimental group were determined by quantitation of the western blot results. Unpaired \( t \) tests were used for statistical analysis (\( t_{(4)} = 4.475, P = 0.011 \)).
were revealed by unpaired *t* tests (*t*\(_{4}\) = 4.475, *P* = 0.011) (Fig. 1f).

**Fig. 2** NF-κB signalling pathway inhibitor (BAY 11–7082) inhibited liver fibrosis in HSCs caused by MBP-CssPLA2. a The relative mRNA levels of TNF-α in the four groups of LX-2 cells incubated with 25 μg/ml MBP, 25 μg/ml MBP-CssPLA2, 25 μg/ml FABP or 25 μg/ml MBP-CssPLA2 + 10 μM NF-κB inhibitor. b The relative mRNA levels of collagen III in the four groups of LX-2 cells incubated with 25 μg/ml MBP, 25 μg/ml MBP-CssPLA2, 25 μg/ml FABP or 25 μg/ml MBP-CssPLA2 + 10 μM NF-κB inhibitor. c The absorbance of TNF-α at 450 nm in the four groups of LX-2 cells incubated with 25 μg/ml MBP, 25 μg/ml MBP-CssPLA2, 25 μg/ml FABP or 25 μg/ml MBP-CssPLA2 + 10 μM NF-κB inhibitor. d The absorbance of collagen III at 450 nm in the four groups of LX-2 cells incubated with 25 μg/ml MBP, 25 μg/ml MBP-CssPLA2, 25 μg/ml FABP or 25 μg/ml MBP-CssPLA2 + 10 μM NF-κB inhibitor.

**The NF-κB signalling pathway inhibitor (BAY 11–7082) inhibited liver fibrosis in HSCs caused by MBP-CssPLA2**

The levels of markers of NF-κB signalling pathway and HSC activation (TNF-α and collagen III, respectively) in HSCs were tested by quantitative RT-PCR analysis. Unpaired *t* tests were performed, and it was found that the differences in levels between the group of cells incubated with 25 μg/ml MBP-CssPLA2 and the group incubated with 25 μg/ml FABP were significant: *t*\(_{4}\) = 3.335, *P* = 0.0290 (Fig. 2a) and *t*\(_{4}\) = 6.113, *P* = 0.0036 (Fig. 2b). Additionally, the differences in levels between the group...
of cells incubated with 25 µg/ml MBP-CssPLA2 and the group incubated with 25 µg/ml MBP were significant: \( t_{(4)} = 3.060, P = 0.0376 \) (Fig. 2a) and \( t_{(4)} = 9.043, P = 0.0008 \) (Fig. 2b), and the differences between the group of cells incubated with 25 µg/ml MBP-CssPLA2 and the group incubated with 25 µg/ml MBP-CssPLA2 + 10 µM NF-κB inhibitor (BAY 11-7082) were significant: \( t_{(4)} = 3.502, P = 0.0249 \) (Fig. 2a) and \( t_{(4)} = 15.81, P < 0.0001 \) (Fig. 2c) and \( t_{(4)} = 7.767, P = 0.0015 \) (Fig. 2d).

**Discussion**

NF-κB signalling regulates activation or apoptosis of HSCs by regulating inflammatory factors such as TNF-α. Expression of NF-κB signalling markers is upregulated in liver fibrosis [18–20]. Therefore, we hypothesized that CssPLA2 may activate HSCs to cause liver fibrosis through activation of the NF-κB signalling pathway and that the receptor of CssPLA2, TM7SF3, plays an important role in this process.

This study will contribute to elucidating the mechanism of HSC activation by CssPLA2 and clarify its role in the process of liver fibrosis caused by *Clonorchis sinensis* infection. Additionally, interfering with the signalling pathway involved in HSC activation by CssPLA2 may be
a potential treatment strategy for liver fibrosis caused by Clonorchis sinensis infection.

CsPLA2-induced activation of human HSCs may involve multiple pathways. For example, the activation of the NF-κB signalling pathway is closely associated with liver fibrosis [21, 22]. The levels of phosphorylated NF-κB were obviously increased in the livers of mice infected with Clonorchis sinensis [23]. The release of cytokines such as TNF-α and IL-1β [24–27] is closely related to the activation of the NF-κB signalling pathway. The NF-κB signalling pathway is associated with a variety of inflammatory responses, such as TNF-α secretion, which regulates the activation or apoptosis of HSCs during liver fibrosis. The levels of TNF-α, IL-1β, phospho-p65 and other NF-κB signalling markers were increased in the progression of liver fibrosis [18–20]. CsPLA2 is an important ESP of Clonorchis sinensis. A multitude of reports have shown that the ESPs of Clonorchis sinensis promote the release of cytokines and induce inflammation [24–27]. CsESP are the main causes of liver fibrosis caused by Clonorchis sinensis, which can induce HSC activation and proliferation and lead to pathological changes in biliary epithelial cells [28]. Therefore, activation of the NF-κB signalling pathway and cytokine release play important roles in the progression of liver fibrosis induced by Clonorchis sinensis infection [29–32]. Based on the above findings, it can be concluded that CsPLA2-induced activation of HSCs is not the result of the direct action of a single JNK signalling pathway on HSCs, but may also be caused by the NF-κB signalling pathway.

It is generally believed that the activation of signalling pathways is caused by the binding of proteins to their receptors on the cell surface [33–36]. TM7SF3 is located on the surface of HSCs and plays an important role in hepatic fibrosis caused by CsPLA2. Therefore, TM7SF3, which is the receptor of CsPLA2, contributes substantially to liver fibrosis caused by CsPLA2.

Conclusion
The NF-κB signalling pathway is involved in the activation of HSCs by CsPLA2. The expression level of the marker of NF-κB signalling pathway activation, phospho-p65, is upregulated in the context of liver fibrosis. Additionally, TM7SF3, the receptor of CsPLA2, which is located on the surface of HSCs, is related to liver fibrosis caused by CsPLA2, and inhibition of TM7SF3 with a TM7SF3 antibody relieves liver fibrosis caused by CsPLA2.
Abbreviations

Clonorchis sinensis, CspLa2: Secreted phospholipase A2 of Clonorchis sinensis; iPLA2: Secreted phospholipase A2; ESPs: Excretory and secretory products; MBP: Maltose-binding protein; TM7SF3: Transmembrane 7 superfamily member 3.

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Authors’ contributions

YJW and XRL conceived and designed the experiments; YJW, QH and YL performed the experiments; XRL, YJW, MS, YXY and XD analysed the data; and YJW, XRL and QH wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The nucleic acid sequence of CspLa2 supporting the conclusions of this article is available in the GenBank repository (accession numbers: DQ 974199) at https://www.ncbi.nlm.nih.gov/ncbi.nlm.nih.gov. The nucleic acid sequence of TM7SF3 supporting the conclusions of this article is available in the Gene repository (Gene ID: 51768) at https://www.ncbi.nlm.nih.gov/gene/51768.

Declarations

Ethics approval and consent to participate

All work was conducted with appropriate permits and permissions. All animals were housed in accordance with guidelines from the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal protocols were approved by the Institutional Review Board and the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University (permit number SCXK Guangdong, 2009–2011).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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