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GGPPS deficiency aggravates CCl_4-induced liver injury by inducing hepatocyte apoptosis

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ABSTRACT

GGPPS catalyses the expression of GGPP, a key protein in the mevalonate metabolic pathway. HMG-CoA reductase inhibitor statins can induce liver injury by inhibiting GGPP. However, the function of *GGPPS* in liver injury has not yet been revealed. In this study, we found that *GGPPS* increased in liver injury and that *GGPPS* deletion augmented liver injury and fibrosis. *GGPPS* inhibition induced hepatocyte apoptosis, inflammation and TGF-β1 secretion, which activated hepatic stellate cells. Our findings imply that *GGPPS* deletion induces hepatocyte apoptosis, which makes the liver vulnerable to hepatotoxicity.

1. Introduction

Liver fibrosis is characterised by the excessive accumulation of extracellular matrix (ECM) proteins, which occurs in most types of chronic liver diseases [1]. Toxicity and damage, including that caused by chronic hepatitis C virus (HCV) infection, alcohol, non-alcoholic steatohepatitis (NASH) and drugs, injure the liver and promote the generation and release of growth factors (platelet-derived growth factor (PDGF), transforming growth factor-beta 1 (TGF-β1), etc.) and cytokines that activate HSCs, the main ECM-producing cells in the injured liver. After liver injury, the activated form of HSCs play a vital function in the production and degradation of ECM. The imbalance between ECM production and degradation ultimately leads to liver fibrosis.

Geranylgeranyl pyrophosphate (GGPP) is a key protein in the mevalonate metabolic pathway and functions in the protein prenylation of small GTPases, which is necessary for their activation [2]. GGPP has been reported to have a vital function in the restoration of statin-induced liver injury, which is an unavoidable type of liver injury [3]. Many articles have reported severe hepatotoxicity attributed to statins due to over-dose or idiosyncrasy [4]. A higher dose of statin ($\geqslant$1 defined daily dose) and the use of rosuvastatin before the liver injury event are reported to be associated with liver injury [5]. While inhibiting 3-hydroxy-3-methylglutarylcoenzyme

Abbreviations: GGPPS, geranylgeranyl diphosphate synthase; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutarylcoenzyme A; CCl4, carbon tetrachloride; ECM, extracellular matrix; COL1A1, type I collagen; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; HSC, hepatic stellate cells; plpC, polyriboinosinic-polyribocytidylic; PARP, poly ADP-ribose polymerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, foetal bovine serum; shRNA, short hairpin RNA; α-SMA, α-smooth muscle actin; WT, wild type; CTGF, connective tissue growth factor; TGF-β1, transforming growth factor-beta 1; PDGF, platelet-derived growth factor; FPP, farnesyl pyrophosphate; PCNA, proliferating cell nuclear antigen; TNF-α, tumour necrosis factor-alpha; MAPK, mitogen-activated protein kinase; HCC, hepatocellular carcinoma

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A (HMG-CoA) reductase, statins suppress hepatocyte viability and induce cell death [3,6]. However, the inhibitory effect can be reversed via the supplementation of mevalonate or GGPP [3].

GGPP is biosynthesized from acetyl-CoA through the mevalonate metabolic pathway catalysed by geranylgeranyl diphosphate synthase (GGPPS) [7,8]. The role of the catalytic enzyme GGPPS has been revealed in many diseases. Shen et al. found that *GGPPS* functions in the signalling pathway controlling cigarette smoke-induced pulmonary inflammation [9]. Wang et al. reported that the deletion of *GGPPS* in mouse Sertoli cells caused infertility [10]. The research by Xu et al. suggested that the cardiac-specific knockout of *GGPPS* resulted in hypertrophic cardiomyocyte growth [11]. Our previous study revealed that hepatocellular carcinoma (HCC) patients with cirrhosis had relatively higher expression of *GGPPS* [12]. Based on the understanding that GGPP has a prominent role in the restoration of statin-induced liver injury, we speculate that the catalytic enzyme GGPPS might also play a role in liver injury.

To date, the function of *GGPPS* in liver injury has not yet been reported. In this study, we aim to investigate the role of *GGPPS* in liver injury and to reveal the possible mechanism for its participation in this common disease.

2. Materials and methods

2.1. Reagents

Carbon tetrachloride (CCl_4), olive oil and polyriboinosinic-polyribocytidylic (pIpC) dsRNA were purchased from Sigma Chemicals Co. (USA). Primary antibodies against caspase-3, cleaved caspase-3, caspase-9, cleaved caspse-9, poly ADP-ribose polymerase (PARP), cleaved PARP, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Mcl-2, Bcl-2, p-JNK, JNK, p-ERK, ERK, p-p65 and p65 were purchased from Cell Signalling Technology (Beverly, MA). The primary antibody against GGPPS was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). TGF-β1 neutralization antibody (ab647) was purchased from Abcam (Abcam, Cambridge, UK).

2.2. Cell culture

The human liver cell line LO2 and the hepatic stellate cells (HSC) cell line LX2 were obtained from the Cell Bank of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. LO2 cells were supported in RPMI-1640 medium (Gibco, Gaithersburg, MD) containing 10% foetal bovine serum (FBS) (Gibco, Gaithersburg, MD). HSC-LX2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD) supplemented with 10% FBS. For co-culture experiments, LO2 cells were cultured in DMEM containing 10% FBS. All of the cells were maintained at 37 °C in an incubator supplemented with 5% CO_2 under humidified conditions.

2.3. Patients and samples

Liver specimens from 6 patients with liver cirrhosis and 6 patients with hemangioma from the Department of Hepatobiliary Surgery of Drum Tower Hospital were included in the study. This study was approved by the Scientific Research Ethics Committee of the affiliated Drum Tower Hospital, Medical School of Nanjing University, and informed consents were obtained from all participants.

2.4. Mice and CCl_4 administration methods

Eight-week-old female C57BL/6 mice were used in acute liver injury experiments. Liver-specific *GGPPS* knockout mice ($GGPPS^{-/-}$) were generated as described [13]. Briefly, two LoxP sites were inserted into the *GGPPS* gene, flanking exons 3 and 4. Liver-specific GGPPS knock-out mice were generated by breeding *GGPPS*-floxed mice with Mx1-Cre transgenic mice, which express Cre recombinase. Mx1-Cre $GGPPS^{flox/flox}$ mice were termed $GGPPS^{-/-}$, and $GGPPS^{flox/flox}$ mice obtained from the same breeding were termed as wild type. To induce the deletion of *GGPPS*, $GGPPS^{-/-}$ and control mice were injected intraperitoneally (i.p.) with pIpC dsRNA (20 μg/g body weight) three times at a 2-day interval. All animal procedures were carried out in accordance with the Animal Care and Use Committee of the Model Animal Research Centre of Nanjing University, Nanjing, China.

For CCl_4 administration, CCl_4 was mixed with olive oil at the volume ratio of 1:3. In the acute liver injury model, every mouse was given a single i.p. injection of CCl_4 (1 μl/g body weight) and sacrificed after 0 h, 24 h, 48 h and 72 h. In the liver fibrosis model, every mouse was given two i.p. injections of CCl_4 (1 μl/g body weight) every week on Tuesday and Friday for 7 consecutive weeks and sacrificed 1 week after the last injection. Olive oil (1 μl/g body weight) was given to control mice. Each group contained 8 mice.

2.5. qRT-PCR

The relative mRNA level changes were determined by quantitative real-time PCR. Briefly, total RNA was extracted using TRIzol reagent (TaKaRa, Japan) and the reverse transcribed to cDNA using Primescript RT master mix (TaKaRa, Japan). The cDNA was subjected to real-time PCR using a SYBR Green PCR Kit (TaKaRa, Japan) and an ABI PRISM 7300 Sequence Detector. The internal control was the 18S rRNA gene. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are listed in Table 1.

2.6. Western blotting

Protein levels were determined by Western blotting following published protocols [14]. Protein levels were detected by incubating immunoblots with primary antibody followed by incubation with HRP-conjugated secondary antibody. The signal was developed with ECL (Millipore, Switzerland) and visualised using a Tanon 5200 imaging system (Tanon, China).

2.7. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay

Cell apoptosis in mouse liver was determined by a commercial apoptosis detection kit (DeadEnd™ Fluorometric TUNEL System, Promega) following the manufacturer's instructions. The nuclear was visualised by DAPI staining.

2.8. short hairpin RNA (shRNA) transfection

GGPPS-targeting oligonucleotide sequences were synthesized and inserted into a GV115 lentiviral expression vector (GeneChem, Shanghai, China). *GGPPS* targeting sequence: shRNA 1: TGAGCTAGTAGCCTTAGTA; shRNA 2: TTGAAGCTAAAGCCTATAA. *GGPPS* shRNA 1 and 2 were transfected into LO2 cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Vectors containing scramble sequences were used as a negative control.

2.9. Flow cytometry

Cells were harvested and collected by centrifugation. Cells were washed with PBS and resuspended in 500 μl binding buffer, after which 5 μl of Annexin V-FITC and 5 μl of PI (Beyotime, Nantong, China) were then added. The cells were incubated in the dark for 10 min and then subjected to flow cytometric analysis.

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