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Epigenetic harnessing of HCV via modulating the lipid droplet-protein, TIP47, in HCV cell models

Nada M. El-Ekiaby [a,*], Radwa Y. Mekky [a], Shereen A. El Sobky [b], Noha M. Elemam [a], Mohammed El-Sayed [c], Gamal Esmat [c], Ahmed I. Abdelaziz [a,*]

[a] Molecular Pathology Research Group, Department of Pharmacology and Toxicology, German University in Cairo, Cairo, Egypt
[b] Molecular Pathology Research Group, Department of Pharmaceutical Biology, German University in Cairo, Cairo, Egypt
[c] Endemic Medicine and Hepatology, Cairo University, Cairo, Egypt

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ABSTRACT

This study aimed at identifying potential microRNAs that modulate hepatic lipid droplets (LD) through targeting the Tail interacting protein of 47kDa (TIP47) in HCV infection.

Bioinformatics analysis revealed that miR-148a and miR-30a potentially target TIP47. Expression profiling showed that both microRNAs were downregulated, while TIP47 was upregulated in liver biopsies of HCV-infected patients. Forcing the expression of both microRNAs in JFH-I infected, oleic acid-treated Huh7 cells, significantly suppressed TIP47 expression and reduced cellular LDs with marked decrease in viral RNA. This study shows that miR-148a and miR-30a, regulate TIP47 expression and LDs in HCV infected cells.

1. Introduction

The accumulation of lipid droplets (LDs) in the liver of HCV infected patients suggests an association between HCV infection and numerous disorders of lipid metabolism [1,2]. Moreover, cultured cells infected with HCV showed a high LD content [3]. Various viral components important for the production of new virions were found to accumulate at the surface of lipid droplets, such as the core protein, NS5A as well as +ve and −ve RNA strands [3–6]. Disruption of the association of core protein or NS5A to LDs prevents the assembly and production of infectious viral particles, which highlights the importance of the HCV–LD interaction in the viral life cycle [3,7].

Proteins associated to LDs surface, regulating their structure and function, include the family of PAT proteins, named after its three first discovered members, **P**erilipin, **A**dipocyte differentiation-related protein (ADRP), and **T**ail-interacting protein of 47 kilo Daltons (TIP47), in addition to S3-12 and OXPAT/MLDP [8–10]. The expression of PAT proteins is affected by HCV infection. In HCV core expressing cells, ADRP was found to be significantly lower, while TIP47 was significantly higher compared to cells that are not expressing the core protein [5]. However, another study showed that TIP47 protein levels were lower in HCV replicating cells compared to control cells, while its mRNA expression was higher [11]. While HCV alters TIP47 expression, TIP47 in turn is essential for HCV life cycle. Overexpression of TIP47 increased replication and release of HCV particles, while it's silencing markedly decreased the release of infectious particles. The interaction between HCV and TIP47 was reported to be through the association of NS5A to the N-terminus of TIP47 [11,12]. This interaction possibly integrates LD membranes into the HCV membranous web, facilitating HCV replication and assembly [12]. TIP47 was even found to associate to the released viral particle [13]. Recently, HCV was shown to induce hepatic LD accumulation by suppressing peroxisome proliferator-activated receptor (PPAR)-α and angiopoietin-like protein 3 (ANGPTL3), known regulators of

Abbreviations: LD, lipid droplet; ADRP, Adipocyte differentiation-related

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* Corresponding authors at: Office: B5.133, German University in Cairo, The German University in Cairo – GUC, New Cairo City – Main Entrance Al Tagamoa Al Khames, 11835 Cairo, Egypt. Fax: +20 2 27581041 (A.I. Abdelaziz).

E-mail address: ahmed.abdel-aziz@guc.edu.eg (A.I. Abdelaziz).

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triglyceride homeostasis. This suppression was mediated by HCV induced upregulation of miR-27b, representing a novel mechanism contributing to the development of HCV induced hepatic steatosis [14]. Moreover, miR-27a was found to suppress HCV infection as well as cellular lipid storage in Huh7.5 cells through targeting and suppressing various lipogenic genes [15]. These data show that there is a noticeable interplay between HCV, lipid droplets along with their associated PAT proteins, and microRNAs. To date only very limited data concerning the role of microRNAs in regulating lipid droplets through regulating PAT proteins exist. Hence, this study aimed at examining the effect of microRNAs on hepatic TIP47 expression in an attempt to suppress hepatic lipid droplet formation and subsequently HCV infection.

2. Patients and methods

2.1. Patients

Liver needle biopsies were taken from 21 chronic HCV patients. All patients included are HBV negative and did not receive any treatment. Healthy liver biopsies were obtained from nine donors during liver transplantation. All patients were recruited from Al Kasr Al Aini Hospital, Cairo University Medical School. The study followed the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from all patients included in the study. Clinical parameters of the patients are presented in Table 1.

2.2. Cell culture

Human Hepatocellular Carcinoma cells (Huh-7) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L Glucose, L-Glutamine, 1% Penicillin/Streptomycin and 10% Fetal Bovine Serum (FBS). Cells were incubated in a CO_2 incubator adjusted to 5% CO_2 at 37 °C.

2.3. In-vitro transcription and transfection of HCV RNA

pJFH-I harboring the full-length HCV genotype 2a genome (kindly provided by Prof. Wakita) was used to generate HCV replicon cells. Briefly, pJFH-I was linearized using XbaI restriction enzyme, followed by purification of linearized DNA with phenol/chloroform. 1 μg of purified DNA was in vitro transcribed using MEGAscript® T7 Transcription Kit (Ambion, USA) yielding full length HCV RNA. 10 μg of HCV RNA were transfected into Huh7 cells using Superfect transfection reagent. 3 days post transfection, cell culture supernatant was collected, filtered using 0.45 μm filter and stored at −80 °C for later infection of naïve Huh7 cells.

Table 1
Patients' clinical data.

Patient #	Age	Gender	VL	ALT	AST	Metavir score
P1	43	M	117744	28	30	F1
P2	37	M	1180492	74	74	F1
P3	36	M	215500	80	62	F1
P4	28	F	169000	32	25	F1
P5	28	M	106000	58	37	F1
P6	50	M	1060000	56	60	F2
P7	35	F	1650000	18	21	F3
P8	25	M	45190	105	55	F1
P9	47	M	3230000	26	28	F3
P10	28	F	119000	33	26	F4
P11	46	M	1654000	78	101	F3
P12	55	M	179000	76	80	F3
P13	32	M	3120000	52	47	F1
P14	35	M	4020000	74	65	F1
P15	34	M	1790000	62	53	F1
P16	52	M	1120000	34	53	F4
P17	68	F	15432000	54	43	F2
P18	34	M	403200	42	55	F1
P19	40	F	53200000	60	44	F1
P20	38	F	7670000	15	18	F1
P21	56	F	56300	67	53	F4

2.4. RNA extraction and quantification

Total RNA was extracted from liver biopsies or Huh7 cells using Biozol reagent according to the manufacturer's instructions (Bioer Technology, China). The relative expression of TIP47 was quantified relative to the housekeeping gene β2-microglobulin (B2M) and the relative expression of miR-148a and miR-30a were quantified relative to the housekeeping gene RNU6B using TaqMan real-time quantitative polymerase chain reaction (PCR) (StepOne, Applied Biosystems).

2.5. Transfection of oligos

Huh7 cells were transfected with 25 nM mimics, antagomirs or siRNAs using Hiperfect transfection reagent (Qiagen) according to the manufacturer's instructions. Transfection was performed in 96-well plates for TIP47 mRNA quantification, intracellular LDs imaging and viral RNA quantification or in 24-well plates to examine TIP47 protein expression.

2.6. Fatty acid treatment

24 h post transfection with oligos; lipid loading of Huh7 cells was achieved by incubating the cells with 600 μM bovine serum albumin-coupled oleic acid (OA) (Sigma Aldrich). Cells were either harvested 48 h. after OA treatment for viral load and TIP47 mRNA quantification or after 72 h for LDs staining or protein expression examination using western blotting.

2.7. Western blotting

Cells were washed with PBS and proteins were extracted using laemmli buffer. Protein concentration was measured using Modified Lowry Protein Assay Kit, (Pierce Biotechnology, Inc.) according to the manufacturer's instructions. 30 μg of proteins were separated using SDS–PAGE. Proteins were transferred to nitrocellulose membrane and the expression of TIP47 and β-actin was evaluated using mouse anti-TIP47 (B3) antibody and mouse anti-β-actin (C4) antibody (Santa Cruz Biotechnology Inc., USA), respectively. Goat anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc., USA) along with TMB Membrane Peroxidase Substrate System (KPL Inc., USA) was used for colorimetric detection of the protein bands.

2.8. Lipid droplet staining

A 0.35% oil-red-O (ORO) stock solution (Serva, Germany) was prepared and filtered using a 0.22 μm filter. A working solution of ORO was freshly prepared by diluting the stock solution with double distilled water at a 6:4 ratio, was left to stand for 20 min and filtered using a 0.22 μm filter. Cultured cells were fixed with 4% formaldehyde in PBS for 10 min. Fixed cells were washed with 3 changes of PBS. Cells were permeabilized by incubating with 0.05% Tween 20 in PBS for 15 min followed by 3 washes with PBS then once with 60% isopropanol. Afterwards, cells were incubated with ORO working solution for 10 min followed by 4 washes with distilled water and then mounted with a mounting solution containing DAPI to stain the cell nuclei. LDs and nuclei were visualized using fluorescence microscopy with a 100× objective (Axiom Zeiss).