Original Article

Two Triacylglycerol Pathway Genes, CTDNEP1 and LPIN1, are Down-Regulated by hsa-miR-122-5p in Hepatocytes

Mahmood Naderi PhD[1], Abdolreza Pazouki MD[2], Ehsan Arefian PhD[3], Seyed Mahmoud Hashemi PhD[4], Fatemeh Jamshidi-Adegani PhD[5], Omid Gholamalamdari PhD[5], Sara Soudi PhD[6], Kayhan Azadmanesh MD PhD[7], Siamak Mirab Samiee PhD[8], Shahin Merat MD[1], Mohammad Gholami Fesharaki PhD[9], Mahdieh Mondanizadeh PhD[10], Mohammad Vasei MD•[1,11], Masoud Soleimani PhD•[12]

Abstract

Background: Expression of miR-122 is highly specific to hepatocytes of the liver. This miRNA is involved in lipid hemostasis of the tissue; however, there is no comprehensive understanding of its function in lipid hemostasis.

Materials and Methods: Since hepatocytes are responsible for part of Triacylglycerol (TAG) synthesis in the body, we hypothesized that miR-122, as the most abundant miRNA in the tissue, might regulate TAG metabolism by targeting key enzymes that are involved in its production pathway. A systematic computational analysis of putative targets of miR-122 identified *CTDNEP1* and *LPIN1* genes in the TAG pathway. We used dual-luciferase reporter assay, quantitative RT-PCR as well as western blot to confirm the repressive effect of miR-122 on *CTDNEP1* and *LPIN1* in TAG pathway.

Results: Real time PCR on liver needle biopsies with hepatosteatosis showed that miR-122 is up-regulated in hepatosteatosis. Surprisingly, the protein and RNA level of identified targets of miR-122 are also up-regulated in clinical samples, probably as a disproportionate feedback response to the high level of miR-122.

Conclusion: Our findings suggest that up-regulation of miR-122 can trigger the compensatory response of *LPIN1* and *CTDNEP1* in hepatosteatosis.

Keywords: Lipid metabolism, miR-122, NAFLD, triglycerides

Cite this article as: Naderi M, Pazouki A, Arefian E, Hashemi SM, Jamshidi-Adegani F, Gholamalamdari O, Soudi S, Azadmanesh K, Mirab Samiee S, Merat S, Gholami Fesharaki M, Mondanizadeh M, Vasei M, Soleimani M. Two triacylglycerol pathway genes, CTDNEP1 and LPIN1, are down-regulated by hsa-miR-122-5p in hepatocytes. *Arch Iran Med*. 2017**; 20(3):** 165 – 171.

Introduction

MicroRNAs are small (~22 nucleotides) non-coding regulatory molecules that play their role by binding to 3' untranslated region of mRNAs to either repress their translation or induce their degradation.[1] These small molecules are able to simultaneously repress multiple targets even within one biological pathway;[2] therefore, they are effective switch controllers within intrinsic biological processes such as metabolism, cell growth, differentiation and so on.[3]

Authors' affiliations: [1]Liver and Pancreatobiliary Diseases Research Center, Digestive Disease Research Institute, Tehran University of Medical Sciences, Tehran, Iran, [2]Minimally Invasive Surgery Research Center, Iran University of Medical Sciences, Tehran, Iran, [3]Department of Microbiology, School of Biology, College of Science, University of Tehran, Tehran, Iran, [4]Department of Immunology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [5]Stem Cell Technology Research Center, Tehran, Iran, [6]Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, [7]Department of Virology, Pasteur Institute of Iran, Tehran, Iran, [8]Food and Drug Laboratory Research Center, Tehran, Iran, [9]Department of Biostatistics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran, [10]Department of Biotechnology and Molecular Medicine, Arak University of Medical Sciences, Arak, Iran, [11]Department of Pathology, Tehran University of Medical Sciences, Shariati Hospital, Tehran, Iran, [12]Department of Hematology, Tarbiat Modares University, Tehran, Iran.

•**Corresponding authors and reprints:** Mohammad Vasei MD, Department of Pathology and Digestive Disease Research Institute, Tehran University of Medical Sciences, Shariati Hospital, Tehran, Iran. Tel: +98-21-66582505, E-mail: mvasei@tums.ac.ir.

Masoud Soleimani PhD, Department of Hematology, Tarbiat Modares University, Tehran, Iran. Tel: +98-21-82884508, E-mail: soleim_m@modares.ac.ir.

Accepted for publication: 25 January 2017

Among different types of miRNAs in liver, miR-122 is the most abundant and tissue-specific miRNA,[4] accounting for > 70% of the total miRNA in the tissue.[5] MiR-122 is known to be involved in lipid hemostasis in liver. Germline deletion of miR-122 in mice results in hepatosteatosis with marked reduction of VLDL secretion.[6] In a separate study, similar observations were found in which miR-122 deletion caused steatohepatitis, fibrosis and high incidence of hepatocellular carcinoma.[7] Esau, et al. found that inhibition of miR-122 by antisense oligonucleotide in normal mice caused hepatic and plasma cholesterol reduction and increased hepatic fatty acid oxidation. Their work for the inhibition of miR-122 on a diet-induced obesity mouse model resulted in reduced plasma cholesterol level and liver steatosis.[8] Despite the importance of hsa-miR-122 in regulation of fatty acid metabolism, the molecular mechanisms by which hsa-miR-122 regulates TAG pathway and hepatosteatosis are under debate. For instance, inhibition of hsa-miR-122 by antisense oligonucleotides by Esau, et al. showed reduced state of hepatosteatosis[8] while the loss of function of the miRNA by two aforementioned researches[6,7] resulted in elevated hepatosteatosis. These data highlight the importance of elucidating the role of miR-122 more comprehensively.

Considering the fact that hepatosteatosis is predominately formed by TAG accumulation in the liver[9] along with the reported reverse link between miR-122 expression and hepatosteatosis,[6,7] we hypothesized that hsa-miR-122 might regulate TAG metabolism by targeting key enzymes that are involved in its production

cascade. A systematic computational analysis of putative targets of miR-122 identified *CTDNEP1* and *LPIN1* genes in the TAG pathway. *LPIN1* catalyzes the conversion of phosphatidic acid (PA) to diacyglycerol (DAG), also known as phosphatidate phosphatase 1 (PAP1) activity, in glycerol phosphate pathway. *CTDNEP-1* is shown to dephosphorylate lipin-1,[10] causing endoplasmic reticulum over cytosolic localization of lipin-1 which may in turn increase the physiological expression of PAP1 activity and, hence elevated amount of TAG synthesis.[11]

To have an in-depth understanding of the repressive role of miR-122 on *CTDNEP1* and *LPIN1*, we used the hepG2 cell line as a model. Dual-luciferase reporter assay, quantitative RT-PCR as well as western blot were used to inspect the repressive role of miR-122 in transcriptomic and proteomic levels. We also measured the level of this miRNA and its relation with *CTDNEP1* and *LPIN1* expression in the liver of obese patients undergoing laparoscopic gastric bypass for the purpose of weight loss.

Materials and Methods

Bioinformatics analyses

All target predictions were done with mirSVR predicted target site scoring method,[12] provided on www.microrna.org website. In brief, all the predicted targets of hsa-miR-122 were extracted from the website. At the time, this approach resulted in 1737 predicted genes. The predicted list was computationally crosschecked with the list of involved genes in TAG pathway, downloaded and subsequently extracted from "Glycerolipid Metabolism" section of KEGG. All the crosscheckings were based on comparing Entrez Gene IDs of the genes as the unique identifier. Excel spreadsheet was programmatically used to do all the crosscheckings.

Cell culture

Human hepatocytes (hepG2) were cultured on F12 Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL), and maintained at 37ºC with 5% CO_2 in a humidified air. All the cell lines were authenticated using Short Tandem Repeat PCR. For transduction, hepG2 cells were plated at 5×10^5 cells/60 mm plate 24 hours prior to transduction. Then, the cells were transduced with control (empty backbone virus) and miR-122 virus with the multiplicity of infection (MOI) of two along with 2 µg/mL of polybrene. The cells were selected with 2 µg/mL of puromycin 48 hours after transduction for three consecutive days.

Vector construction

The pLEX-JRED-mir-122 plasmid was constructed by cloning a fragment, containing the pri-mir-122 (from -221 to +343 bp relative to the 5'-end of pri-mir-122), into the pLEX-JRED plasmid. The pri-miRNA was amplified by PCR on human genomic DNA extracted from white blood cells using blood & cell culture DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The ligation of digested PCR products and the vectors was carried out using T4 DNA ligase enzyme (Fermentas, Burlington, VT, USA), based on the manufacturer's instructions. In addition, 3'UTR regions of *LPIN1* and *CTDNEP1* were amplified from human genome and then cloned in psicheck-2 vector (Promega) by Xho-1/Not-1 overhangs, based on the manufacturer's instructions. Finally, the authenticity of the cloned vectors was approved by sequencing. Table 1 describes all the cloning primers and enzymatic overhangs for the cloning of the vectors.

Construct expression verification

To assess the functionality of the construct, pLEX-JRED-mir-122 was transduced into hepG2 cell line and after selection with 2 µg/mL of puromycin, the amount of miR-122 overexpression was measured in comparison with the control pLEX-JRED group using real-time RT PCR.

Virus packaging and concentration

Lentiviruses were produced by Trono methodology through calcium phosphate (CaPo4) transfection of transfer vector, psPAX2 and PMD2G into 293T cells.[13] Virus concentration was performed based on the article by Fallah, et al. in which polyethylene glycol and NaCl were used for viral concentration.[14]

Dual-Luciferase Reporter assay

Twenty-four hours prior to transduction, 2×10^5 hepG2 cells were seeded in 24-well plates. Then, the cells in control and test groups were respectively transduced with control and miR-122 virus as described in Cell Culture section. The cells were selected with 2 µg/mL of puromycin 48 hours after transduction for three consecutive days. Afterwards, each well of control and miR-122 groups was transfected with either psi-CHECK2-control vector or psi-CHECK2-3'UTR vector using Lipfectamine 2000 (Invitrogen, Carlsbad, Calif, USA). All the experiments were done in quadruple. Then, luciferase assay was performed 24 hours after transfection using the Dual-Luciferase reporter

Table 1. All the cloning oligos are listed.

Name of the primers	Sequence	Enzymatic overhang	Vector Name
Clo-h-pri-mir-122 (F)	CCGCTCGAGTGGAACACAGGCAGTGGAC	XhoI	pLEX-JRED
Clo-h-pri-mir-122 (R)	CGACGCGTAAAGTGCTGGGATTTCAGG	MluI	
Clo –h-LPIN1- 3' UTR region (F)	CCGCTCGAGTAACTTCACCTTTTGGAGAGAG	XhoI	psiCHECK-2
Clo –h-LPIN1- 3' UTR region (R)	AAGGAAAAAAGCGGCCGCTAGAATATGCTTGATAACACACC	NotI	
Clo –h-CTDNEP1- 3' UTR region (F)	CCGCTCGAGCAGCTGCTCCCCCTCCAC	XhoI	psiCHECK-2
Clo –h-CTDNEP1- 3' UTR region (R)	AAGGAAAAAAGCGGCCGCCCCAGAGAAGGACCTAAGGC	NotI	
"Clo" stands for cloning and "h" stands for human in the naming of the cloning oligos			

assay system (Promega, Madison, Wis) and Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer's instructions. The Renilla/firefly luciferase ratios were normalized against the control psicheck-2 (empty psicheck-2) vector.

Seed region mutagenesis

The seed region of hsa-miR-122-5p was mutated in two positions. For this purpose, the third and fifth guanines where substituted with cytosine and therefore, the final mutated mature sequence TGCACTGTGACAATGGTGTTTG was produced.

B-actin & Lipin-1 Western Blotting

For protein extraction, 5×10^5 hepG2 cells were seeded into 60 mm plates 24 hours prior to transduction. Then, the cells were transduced with either control or miR-122 virus. Then, the cells were selected with 2 μg/mL of puromycin 48 hours after transduction for three consecutive days and then the cells were lysed with RIPA buffer containing protease inhibitor. Protein concentration was determined using a BCA Protein Assay kit (Pierce, Thermo scientific). The cell lysates from control vector and miR-122 over-expressed hepG2 cells were resolved by 6% SDS-PAGE gel. For this purpose, 30 μg per lane of total protein was loaded and then ran with 100 V for 2 hours. The gel was transferred with 30 V for 16 hours into methanol-activated PVDF membrane. Control and miR-122 groups were tested in triplicate. Following transfer, the membrane was blocked with 3% BSA, diluted in TBS buffer for 2 hours. Then, overnight incubation with the primary antibodies, diluted in TBST buffer with 1% BSA, was performed for b-actin (Abcam Cat No: ab8227, 1/2000 dilution) and lipin-1 (Abcam Cat No: ab92316, 1/1000 dilution) at 4ºC. Afterwards, specific reactive bands were detected using an anti-rabbit IgG (Abcam Cat No: ab6721, 1/3000 dilution), conjugated with HRP. The immune-reactive bands were visualized by the ECL western blotting detection kit (Pars Tous Biotechnology Co, Mashhad, Iran) and then captured by Canon EOS 60D with Focal Length (FL) of 50 mm. The camera was set into manual focus (MF) in Bulb mode with aperture of 1.8 and the ISO of 400 in a completely dark room for 30 s and 5 min for b-actin and lipin-1, respectively.

Clinical samples

A total of 16 liver biopsies were taken from female obese patients during laparoscopic gastric bypass surgery. All the clinical specimens were obtained with informed consent from the patients, and approved by the Institutional Review Board of Hazrat Rasoul Hospital. During sampling, all the patients with a history of steroid drug consumption, viral hepatitis or alcohol consumption were excluded from the project. Prior to operation, fasting blood sugar (FBS), cholesterol, HDL, LDL, TG, age, diabetes status and body mass index (BMI) were determined. Two needle biopsies were taken from each patient; one was used for histopathological study and the other was utilized for gene quantification. All the liver biopsies were categorized into two groups including normal obese (three samples) and nonalcoholic fatty liver disease (NAFLD, thirteen samples), based on pathological examination. A biopsy was put in Normal Group when no fat accumulation was observed on inspection of H&E stained tissues. Liver biopsies with more than 5% of fat accumulation were put in NAFLD group while no sign of inflammation and damage were found in liver slides. Samples with fat accumulation, inflammation and damage to liver cells were considered as Nonalcoholic Steatohepatitis (NASH) and excluded from this experiment.

Immunohistochemistry (IHC)

Four-micron sections of the paraffin blocks were prepared on Poly L-Lysine-coated slides. After deparaffinization by xylol and heating, antigen retrieval was performed by putting the slides in boiling citrate buffer for 20 minutes. The slides were subsequently incubated with 1/40 Rabbit antihuman monoclonal antibody to Lipin 1 (Abcam Cat No: ab92316). After washing, the slides were primarily treated by Envision visualization/detection method (DAKO, Denmark), and then, they were stained with diaminobenzidine tetrahydrochloride (DAKO, Denmark), and counter stained with hematoxylin. All the samples were scored based on the amount of staining by an expert pathologist.

RNA Quantitation

Total RNA was isolated from liver samples of obese patients or cultured hepG2 cells with QIAzol (Qiagen), and, then treated with amplification grade DNase I (Sigma-Aldrich Cat No. AMP-D1) to remove any contamination with genomic DNA. First-strand cDNA synthesis for SYBR-Green based series was performed using random hexamer. In contrast, for miR-122 quantification a stem-loop methodology was used.[15] In brief, 100 ng of extracted RNA in two separate tubes was used to reverse-transcribe snord-47, as house-keeping gene, and miR-122 by the use of snord-47RT and miR-122RT primers, as depicted in Table 2. Real-time PCR reactions of 15 μL were prepared for SYBR-Green based series (Table 1) on Rotor Gene 6000 (Corbett life science) as follows : 7.5 μL 2X SYBR Premix Ex Taq II (Takara, Cat No: RR820), 0.4 μM of final concentration for each primer, 2 μL template and distilled water to reach the volume of 15 μL. Real-time PCR was performed in three-step with the following thermal setting: 3 min at 95°C for initial enzyme activation followed by 40 amplification cycles (each 5 seconds at 95°C, 20 seconds at 60°C, and 30 seconds at 72°C with fluorescence detection). Finally, a melting curve analysis was done for each SYBR-Green based run. Real-time PCR reactions of 20 μL were prepared for probe-based series (miR-122 quantification) in Rotor Gene 6000 (Corbett life science) as follows: 10 μL 2X QuantiFast probe PCR kit (Qiagen), 0.4 μM of final concentration for each primer, 0.2 μM of final concentration of each probe, 2 μL template and distilled water to reach the volume of 20 μL. Real-time PCR was performed in two steps with the following thermal setting: 15 min at 95°C for initial enzyme activation followed by 50 amplification cycles (each 15 seconds at 95°C, 60 seconds at 60°C with fluorescence detection). All the real time PCR calculations were done with $2^{-\Delta\Delta Ct}$ using the REST software.[16] All the primer and probe sets are listed in Table 3.

Statistical Analysis

Each experiment was performed in triplicate and data was expressed as mean ± standard error of mean (S.E.M). Student *t*-test for unpaired two groups and one-way ANOVA for three groups were executed using SPSS 16.0. $P < 0.05$ was considered statistically significant. One or two asterisks in the figures (*, **) indicate that the result is significant with ($P < 0.05$) and ($P < 0.001$), respectively.

Table 2. All the patients' information.

Patient	Liver's state	FBS	Cholesterol	HDL	LDL	TG	Age	Diabetes	BMI
1	Normal	83	151	34	74	88	33	no	56.44
2	Normal	98	192	46	116	149	34	no	43.87
3	Normal	93	194	58	121	84	33	no	41.02
4	NAFLD	105	237	46	148	198	45	Yes	53.15
5	NAFLD	88	216	43	108	244	55	Yes	44.99
6	NAFLD	100	149	38	84	185	31	Yes	47.67
7	NAFLD	93	173	36	114	117	43	Yes	40.77
8	NAFLD	92	281	62	197	126	48	Yes	46.47
9	NAFLD	103	182	47	85	222	48	Yes	38.63
10	NAFLD	98	193	44	131	88	45	Yes	37.8
11	NAFLD	87	231	38	156	184	31	Yes	34.85
12	NAFLD	109	195	67	103	135	39	Yes	37.89
13	NAFLD	119	276	36	195	264	33	Yes	42.99
14	NAFLD	101	236	59	133	247	44	Yes	39.76
15	NAFLD	NA	NA	NA	NA	NA	44	Yes	53.52
16	NAFLD	107	207	61	90	282	32	Yes	35.96

FBS: fasting blood sugar, TG: triglyceride, BMI: body mass index

Table 3. Oligos for the purpose of gene expression.

Primer name	Sequence
Probe-based	
h-miR-122RT	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACCAAACA
h-miR-122 (F)	AGGCTGGAGTGTGACAATG
h- miR -122 (R)	GAGCAGGGTCCGAGGT
h- miR -122 probe	FAM-TGTTTGGTCGTATGCAGTGCG-BHQ1
Probe-based	
h-SNORD-47RT	GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACCTC
h-SNORD-47 (F)	ATCACTGTAAAACCGTTCCA
h- miR -122 (R)	GAGCAGGGTCCGAGGT
h-SNORD-47 probe	FAM-TGATTCTGAGGTTGTCGTATGCA-BHQ1
SYBR-Green based	
h-LPIN-1(F)	ACCACAATCAAGGAGGAAAG
h-LPIN-1(R)	AGCTGACATTAGGCAGAAGAG
h-CTDNEP1 (F)	CCATCCAGACAATGCCATC
h-CTDNEP1 (R)	CACGGAACGAACATCAGC
h-TBP (F)	CGAAACGCCGAATATAATC
h-TBP (R)	TGGACTGTTCTTCACTCTTGG

Results

To test our hypothesis concerning the repressive effect of hsa-miR-122 on key enzymes in TAG pathway, we used bioinformatics and experimental approaches to elucidate the case (Figure 1). Our bioinformatics analyses showed that 3' UTR regions of *CTDNEP1* and *LPIN1* are targets of hsa-miR-122-5p in two and one regions, respectively (Figure 1A). Dual-luciferase reporter assay, quantitative RT-PCR and western blot on hepG2 cell line collectively confirmed the repressive effect of hsa-miR-122-5p on *CTDNEP1* and *LPIN1*. Luciferase ratio for *LPIN1* and *CTDNEP1* was significantly decreased in the test groups (transduced miR-122 plus either 3'UTR of *LPIN1* or *CTDNEP1*) compared with the controls with respectively 41% and 33% reduction (Figure 1C). This indicates that hsa-miR-122-5p is able to target 3' UTR regions of the genes. Of note, the use of mutated seed region of hsa-miR-122-5p did not result in any reduction in luciferase ratio (data not shown). Real time RT-PCR showed a significant decrease in *LPIN1* and *CTDNEP1* trascriptomic levels in the test groups compared to the corresponding controls with 56% and 54% down-regulation, respectively (Figure 1D). Our data from our hepG2 cell line was further implemented with western blot for lipin-1 in which we showed a significant reduction of the protein in the test group compared with the respective control (Figures 1E and 2F).

In clinical specimens, we did not observe any significant difference (P-value> 0.05) between normal obese and NAFLD groups in terms of FBS, cholesterol, HDL, LDL, TG, age, diabetes state or body mass index (BMI). Real time PCR for hsa-miR-122-5p expression showed significant overexpression of hsa-miR-122-5p in NAFLD group in comparison with normal obese patients (Figure 2A). *CTDNEP1* and *LPIN1* were similarly overexpressed (Figure 2B). IHC for lipin-1 showed significant overexpression in lipin-1 protein level in NAFLD compared to normal obese control (Figure 2C).

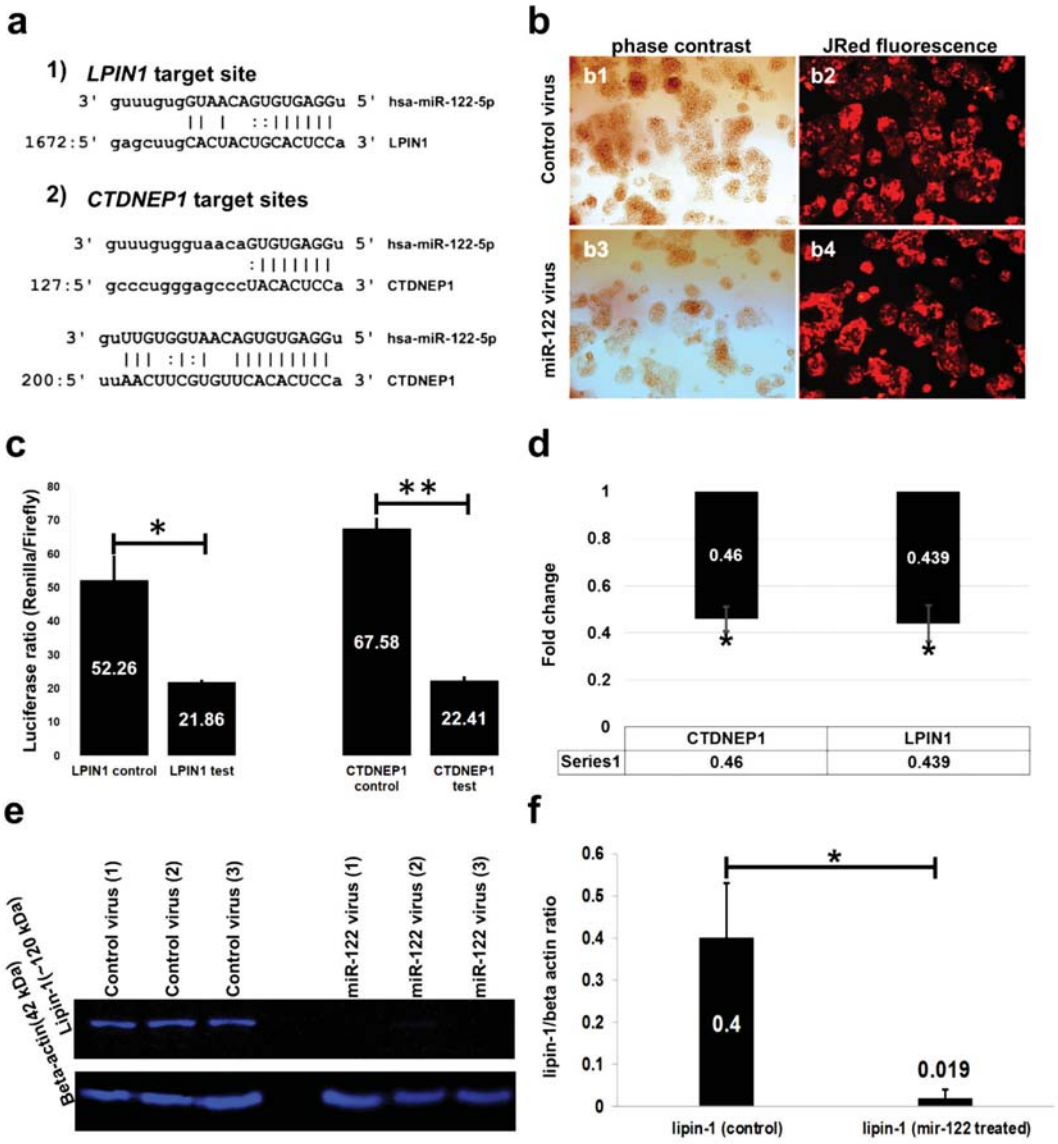

Figure 1. **a1, a2**) hsa-miR-122-5p target site prediction in 3' UTR region of *LPIN1* and *CTDNEP1*; **b1–b4**) This picture shows viral transduction with either mir-122 or control virus on hepG2 cells. Phase contrast and fluorescent pictures are shown; **c)** Dual Luciferase Reporter assay for *LPIN-1* and *CTDNEP-1*. As shown, both genes have significant decrease in luciferase ratios; **d)** Real-time PCR on miR-122 transduced hepG2 cells in comparison with the respective control. As shown, both *LPIN1* and *CTDNEP1* are downregulated; **e, f**) Western blot analysis of lipin-1 protein in miR-122-transduced hepG2 cells compared with respective controls. Section e depicts captured picture of lipin-1 and beta-actin protein levels. Section f depicts the numerical calculation and statistical presentation of section E captured bands. Asterisk (*) shows ($P < 0.05$) and double asterisks show ($P < 0.001$).

Discussion

In this study, we depicted that hsa-miR-122-5p is able to suppress the expression of CTDNEP1 and LPIN1 by targeting the 3' UTR region of the genes. Hsa-miR-122 is a liver specific microRNA that has been linked to lipid homeostasis.[8] However, the mechanisms by which miR-122 can affect TAG homeostasis in liver are not fully understood. To this aim, we inspected the effect of hsa-miR-122-5p on TAG biosynthesis genes. Primary *in silico* analyses depicted that hsa-miR-122-5p had target site(s) within 3'UTR regions of *LPIN1* (encoding lipin-1) and *CTDNEP1* (also known as *DULLARD*) genes. These bioinformatic predictions were experimentally validated in miR-122-transduced hepG2 cell line. The hepG2 cell line was chosen due to its unique characteristic of expressing no or little miR-122,[17] and, therefore, it provided a non-noisy background for the measurement of miR-122 suppression effects on 3' UTR region of inspected genes. Dual luciferase reporter assay in miR-122-transduced hepG2 significantly depicted the suppression of *CTDNEP1* and *LPIN1* by hsa-miR-122-5p (Figure 1C) while the use of mutated seed region of hsa-miR-122-5p did not repress the genes (data not shown). Real-time PCR in miR-122-transduced hepG2 cell line showed that both *LPIN1* and *CTDNEP1* were significantly downregulated (Figure 1D). Furthermore, western blot analysis of lipin-1 protein in miR-122-transduced hepG2 cell line further confirmed the repressive role of hsa-miR-122-5p on lipin-1 in protein level

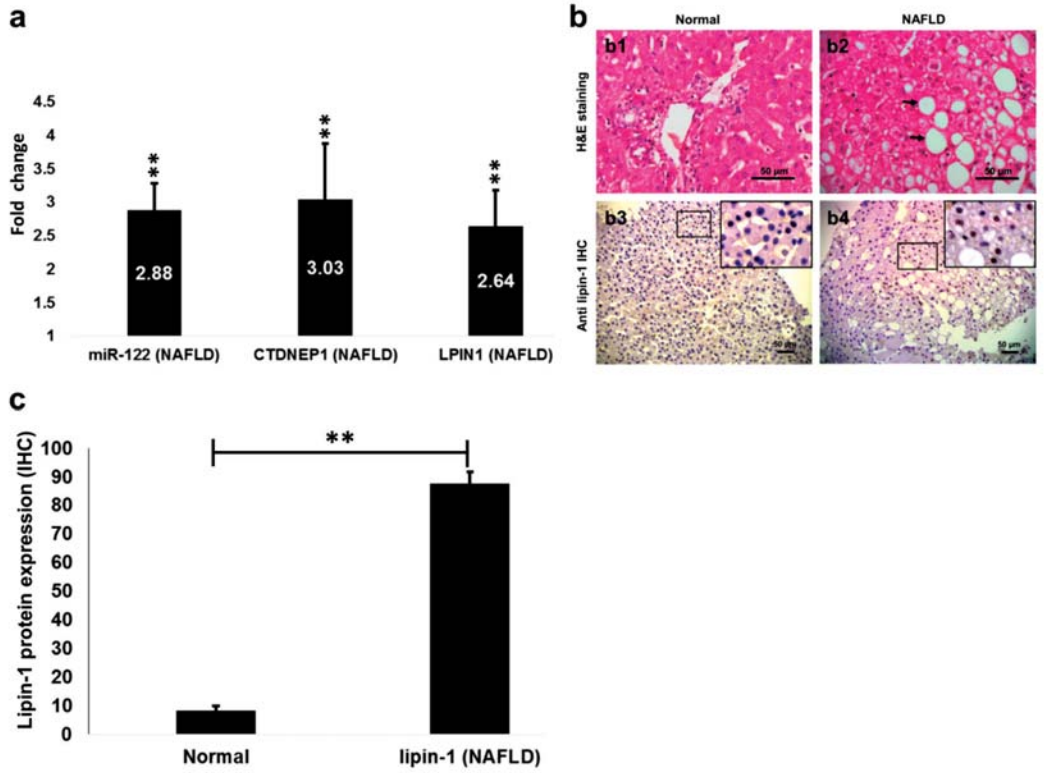

Figure 2. This picture depicts different experiments on two clinical groups including normal obese and NAFLD; **a**) Real-time PCR results for hsa-miR-122-5p, *CTDNEP1* and *LPIN1* in NAFLD in comparison to respective controls; **b1–b4**) H&E staining on representative slides of normal obese (b1) and NAFLD (b2). Black arrows in (b2) are indicative of fat droplets; **b3, b4**) Immunohistochemistry (IHC) for lipin-1 protein for the same patients of H&E staining in normal obese and NAFLD groups. These pictures present merely a part of a slide and scoring for each sample was performed based on the whole slide area; **c)** Semi-quantitative IHC scores for lipin-1 protein, based on two groups including normal obese and NAFLD (Abbreviations; NAFLD: nonalcoholic Fatty Liver Disease. Asterisk (*) shows ($P < 0.05$) and double asterisks show ($P < 0.001$).

(Figures 1E and 2F). Collectively, this data confirmed that *LPIN1* and *CTDNEP1* were targeted and subsequently suppressed by mature hsa-miR-122-5p.

Real time PCR on clinical samples showed significant over-expression of miR-122 in NAFLD group compared with normal obese controls. Surprisingly, *CTDNEP1* and *LPIN1* as the targets of hsa-miR-122-5p were also up-regulated. IHC results for lipin-1 protein in NAFLD compared with normal obese controls had a similar upward trend with the real-time PCR results of the gene (Figure 2C). This phenomenon is probably a disproportionate feedback response to the high level of miR-122 during hepatosteatosis. This observation suggests that upregulation of miR-122 in hepatosteatosis can triggers compensatory response of *LPIN1* and *CTDNEP1*. It is noteworthy that in both hepG2 cell line model and clinical samples, we observed the same downward or upward trend in the expression of *CTDNEP1* and *LPIN1*. This finding is in accordance with the report by Han, et al. in which they observed that the expression pattern of *CTDNEP1* closely mirrors that of *LPIN1* in human and murine tissues,[18] suggesting either a potential relationship between these two proteins[19] or a master regulator for both of the genes.

The relation between miR-122 and *LPIN1* can also be viewed from the perspective of liver physiology. Indeed, due to the suppressive effect of miR-122 on *LPIN1* and the abundance of this miRNA in liver (70%), it is expected to have lower expression of *LPIN1* in liver in comparison with its two other family members, *LIPIN-2* and *LIPIN-3*. Actually, Donkor, et al. showed that *LPIN2* and *LPIN3* are more abundant than *LPIN1* in liver. The reason for this type of tissue expression pattern is still to be inspected.[20,21]

Collectively, we propose that miR-122 suppresses *LPIN1* and *CTDNEP1* by targeting 3' UTR region of the genes. Therefore, harmonic performance of *LPIN1* and *CTDNEP1* could be possibly justified with the action of miR-122 even though this information needs to be further elucidated.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors thank Dr. Somayyeh Mokhber for providing patients' information. Moreover, we would like to thank Ms. Mansoureh Barzegar for her technical assistance.

References

1. Bartel DP. MicroRNAs: Target recognition and regulatory functions. *Cell*. 2009; 136: 215 – 233.
2. Small EM, Olson EN. Pervasive roles of microRNAs in cardiovascular biology. *Nature*. 2011; 469: 336 – 342.
3. Willeit P, Skroblin P, Kiechl S, Fernández-Hernando C, Mayr M.

Liver microRNAs: potential mediators and biomarkers for metabolic and cardiovascular disease? *Eur Heart J*. 2016; ehw: 146.
4. Girard M, Jacquemin E, Munnich A, Lyonnet S, Henrion-Caude A. miR-122, a paradigm for the role of microRNAs in the liver. *J Hepatol*. 2008; 48: 648 – 656.
5. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol*. 2012; 12: 735 – 739.
6. Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R, et al. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. *J Clin Inves.* 2012; 122: 2884 – 2897.
7. Hsu Sh, Wang B, Kota J, Yu J, Costinean S, Kutay H, et al. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. *J Clin Inves.* 2012; 122: 2871 – 2883.
8. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab*. 2006; 3: 87 – 98.
9. Fabbrini E, Sullivan S, Klein S. Obesity and nonalcoholic fatty liver disease: Biochemical, metabolic, and clinical implications. *Hepatol*. 2010; 51: 679 – 689.
10. Csaki LS, Reue K. Lipins: Multifunctional lipid metabolism proteins. *Annu Rev Nutr*. 2010; 30: 257 – 272.
11. Reue K, Brindley DN. Thematic Review Series: Glycerolipids. Multiple roles for lipins/phosphatidate phosphatase enzymes in lipid metabolism. *J Lipid Res*. 2008; 49: 2493 – 2503.
12. Betel D, Koppal A, Agius P, Sander C, Leslie C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.* 2010; 11: R90.
13. Barde I, Salmon P, Trono D. Production and titration of lentiviral vectors. *Curr Prot Neurosci.* 2010; 4(21): 1 – 23.
14. Fallah P, Arefian E, Naderi M, Aghaee-Bakhtiari SH, Atashi A, Ahmadi K, et al. miR-146a and miR-150 promote the differentiation of CD133+ cells into T-lymphoid lineage. *Mol Biol.* 2013; Reports: 1 – 7.
15. Naderi M, Abdul TH, Soleimani M, Shabani I, Hashemi SM. A Home-brew Real-time PCR Assay for Reliable Detection and Quantification of Mature miR-122. *Appl Immunohistochem Mol Morphol.* 2015; 23: 601 – 606.
16. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research*. 2002; 30: e36 – e39.
17. Coulouarn C, Factor VM, Andersen JB, Durkin ME, Thorgeirsson SS. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. *Oncogene*. 2009; 28: 3526 – 3536.
18. Han S, Bahmanyar S, Zhang P, Grishin N, Oegema K, Crooke R, et al. Nuclear envelope phosphatase 1-regulatory subunit 1 (formerly TMEM188) is the metazoan Spo7p ortholog and functions in the lipin activation pathway. *J Biol Chem.* 2012; 287: 3123 – 3137.
19. Csaki LS, Dwyer JR, Fong LG, Tontonoz P, Young SG, Reue K. Lipins, Lipinopathies, and the Modulation of Cellular Lipid Storage and Signaling. *Prog Lip Res.* 2013; 52: 305 – 316.
20. Donkor J, Sariahmetoglu M, Dewald J, Brindley DN, Reue K. Three mammalian lipins act as phosphatidate phosphatases with distinct tissue expression patterns. *J Biol Chem.* 2007; 282: 3450 – 3457.
21. Donkor J, Zhang P, Wong S, O'Loughlin L, Dewald J, Kok BP, et al. A conserved serine residue is required for the phosphatidate phosphatase activity but not the transcriptional coactivator functions of lipin-1 and lipin-2. *J Biol Chem.* 2009; 284: 29968 – 29978.