

Received: 2016.05.16  
Accepted: 2016.06.14  
Published: 2016.07.06

# Hepatitis C Virus Increases Free Fatty Acids Absorption and Promotes its Replication Via Down-Regulating GADD45 $\alpha$ Expression

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Statistical Analysis C  
Data Interpretation D  
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**Source of support:** The present study was supported by Natural Science Foundation of Hubei Province Grant 2012FFB04411

**Background:** Hepatitis C virus (HCV) infection, as a major cause of chronic hepatic diseases, is always accompanied with an abnormality of lipid metabolism. The aim of this study was to investigate the pathogenic role of free fatty acids (FFA) in human HCV infection.

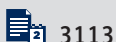
**Material/Methods:** Peripheral blood lipid indexes among HCV patients with different viral loads (199 samples) and healthy donors (80 samples) were detected by clinical biochemistry tests. HCV replication and the expression of growth arrest and DNA-damage-inducible gene 45- $\alpha$  (GADD45 $\alpha$ ) in Huh7 cells and clinical samples were quantified by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting. Lipid accumulation in Huh7 cells was detected by immunofluorescence.

**Results:** In this study, we found that FFA showed a significant positive correlation with viral load in peripheral blood of HCV patients, but not total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), or low-density lipoprotein cholesterol (LDL-C). GADD45 $\alpha$  expression in HCV patients dramatically decreased with the increase of viral load. In Huh7 cells, FFA treatment significantly enhanced HCV replication. HCV infection inhibited GADD45 $\alpha$  expression, and this effect was further enhanced with the presence of FFA treatment. Ectopic expression of GADD45 $\alpha$  in HCV-infected Huh7 cells markedly inhibited the absorption of FFA and HCV replication. However, FFA significantly elevated GADD45 $\alpha$  expression without HCV infection.

**Conclusions:** These results demonstrated that HCV down-regulates GADD45 $\alpha$  expression to enhance FFA absorption and thus facilitate its replication. GADD45 $\alpha$  is an essential mediator for the pathogenesis of HCV infection. Thus, our study provides potential clues in the search for novel therapeutics and fatty lipid control options for HCV patients.

**MeSH Keywords:** **Fatty Acids, Nonesterified • Hepatitis C Antibodies • Lipid Accumulation Product**

**Full-text PDF:** <http://www.medscimonit.com/abstract/index/idArt/899591>



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## Background

Human hepatitis C virus (HCV) infects approximately 170 million persons and adds about 3 million new infections each year worldwide [1,2]. In China, a recent survey showed that total HCV infection rate was 0.4% in the human population in six regions during 2006–2008 [3]. Among HCV-exposed individuals, up to 60–85% will develop chronic infection, which can ultimately lead to hepatic steatosis, fibrosis, cirrhosis, hepatocellular carcinoma, and even death [4]. Due to the high prevalence of HCV chronic infection and its relation to liver diseases, studies in recent years have confirmed that HCV hijacks cellular lipid metabolism to facilitate its replication [5–7]. HCV core protein induces lipid accumulation in hepatocytes by inhibiting microsomal triglyceride transfer protein (MTP) activity [8]; HCV enhances the production of reactive oxygen species (ROS), which results in the peroxidation of membrane lipids, and coopts the very low density lipoprotein (VLDL) secretion pathway for viral maturation or secretion [9]. In addition, HCV also promotes its own replication and inhibits the antiviral effects of IFN- $\alpha$  via inducing the *de novo* synthesis of fatty acids and impairs fatty acid oxidation [10–12]. Importantly, inhibitors of fatty acid biosynthetic pathways have been effectively used to inhibit HCV replication [13,14]. These research results suggest a strong contribution of lipid metabolism to HCV replication in hepatocytes.

Altered serum lipid profiles are common histologic features of chronic infection with HCV [15]. Chronic HCV patients exhibit low levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) compared with uninfected controls [16]. Free fatty acids (FFA), mainly composed of palmitic and oleic acids, are the major components of liver triglycerides that overflow to hepatocytes and cause hepatic steatosis [17]. Recently, it was found that FFA promote HCV RNA replication in HCV replicon cells and attenuate the antiviral response of IFN- $\alpha$  in a cell culture model [12]. More importantly, FFA treatment also induces an increased amount of intracellular ROS in Huh7 cells [18]; enhanced ROS production has been thought to injure liver cells and cause alanine transaminase (ALT) secretion. Most patients with chronic HCV have elevated or fluctuating levels of serum ALT [19,20], and the presence of elevated ALT levels in HCV patients is frequently used as a guideline for commencing treatment [21,22].

Genome-wide expression analysis found that FFA treatment induced a relatively high level of GADD45 $\alpha$  mRNA expression [23]. GADD45 $\alpha$  is a member of the GADD45 family, which mainly serves as a tumor suppressor with the abilities to regulate cell proliferation, DNA repair, and apoptosis [24–26]. Overexpression of GADD45 $\alpha$  leads to growth suppression in numerous cell types; however, when GADD45 $\alpha$  is depleted or

repressed, cells show uncontrolled proliferation [27]. As for the role of GADD45 $\alpha$  in HCV infection, several studies have found that the HCV non-structural 5A (NS5A) protein depends on p53 to down-regulate GADD45 $\alpha$  expression and subsequently triggers abnormal cellular proliferation in an HCV-infected cell model [28,29]. Furthermore, patients with HCV-infected hepatocellular carcinoma also exhibit a low level of GADD45 $\alpha$  expression in tumor tissues [30,31]. However, HCV proteins including core, NS3, NS5A, and HCV subgenomic replicon induce ROS production in human hepatoma cells [32], and enhanced ROS production has been found to have the ability to stimulate GADD45 $\alpha$  expression via activating the JNK/p53 pathway [33]. Thus, the interaction between HCV and GADD45 $\alpha$  is complicated. In the present study, we investigated the role of FFA and GADD45 $\alpha$  in HCV infection and found that HCV increases FFA absorption and promotes its own replication via down-regulating GADD45 $\alpha$  expression.

## Material and Methods

### Huh7 cell line culture and materials

The Huh7 cell line was acquired from the Basic Medicine School of Wuhan University and was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) containing 10% fetal bovine serum (BCS) (Hyclone), 1% penicillin, and 1% streptomycin (Beyotime, China). HCVcc-JFH-1 was provided by the laboratory of Wen-Zhe Ho (Wuhan University, Wuhan). The HCV RNA polymerase chain reaction (PCR) commercial kit was from Daan Gene Co. Ltd., Guangzhou, China; FFA detection kits, HDL-C kit, and LDL-C kit were purchased from Sekisui Medical Technology (Japan). The TC, triglyceride (TG), and alanine aminotransferase (ALT) test kit was from Beijing Lab Biotech Co. (Beijing, China). Oleate (No.0-7501) and palmitate (No.P-0500) were from Sigma. Oleate was mixed with palmitate in a 2:1 ratio to form the FFA mixture, as described by others [12,34].

### Participants

All HCV-positive patients were enrolled from the Department of Infectious Diseases of Zhongnan Hospital of Wuhan University from 2011 to 2013. Informed consent was obtained from each subject before inclusion in this study, and this study was approved by the hospital's ethics committee. Serum samples from 199 untreated HCV patients were collected before therapy. The diagnosis of chronic hepatitis was based on the criteria from the Hepatitis C Prevention Guide, which was created by the Parasitic Epidemiology branch of The Chinese Medical Association Society of Liver Diseases (<http://www.heporg.com>). The age range of the patients was 15 to 81 years; the average age was 47 years. Among them, 114 were male, and 85 were female. A total of 80 healthy subjects without liver diseases,

cerebrovascular diseases, and dyslipidemia were enrolled as a control group, who passed radiographic examination and serological detection in the physical examination center of the same hospital. The age range of the control group was 18 to 72 years; the average age was 43.7 years. Among them, 45 were male and 35 were female.

### Serum index detection

Serum samples of fasting venous blood from the 199 HCV patients were collected before treatment. Fasting venous blood from the control group also was collected. All blood samples were centrifuged for 10 min at 3000 rpm/min, and the collected serum was tested or stored at -20°C if the testing was not timely; serum must be tested within a week. First, HCV-RNA virus load was detected according to the manufacturer's protocol. Based on the result, the patients were divided into low ( $5.0 \times 10^2 \leq \text{HCV-RNA copy}$ ,  $< 10^3$  copies/mL,  $n=82$ ), medium ( $10^3 \leq \text{HCV-RNA copy}$ ,  $\leq 10^5$  copies/mL,  $n=61$ ) and high (HCV-RNA copies  $> 10^5$  copies/mL,  $n=56$ ) virus load groups according to the criteria outlined in the Hepatitis C Prevention Guide by the Parasitic Epidemiology branch of The Chinese Medical Association Society of Liver Diseases. Then the levels of serum FFA, ALT, TC, TG, HDL-C, and LDL-C were measured in both the patient and control groups. HCV-RNA viral loads were detected by the fluorescence quantitative real-time polymerase chain reaction (qRT-PCR) (ABI 7300 fluorescent quantitative PCR analyzer); levels of serum FFA, ALT, TC, and TG were measured by an enzymatic method; and HDL-C and LDL-C were measured by a direct method (Beckman 5400 automatic biochemical analyzer). To ensure the accuracy and reproducibility of test results, all above tests were controlled by the hospital internal quality control department and assessed by Ministry of Health Clinical Laboratory Center external quality assessment.

### Human liver tissues

Tumor tissues from liver cancer patients coinciding with chronic HCV infection were examined. Similar tissues from uninfected patients with liver cancer were used as controls.

### RNA quantification by fluorescence qRT-PCR

HCV RNA was quantified by fluorescence qRT-PCR. The HCV RNA from HCV-infected Huh7 cells was extracted using the TRIzol reagent (Invitrogen, Carlsbad, California, USA). The HCV primers, forward (5'-AGCCATGGCGTTAGTATGAGTGTC-3') and reverse (5'-ACAAGGCCTTTCGCAACCCAA-3'), were designed based on the HCV sequence (GenBank accession no. M67463) that was complementary to a sequence within the 5' untranslated region of the HCV genome, according to a previous method [35]. RNA was quantified using RT-PCR with the SYBR Green Real-Time PCR Master Mix kit (Toyobo). GAPDH was the endogenous

control gene, forward primer (5'-ACCACAGTCCATGCCATCAC-3') and reverse primer (5'-TCCACCACCTGT TGCTGTA-3').

### siRNA and overexpression assays

siRNA inhibition of GADD45 $\alpha$  was purchased from Qiagen (Milano, Italia): Hs\_GADD45 $\alpha$ \_5\_HP validated siRNA (NM\_001924). Huh7 cells were transfected with 5 nM double-stranded siRNA using HiPerfect transfection reagent (Qiagen) according to the manufacturer's protocol. GADD45 $\alpha$  depletion was evaluated through Western blot. Full length of GADD45 $\alpha$  cDNA (GenBank accession No. M60974) was amplified from human normal liver tissue and then subcloned in-frame into pcDNA3.1 to generate the recombinant GADD45 $\alpha$  eukaryotic expression plasmid forward primer (5'-CGTAAGCTTCTGCAGTTTGCAATATG-3') and reverse primer (5'-GCTAGATATCGATGC CATCACCGTTTCAG-3'). Huh7 cells were transfected with 2  $\mu\text{g}$  of total DNA of pcDNA3.1-GADD45 $\alpha$  using Lipofectamine 2000 (Invitrogen). Expression of GADD45 $\alpha$  was identified by Western blotting 48 h later.

### Western blotting

Huh7 cells ( $2 \times 10^5$ ) were cultured in a 24-well plate to 80–85% confluence and then co-cultured with different concentrations of FFA with or without HCV. Cells were washed with phosphate-buffered saline (PBS) after 4 h, and then fresh DMEM containing FFA was added and cells were cultured for another 72 h. HCV-NS3 protein expression was detected by Western blot using NS3 mAb (8G-2, Abcam); GADD45 $\alpha$  antibody was from Santa Cruz Biotechnology (Santa Cruz, California, USA). Total cell extracts were prepared at 4°C in 135 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM CaCl<sub>2</sub>, and 1% Triton X-100 in the presence of phosphatase and protease inhibitors. Protein concentrations were measured colorimetrically with a BCA Protein Assay kit (Pierce) and then subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### ROS level detection

Huh7 cells were transfected with pcDNA3.1-GADD45 $\alpha$  or siRNA, and then treated with HCV with FFA for 72 hours. Cell samples were incubated with 5 M redox sensitive dye DCF-DA (Beyotime, Shanghai, China) for 30 minutes at 37°C. Reactive oxygen species-mediated oxidation of the fluorescent compound DCF was measured by excitation at 488 nm and an emission wavelength of 525 nm using a flow cytometer (BD Accuri C6).

### Immunofluorescence

Infected cells were grown on glass coverslips, washed twice with PBS supplemented with 1% BSA, and fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were then

**Table 1.** Comparison of liver function index and lipid-related indicators in each group (mean  $\pm$ SD).

HCV-RNA copy group	No.	FFA ( $\mu$ mol/L)	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	ALT (U/L)
Health control	80	132.14 $\pm$ 7.62	5.2 $\pm$ 1.15	1.49 $\pm$ 0.66	1.19 $\pm$ 0.22	3.26 $\pm$ 1.12	27.75 $\pm$ 1.74
High	56	651.33 $\pm$ 92.54 <sup>3,5</sup>	4.58 $\pm$ 1.03 <sup>2,5</sup>	1.29 $\pm$ 0.77 <sup>2,5</sup>	1.35 $\pm$ 0.44 <sup>2,5</sup>	2.77 $\pm$ 0.58 <sup>1,5</sup>	85.64 $\pm$ 5.75 <sup>1,4</sup>
Medium	61	590.99 $\pm$ 39.79 <sup>3</sup>	4.32 $\pm$ 1.17 <sup>1</sup>	1.34 $\pm$ 1.13 <sup>2</sup>	1.2 $\pm$ 0.50 <sup>2</sup>	2.5 $\pm$ 0.64 <sup>3</sup>	55.67 $\pm$ 3.47 <sup>1</sup>
Low	82	301.7 $\pm$ 49.31 <sup>1</sup>	4.41 $\pm$ 1.09 <sup>2</sup>	1.37 $\pm$ 1.11 <sup>2</sup>	1.25 $\pm$ 0.45 <sup>2</sup>	2.56 $\pm$ 0.61 <sup>3</sup>	41.45 $\pm$ 5.51 <sup>1</sup>
F value		123.5	2.89	1.91	0.91	6.03	109.8
P value		<0.01	>0.05	>0.05	>0.05	>0.05	<0.01

<sup>1</sup> Compared with control group,  $p<0.05$ ; <sup>2</sup> Compared with control group,  $p>0.05$ ; <sup>3</sup> Compared with control group,  $p<0.01$ ; <sup>4</sup> Compared with groups of copy  $<10^5$ ,  $p<0.05$ ; <sup>5</sup> Compared with groups of copy  $<10^5$ ,  $p>0.05$ ; <sup>6</sup> Compared with groups of copy  $<10^5$ ,  $p<0.01$ .

**Table 2.** Correlation analysis of HCV RNA virus load and levels of serum lipid-related indicators in patient groups.

Groups	P value	r value	Groups	P value	r value
FFA and RNA copy	0.04 <sup>1</sup>	0.76	FFA and TG	0.00 <sup>2</sup>	0.42
FFA and ALT	0.03 <sup>1</sup>	0.68	FFA and HDL-C	0.12	-0.18
FFA and TC	0.94	0.19	FFA and LDL-C	0.15	0.16

<sup>1</sup>  $p<0.05$ ; <sup>2</sup>  $p<0.01$ .

washed three times. For staining lipid droplets, Bodipy-493/503 1  $\mu$ g/mL (Thermo Fisher Scientific, Inc.) was used; the nuclei were counterstained by Hoechst 33258.

### Statistical analysis

SPSS 19.0 was used for clinical data analysis, and results from cellular studies were analyzed by GraphPad Prism 5.0. Quantitative data are indicated by mean  $\pm$ SD. Differences among different groups were tested by one-way analysis of variance (ANOVA) followed by the Neuman-Keuls post hoc test, and a Spearman correlation analysis was performed to examine factors related to HCV-RNA viral load. A  $p$  value of  $<0.05$  was considered statistically significant.

## Results

### FFA concentration positively correlates with HCV viral load in peripheral blood of HCV patients

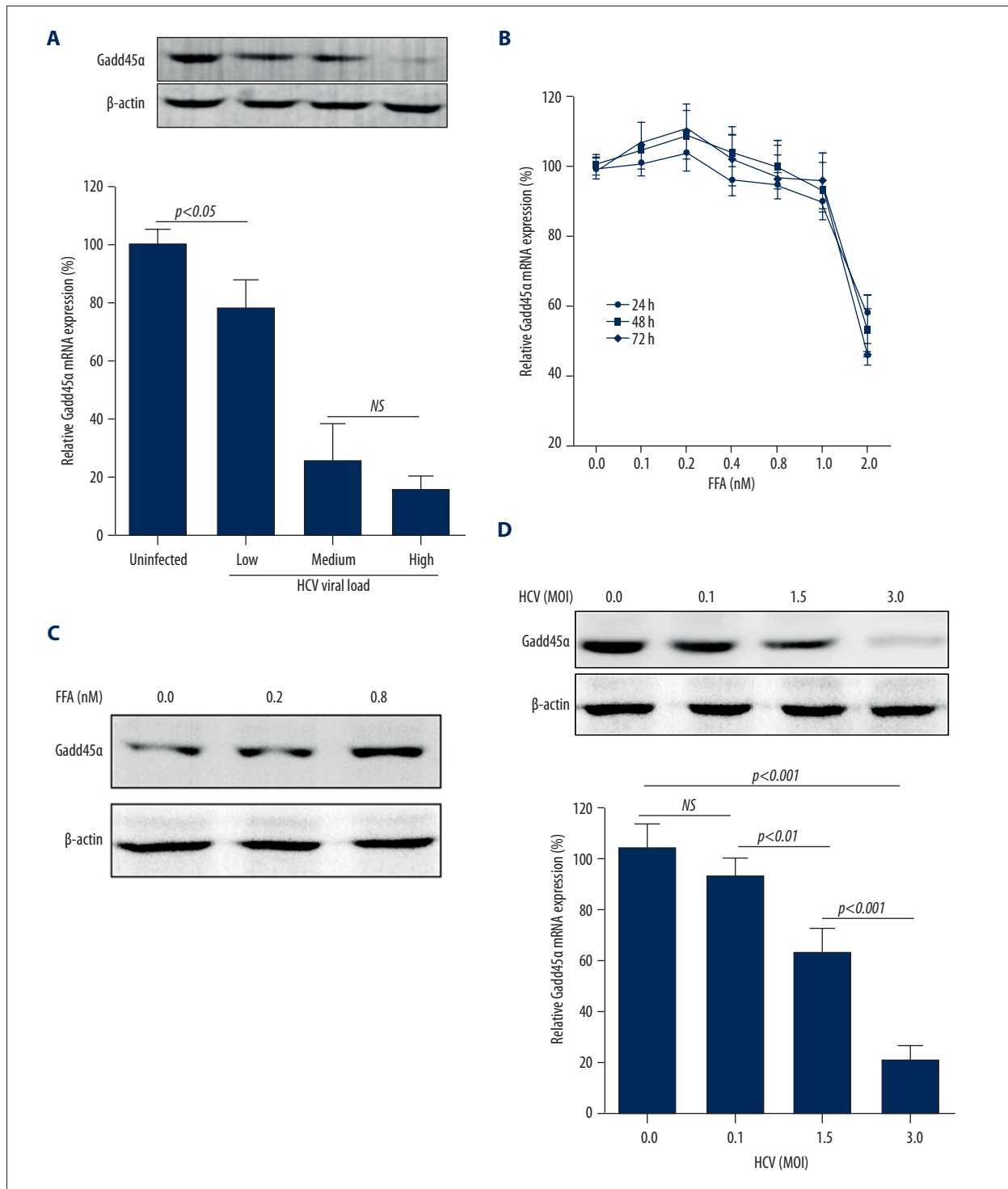
HCV-positive patients were divided into different groups according to HCV RNA load in peripheral blood, as shown in Table 1. The levels of FFA in the low, medium, and high viral load groups were significantly higher than the level in the control group ( $p<0.05$ ,  $p<0.01$ , and  $p<0.01$ ). Concentrations of TC,

TG, HDL-C, and LDL-C did not exhibit a consistent trend with HCV RNA load, although TG decreased with HCV RNA load, but there was no marked difference on the whole. We also evaluated the hepatocellular injury index, ALT in peripheral blood. ALT is the principal reference standard biomarker to diagnose liver dysfunction [36]. Our data revealed that HCV significantly increased the degree of hepatocellular injury with the increase in HCV viral load (Table 1).

The Spearman correlation analysis of levels of serum FFA with other indicators in patient groups is summarized in Table 2. Levels of serum FFA were positively correlated with HCV viral load and serum ALT concentration ( $p<0.05$ ). Levels of serum FFA were highly correlated with serum TG ( $p<0.01$ ); although they were not correlated with serum TC, HDL-C, and LDL-C ( $p>0.05$ ), this may be due to the fact that FFA are metabolic intermediates of TG [37].

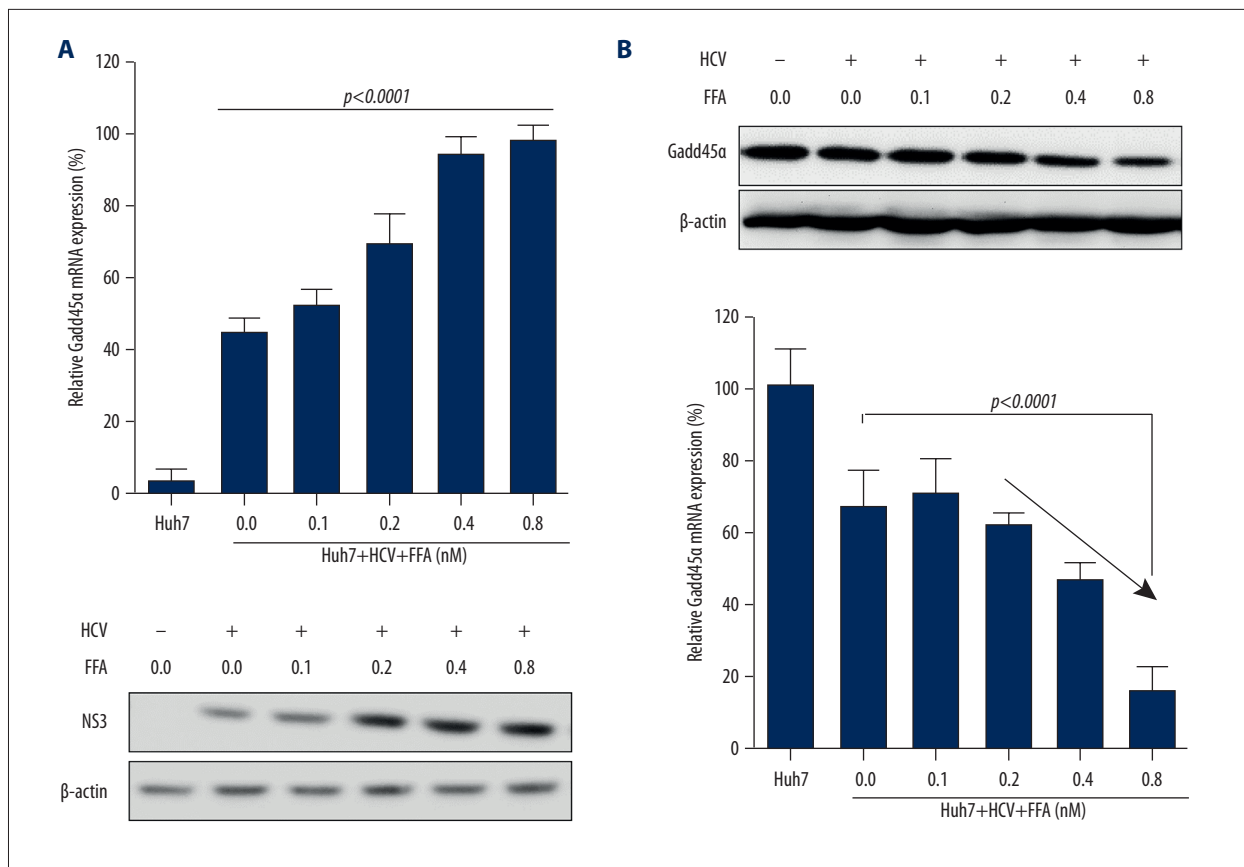
### GADD45 $\alpha$ expression is inhibited in liver cancer tissues from HCV-infected patients

We measured GADD45 $\alpha$  expression in tumor tissues from liver cancer patients with chronic HCV infection. Uninfected liver cancer patients were used as controls. GADD45 $\alpha$  protein expression and mRNA level in tumor tissues were significantly decreased with the increase of serum viral load (Figure 1A).



**Figure 1.** The role of FFA and HCV in GADD45α expression. **(A)** GADD45α protein expression and mRNA level were detected in tumor tissues from liver cancer patients coinciding with chronic HCV infection. Low, medium, and high mean different serum viral loads. **(B)** CCK-8 assay showing the effect of different concentrations of FFA (up to 2 mM) on cell viability of Huh7 cells. Cell viability was expressed as percentage of untreated cells. Huh7 cells ( $2 \times 10^5$ ) were cultured in a 24-well plate to 80%-85% confluence and then treated with different concentrations of FFA **(C)** or infected with HCV **(D)** for 72 hours. mRNA level of GADD45α was quantified by RT-PCR, and protein expression was detected by Western blotting. Results are shown as mean  $\pm$ SD of three independent experiments.





**Figure 2.** FFA promotes HCV replication in Huh7 cells. Huh7 cells ( $2 \times 10^5$ ) were cultured in a 24-well plate to 80%-85% confluence and then co-cultured with different concentrations of FFA with or without HCV (MOI of 1.5) for 72 hours. **(A)** HCV RNA copies were quantified by RT-PCR, and NS3 protein level was detected by Western blotting. **(B)** Quantification of the GADD45α protein levels and mRNA levels in Huh7 cells. Results are shown as mean  $\pm$  SD of three independent experiments.

### FFA promote GADD45α expression

We first evaluated the cell cytotoxic activity of FFA in Huh7 cells. FFA didn't exhibit an obvious adverse effect on cellular viability until 2 mM (Figure 1B), which greatly exceeded the range of concentration of FFA in HCV patients (<0.8 mM). This result hints that FFA alone (at the concentration in HCV patients) may not be enough to impact normal cell function. We then identified the influence of FFA on GADD45α expression; FFA significantly promoted GADD45α expression in Huh7 cells (Figure 1C).

### HCV infection down-regulates GADD45α expression

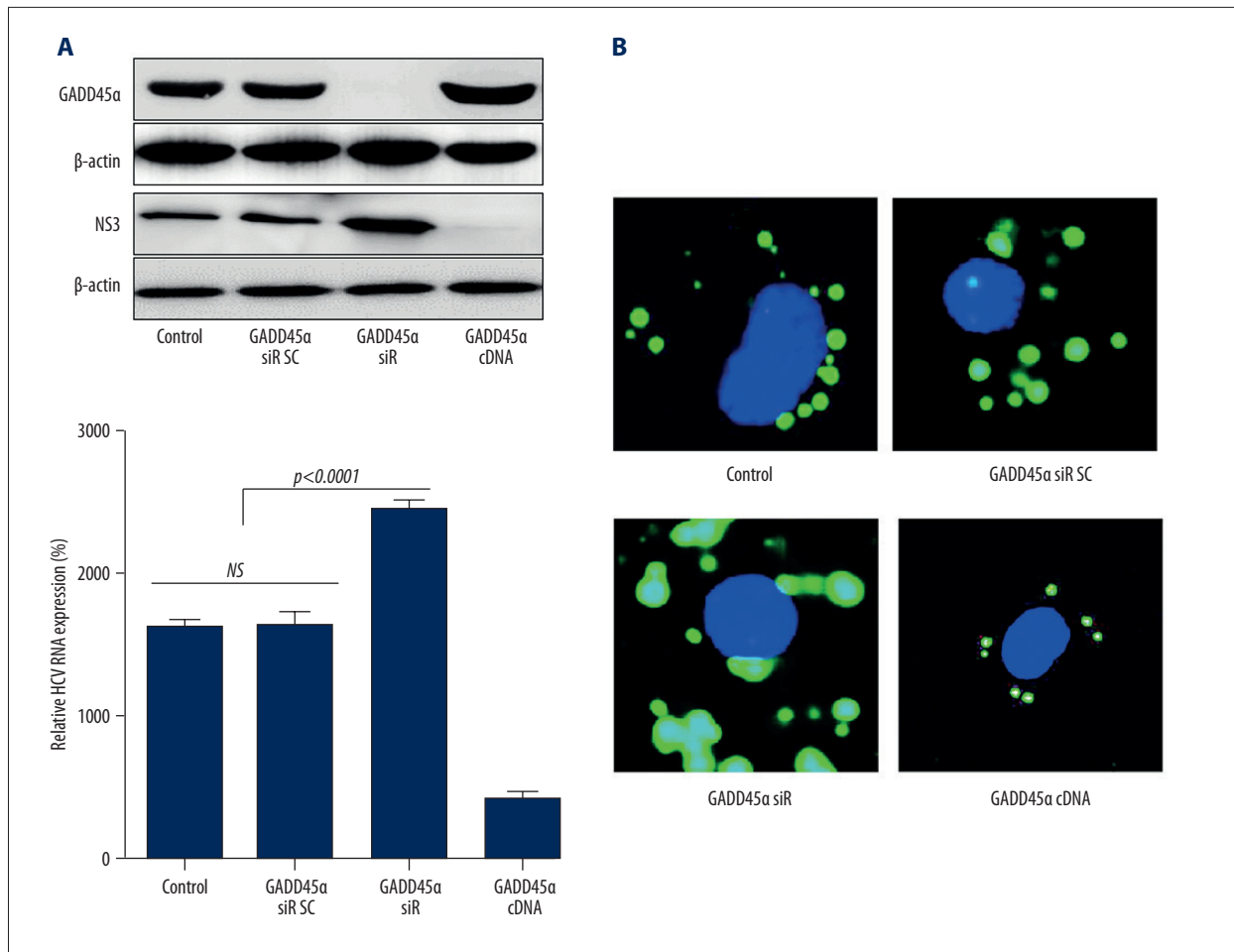
However, in chronic HCV patients, serum concentration showed a contrary change with GADD45α expression, and HCV NS5A protein has been showed to down-regulate GADD45α expression and subsequently trigger aberrant cellular proliferation [29]. The expression of the GADD45α protein and mRNA level were next examined in HCV-infected Huh7 cells. Data revealed that GADD45α expression was reduced in a multiplicity of infection (MOI)-dependent manner (Figure 1D).

### FFA promote HCV replication

To assess the influence of FFA on HCV replication, HCV-infected Huh7 cells (in the condition of same MOI) were stimulated with different doses of FFA. As shown in Figure. 2A, with the increase of FFA concentration, HCV RNA copies and NS3 protein expression were significantly elevated, while protein expression and mRNA levels of GADD45α were markedly decreased (Figure 2B). Thus, these results suggested that FFA promote HCV replication, and then the increased HCV causes the inhibition of GADD45α expression.

### Suppression of GADD45α expression promotes HCV replication and FFA accumulation

To further figure out the correlation of FFA, GADD45α, and HCV infection, HCV replication and FFA accumulation were detected with the presence of GADD45α siRNA or cDNA expressive vector. Huh7 cells were pre-transfected with GADD45α siRNA or pcDNA3.1-GADD45α recombinant eukaryotic expression vector for 48 h, and then infected with HCV and treated by FFA



**Figure 3.** GADD45α plays a vital role in regulating FFA-mediated HCV replication. Huh7 cells were transfected with GADD45α siRNA, a negative control siRNA (scramble), or GADD45α over-expressed plasmid. Forty-eight hours later, GADD45α expression was identified (A), and cells were then infected with HCV and simultaneously treated with FFA (0.8 mM). After 72 h, the relative HCV-NS3 protein and RNA expression were detected by Western blotting and RT-PCR (A); intracellular lipid accumulation was detected by Bodipy-493/503 staining and visualized after 400× amplification (B). Results are shown as mean ±SD of three independent experiments.

(0.8 mM). Depletion of GADD45α significantly enhanced HCV replication (Figure 3A) and intracellular lipid droplet accumulation (Figure 3B); however, overexpression of GADD45α almost totally reversed this phenomenon.

#### GADD45α decreases ROS production induced by HCV infection

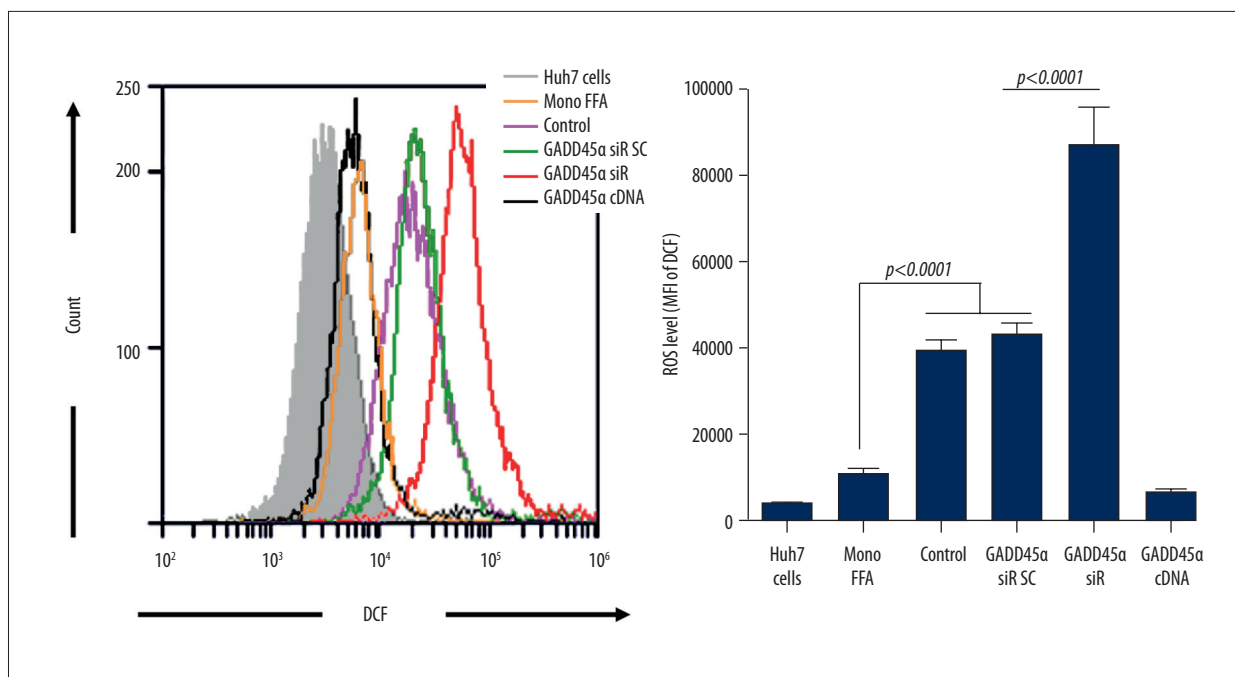
Elevated intracellular ROS production can be induced by FFA treatment and HCV infection, separately, and is usually correlated with an abnormally high level of serum ALT. As we have noticed that ALT is highly elevated in chronic HCV patients, we further evaluated the role of GADD45α in ROS production. As shown in Figure 4, FFA treatment alone caused a minor increase in ROS production; when accompanied with HCV infection, ROS production was greatly raised and further enhanced

after inhibition of GADD45α expression. GADD45α overexpression almost totally cleared up the ROS production induced by FFA and HCV infection.

To the best of our knowledge, this is the first report that reveals the correlation of FFA and GADD45α in HCV infection. FFA promote HCV replication, and enhanced HCV replication in turn accelerates FFA accumulation and ROS production of host cells via repressing GADD45α expression.

#### Discussion

HCV causes chronic liver diseases in 75–85% of the infected individuals; the outcomes include hepatic steatosis and even hepatocellular carcinoma [38]. Regarding the mechanisms



**Figure 4.** GADD45 $\alpha$  inhibits intracellular ROS production of HCV-infected Huh7 cells. Huh7 cells were transfected with GADD45 $\alpha$  siRNA, a negative control siRNA (scramble), or GADD45 $\alpha$  over-expressed plasmid. Forty-eight hours later, levels of intracellular ROS were analyzed by flow cytometry as described in Material and Methods.

involved in HCV-induced liver-related diseases, HCV-induced imbalance in lipid homeostasis seems to be the main cause. HCV infection is associated with enhanced lipogenesis, and reduced secretion and accumulation of lipids in host cells [39,40]. Despite great advances that have been made in our knowledge of HCV interaction with host lipid metabolism, the detailed mechanisms still need to be clarified.

In our study, chronic HCV patients were divided into three groups according to serum HCV viral load, namely, high, medium, and low. Then, blood lipid indexes for HCV patients with different viral loads and healthy donors were detected. We found that levels of FFA in all groups of HCV patients were significantly higher than the level in the healthy control group ( $p < 0.01$ ,  $p < 0.01$ , and  $p < 0.05$ ; Table 1). And importantly, serum FFA level was positively correlated with the level of enzymatic activity of serum ALT and HCV viral load (Table 2). ALT is a kind of enzyme in liver cells that could reflect the degree of liver injury or inflammation to a certain extent [36], which indicated that serum FFA had a close relation with hepatocyte injury and HCV infection.

FFA, which are mainly composed of palmitic and oleic acids, are the main lipid metabolites that are taken up by the liver [41]. Hepatocytes are able to accumulate FFA, and the increased fat content (until 1.2 mM) is not associated with a significant impairment of the cell integrity, in line with clinical and *in vivo* experimental data [42]. Similar results were also observed in

our study: an FFA concentration less than 0.8 mM (the highest level of serum FFA in our experimental populations) exhibited no detectable influence on cellular viability of hepatocytes (Figure 1B, Table 1). In natural conditions, genome-wide expression analysis has identified that FFA induce intracellular ROS production, and then enhanced ROS activates GADD45 $\alpha$  expression to induce cell apoptosis [18,23,43]. GADD45 $\alpha$  is a tumor suppressor and plays an important role in the control of cell cycle checkpoint, cell apoptosis, the DNA repair process, and signaling transduction [44,45]. Importantly, GADD45 $\alpha$  is down-regulated in HCV-infected patients with hepatocellular carcinoma [30,31]. Cellular studies also found that HCV NS5A protein down-regulates GADD45 $\alpha$  expression and subsequently triggers abnormal cellular proliferation in an HCV-infected cell model [29]. We thus collected primary tumor cells from operative tissues of liver cancer patients with HCV infection, and indeed found that GADD45 $\alpha$  expression was down-regulated compared with that in uninfected liver cancer patients and was negatively correlated with serum viral load (Figure 1A). So, serum FFA level showed a contrary tendency with GADD45 $\alpha$  expression in chronic HCV patients, similar to the results observed in type 2 diabetes mellitus (T2DM), in which increased serum FFA levels showed a negative correlation with GADD45 $\alpha$  expression in peripheral blood mononuclear cells from T2DM patients [46].

Next, we took advantage of the HCV cell culture model to study the role FFA and GADD45 $\alpha$  play in HCV infection. In



Huh7 cells, FFA sole treatment induced GADD45 $\alpha$  expression in a dose-dependent manner, and HCV infection without FFA treatment significantly decreased GADD45 $\alpha$  expression in a MOI-dependent manner, similar to the influence of HCV NS5A protein on GADD45 $\alpha$  expression [29]. When Huh7 cells were simultaneously infected with HCV and treated with FFA, we found that FFA promoted HCV replication and decreased GADD45 $\alpha$  expression in a dose-dependent manner (Figure 2). Furthermore, we noticed that interfering GADD45 $\alpha$  expression in HCV-infected cells resulted in multiple effects: increased HCV replication, and enhanced FFA accumulation and ROS production in Huh7 cells. Thus, HCV enhances cellular FFA absorption and then facilitates its own replication via down-regulating GADD45 $\alpha$  expression.

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## Conclusions

We clarified for the first time that serum level of FFA closely correlates with HCV infection and that GADD45 $\alpha$  down-regulation is absolutely required for FFA-induced HCV replication. Our results demonstrated that fatty lipid control may provide important clues for the developing of novel therapeutic targets for HCV infection.

## Conflicts of interest

The authors have no financial conflicts of interest.

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