

MicroRNA-10a Impairs Liver Metabolism in Hepatitis C Virus-Related Cirrhosis Through Deregulation of the Circadian Clock Gene Brain and Muscle Aryl Hydrocarbon Receptor Nuclear Translocator-Like 1

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The circadian rhythm of the liver plays an important role in maintaining its metabolic homeostasis. We performed comprehensive expression analysis of microRNAs (miRNAs) using TaqMan polymerase chain reaction of liver biopsy tissues to identify the miRNAs that are significantly up-regulated in advanced chronic hepatitis C (CHC). We found miR-10a regulated various liver metabolism genes and was markedly up-regulated by hepatitis C virus infection and poor nutritional conditions. The expression of miR-10a was rhythmic and down-regulated the expression of the circadian rhythm gene brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (*Bmal1*) by directly suppressing the expression of RA receptor-related orphan receptor alpha (*RORA*). Overexpression of miR-10a in hepatocytes blunted circadian rhythm of *Bmal1* and inhibited the expression of lipid synthesis genes (sterol regulatory element binding protein [*SREBP1*], fatty acid synthase [*FASN*], and *SREBP2*), gluconeogenesis (peroxisome proliferator-activated receptor gamma coactivator 1 alpha [*PGC1α*]), protein synthesis (mammalian target of rapamycin [mTOR] and ribosomal protein S6 kinase [S6K]) and bile acid synthesis (liver receptor homolog 1 [LRH1]). The expression of *Bmal1* was significantly correlated with the expression of mitochondrial biogenesis-related genes and reduced *Bmal1* was associated with increased serum alanine aminotransferase levels and progression of liver fibrosis in CHC. Thus, impaired circadian rhythm expression of *Bmal1* by miR-10a disturbs metabolic adaptations, leading to liver damage, and is closely associated with the exacerbation of abnormal liver metabolism in patients with advanced CHC. In patients with hepatitis C-related liver cirrhosis, liver tissue miR-10a levels were significantly associated with hepatic reserve, fibrosis markers, esophageal varix complications, and hepatitis C-related hepatocellular carcinoma recurrence. **Conclusion:** MiRNA-10a is involved in abnormal liver metabolism in cirrhotic liver through down-regulation of the expression of the circadian rhythm gene *Bmal1*. Therefore, miR-10a is a possible useful biomarker for estimating the prognosis of liver cirrhosis. (*Hepatology Communications* 2019;3:1687-1703).

The progression of chronic hepatitis to liver cirrhosis (LC) is closely associated with the complication of hepatitis C-related hepatocellular carcinoma (HCC) and concurrent symptoms of liver failure, such as variceal hemorrhage, ascites, jaundice, and hepatic encephalopathy. Therefore, the development of useful biomarkers to predict the complications of LC could facilitate early treatment, thus

Abbreviations: ACTB, beta-actin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMPK, adenosine monophosphate-activated protein kinase; AST, aspartate aminotransferase; BCAA, branched-chain amino acid; *Bmal1*, brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1; CDCA, chenodeoxycholic acid; CHC, chronic hepatitis C; cont, control; CYP7A1, cytochrome P450 family 7 subfamily A member 1; DMEM, Dulbecco's modified Eagle's medium; FASN, fatty acid synthase; FBS, fetal bovine serum; GLuc, Gaussia luciferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVc, cell culture hepatitis C virus; HDL-Chol, high-density lipoprotein cholesterol; hsa-miR, human microRNA;

improving the quality of life of patients with LC and its prognosis.⁽¹⁾

MicroRNAs (MiRNAs) have recently been gaining attention as a useful biomarker for liver disease. Previous reports have shown that expression of miR-21,⁽²⁾ miR-199a, miR-200a,⁽³⁾ and miR-214⁽⁴⁾ is associated with the progression of liver fibrosis. MiR-122 is also reportedly associated with hepatitis C virus (HCV) replication and lipid metabolism and is involved in the inhibition of liver fibrosis, exhibiting an antitumor effect.⁽⁵⁾ Abnormal expression of miRNAs induced in advanced liver fibrosis is possibly associated with abnormal liver metabolism. However, currently there are few reports on miRNAs being closely related to the progression of abnormal liver metabolism.

The circadian rhythm of the liver plays a major role in liver metabolism. In the liver, the metabolism of glucose, bile acid, lipids, and cholesterol is affected by the circadian rhythm, and the expression of metabolism-regulating factors fluctuates in a circadian manner. Reportedly, an abnormal circadian rhythm can lead to genome instability, increased cell proliferation, and be associated with cancer.⁽⁶⁾

In the present study, we found that miR-10a regulates liver metabolism by suppressing circadian rhythm gene expression. We also examined the usefulness of miR-10a as a predictive biomarker for the complications of LC.

Materials and Methods

CLINICAL SAMPLES

We used liver tissue samples from 157 patients with HCV infection (Table 1). miRNA and messenger RNA (mRNA) expression profiling were obtained from 41 patients with chronic hepatitis C (CHC; cohort 1) by TaqMan MicroRNA Assay⁽⁷⁾ and Affymetrix GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA).⁽⁸⁾ mRNA expression profiling was obtained from 50 patients with CHC (cohort 2) to evaluate miR-10a-related circadian rhythm gene expression. The clinical significance of miR-10a was analyzed in 66 patients with LC (Table 1; Supporting Table S2). As

Inh, Inhibitor; *KO*, knockout; *LC*, liver cirrhosis; *LD*, liquid droplet; *LRH1*, liver receptor homolog 1; *miRNA/miR*, microRNA; *MOI*, multiplicity of infection; *mRNA*, messenger RNA; *MT*, mutant; *mTOR*, mammalian target of rapamycin; *N.S.*, not significant; *p*, phosphorylated; *PCK*, phosphoenolpyruvate carboxykinase; *PCR*, polymerase chain reaction; *PEG-IFN*, pegylated interferon; *PGC1 α* , peroxisome proliferator-activated receptor gamma coactivator 1 alpha; *PPAR/A/G*, peroxisome proliferator-activated receptor/alpha/gamma; *RBV*, ribavirin; *ROR*, RA receptor-related orphan receptor; *RORE*, RA receptor-related orphan receptor-responsive element; *RTD-PCR*, real-time detection polymerase chain reaction; *S6K*, ribosomal protein S6 kinase; *SREBP*, sterol regulatory element binding protein; *SVR*, sustained virologic response; *T-Chol*, total cholesterol; *TG*, triglyceride; *TGF- β* , transforming growth factor β ; *UTR*, untranslated region; *WT*, wild type.

Received April 5, 2019; accepted September 4, 2019.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1431/supinfo.

Partially supported by the Japan Agency for Medical Research and Development (grant numbers JP18fk0210046 T.S. and T.S., JP18fk0210012 to M.H., T.S. and T.S., JP18fk0210020 to M.H., JP17fk0210201 to M.H., JP18fk0210005 to M.H. and S.K., and JP18fk0310110 to S.K.).

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep4.1431

Potential conflict of interest: Nothing to report.

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TABLE 1. CLINICAL CHARACTERISTICS OF 157 PATIENTS WITH HCV INFECTION USED IN THIS STUDY

Clinical Category	CHC (Cohort 1)	CHC (Cohort 2)	LC	P Value
No. of patients	n = 41	n = 50	n = 66	
Age (years)	56 (34-71)	56 (30-71)	68* (46-84)	LC vs. cohort1 <0.0001 LC vs. cohort2 <0.0001
Sex (M vs. F)	26 vs. 15	31 vs. 19	34 vs. 32	N.S.
Histology				
F stage (0/1/2/3/4)	15/10/10/6	17/13/15/5	0/0/0/66*	LC vs. cohort1 <0.0001 LC vs. cohort2 <0.0001
A grade (A0-1 vs. A2-3)	26 vs. 15*	21 vs. 29	19 vs. 47	LC vs. cohort1 =0.0018 cohort1 vs. cohort2 =0.04
Laboratory data				
BMI (kg/m ²)	24.5 (19.1-40.6)	23.6 (16.2-29.4)	22.2* (17.2-29.3)	LC vs. cohort1 =0.0018
PLT (×10 ⁴ /mm ³)	15.6 (7-30)	15.4 (8.5-39.4)	9.3* (4.3-24)	LC vs. cohort1 <0.0001 LC vs. cohort2 <0.0001
AST (U/L)	65 (23-283)	48 (21-205)	53 (16-213)	NS
ALT (U/L)	71 (18-326)	71 (19-376)	47* (8-263)	LC vs. cohort1 =0.012 LC vs. cohort2 =0.0026
γ-GTP (U/L)	55 (17-392)	71 (4-307)	40* (13-154)	LC vs. cohort1 =0.0016 LC vs. cohort2 =0.011
TG (mg/dL)	122 (59-276)	101 (30-303)	97* (45-267)	LC vs. cohort1 =0.022
T-Chol (mg/dL)	163 (115-221)	173 (129-237)	133* (88-202)	LC vs. cohort1 <0.0001 LC vs. cohort2 <0.0001
HDL-Chol (mg/dL)	42 (18-70)	47 (27-77)	34* (15-65)	LC vs. cohort1 =0.037 LC vs. cohort2 <0.0001
HCV-RNA (KIU/mL)	2,010 (126-5,000)	2,000 (160-5,000)	N.D.	
IL28B genotype				
TT vs. TG or GG	15 vs. 26	16 vs. 34	N.D.	N.S.
Treatment response (PEG-IFN+RBV)				
SVR vs. TR vs. NR	17 vs. 8 vs. 16	23 vs. 14 vs. 13	N.D.	N.S.

Data represent median (range) or number of patients.

* $P < 0.05$.

Abbreviations: ALB, albumin; BMI, body mass index; γ-GTP, gamma-glutamyl transpeptidase; IL28, interleukin 28; KIU, kallikrein inactivator units; N.D., not detected; NR, no response; PLT, platelet; PT, prothrombin time; T-Bil, total bilirubin; TR, transient response.

control samples, we used healthy liver tissues from 9 patients who underwent surgery for metastatic liver tumor.⁽⁷⁾

The present study was approved by the Institutional Review Board of Kanazawa University Hospital for research on human genome and gene analysis. Written informed consent was obtained from all patients.

miRNA QUANTITATIVE REAL-TIME DETECTION POLYMERASE CHAIN REACTION

We homogenized 1 mg of liver tissue samples preserved in liquid nitrogen using a homogenizer. Total

RNA, including miRNAs, was isolated using the mirVana miRNA isolation kit (Ambion, Austin, TX), as per the manufacturer's instructions, and stored at -80°C until further analysis. miRNA expression levels were measured using the Taqman MicroRNA Assays Human Panel Early Access kit (Applied Biosystems, Foster City, CA).⁽⁷⁾

CELL LINES

We cultured Huh-7 or Huh-7.5 cells containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in Dulbecco's modified Eagle's medium (DMEM; Gibco BRI, Gaithersburg, MD).

OTHER EXPERIMENTAL PROCEDURES

The additional experimental procedures, including miRNA expression analysis, mRNA expression data analysis, HCV replication, fatty acid treatment, western blotting and immunofluorescence staining, quantitative real-time detection polymerase chain reaction (RTD-PCR), and reporter assay, are described in Supporting Experimental Procedures.

STATISTICAL ANALYSIS

Results are expressed as mean \pm SD. A minimum of six samples were tested in each assay. Significance was tested by one-way analysis of variance with Bonferroni's methods, and differences were considered statistically significant at $P < 0.01$.

Results

miRNA EXPRESSION PROFILING OF THE LIVER IN CHC

Among 157 patients with HCV infection, there were no differences in clinical characteristics among CHC patients (cohort 1 vs. cohort 2) except histologic grade (cohort 2 included patients with more active histologic grade). Body mass index, platelet count, alanine aminotransferase (ALT), and serum lipid levels were significantly lower in the patient cohort with LC (Table 1).

miRNA expression profiling was obtained from 41 patients with CHC (cohort 1). To determine the clinical characteristics associated with the differences in miRNA expression, we examined the relationship between the clinical backgrounds of 41 patients with CHC (cohort 1) and miRNA expression (Table 1; Supporting Table S1). The supervised learning method (BRB-ArrayTools; National Center for Biotechnology Information) was used to examine whether 41 patients were classified according to each clinical category using differentially expressed predictor miRNAs ($P < 0.05$). Based on histology (stage, activity, and steatosis), age, sex, levels of ALT, amount of HCV-RNA, interleukin-28B genotype, and treatment response to pegylated interferon (PEG-IFN)+ribavirin (RBV) therapy, each predictor was identified (55, 38, 17, 11, 4, 50, 1, 3, and 7 miRNAs, respectively). However,

only 55 predictors according to histologic progression (F12 vs. F34) could accurately classify the patients ($P < 0.002$) (Supporting Table S1).

Table 2 presents 20 representative miRNAs out of the 55 predictors to differentiate histologic progression (F12 vs. F34). One-way hierarchical clustering of 41 patients with CHC and 9 healthy patients using these 20 predictor miRNAs is shown in Supporting Fig. S1. We previously reported that miR-214 is associated with fibrosis progression⁽⁴⁾ and miR-27a with fatty change and fibrosis.⁽⁹⁾ In the present analysis, we focused on four up-regulated miRNAs (miR-10a, miR-195, miR-199a-3p, and miR-218), and one down-regulated miRNA (miR-19a) with the progression of liver fibrosis. To determine the specific gene expression regulated by these miRNAs, we analyzed the mRNA expression profiles of liver tissues of the 41 patients by Affymetrix GeneChip. Each miRNA candidate target gene was extracted from miRbase (<http://www.mirbase.org>). Gene set expression comparison (F12 vs. F34) based on the Kyoto Encyclopedia of Genes and Genomes pathway was performed using candidate target genes of miRNA ($P < 0.005$) (Table 3). The results revealed that miR-19a, miR-195, and miR-199a-3p regulate genes related to fibrosis and angiogenesis. Interestingly, the regulatory pathway of miR-10a differed from that of other miRNAs; it regulates the metabolic, peroxisome proliferator-activated receptor (PPAR) signaling, and circadian rhythm pathways (Table 3). We could not identify a signaling pathway regulated by miR-218.

miRNA EXPRESSION, HCV INFECTION, AND NUTRITIONAL STATUS

Liver malnutrition develops with the progression of CHC. Therefore, we analyzed the expression of miR-10a, miR-19a, miR-195, miR-199a-3p, and miR-218 under the conditions of HCV infection and poor nutrition. The expression of miR-10a was up-regulated approximately 5-fold in the presence of HCV infection. Furthermore, when the amino acid concentration in the culture medium was decreased to one fifth, miR-10a expression level increased by up to 30-fold (Fig. 1A). In contrast, by adding branched-chain amino acids (BCAAs), this change in the expression disappeared (Fig. 1A). The other miRNAs (i.e., miR-19a, miR-195, and miR-218) except miR-199a-3p exhibited no

TABLE 2. DIFFERENTIALLY EXPRESSED MIRNAS WITH PROGRESSION OF LIVER FIBROSIS

miRNA	Normal	F12	F34	N vs. F12	F12 vs. F34
Up-regulated					
hsa-miR-218	3.44	3.26	7.08	N.S.	2.40E-06
hsa-miR-199a-3p	18.5	17.7	28.9	N.S.	3.30E-06
hsa-miR-195	22.0	21.1	30.5	N.S.	1.97E-06
hsa-miR-214	9.44	8.89	13.8	N.S.	1.48E-04
hsa-miR-10a	2.401	2.81	4.05	N.S.	1.87E-04
hsa-miR-27a	3.19	3.22	4.59	N.S.	2.33E-04
hsa-miR-182	0.0063	0.0040	0.011	N.S.	3.72E-04
hsa-miR-642	0.12	0.22	0.3	5.88E-05	1.23E-03
hsa-miR-200c	0.078	0.035	0.125	N.S.	1.54E-03
hsa-miR-708	0.021	0.022	0.038	N.S.	1.80E-03
hsa-miR-9	0.23	0.17	0.27	N.S.	3.34E-03
hsa-miR-452	0.070	0.10	0.18	N.S.	3.61E-03
hsa-miR-224	1.77	4.04	8.51	1.48E-02	3.77E-03
hsa-miR-338-3p	0.024	0.026	0.053	N.S.	3.89E-03
hsa-miR-429	0.42	0.70	1.41	N.S.	4.15E-03
Down-regulated					
hsa-miR-19a	6.19	4.57	3.46	1.12E-03	2.31E-04
hsa-miR-194	42.9	37.4	25.8	N.S.	3.08E-04
hsa-miR-192	35.2	36.3	25.0	N.S.	8.79E-04
hsa-miR-340	4.08	4.22	3.30	N.S.	2.14E-03
hsa-miR-345	1.11	1.28	1.00	N.S.	2.41E-03

Expression values of miRNAs are shown as relative fold to control RNU6B. Abbreviations: N, normal; RNU6B, RNA U6 small nuclear 6, pseudogene.

change in their expression levels from HCV infection and poor nutritional status (Fig. 1A). The effect of HCV infection and low amino acid levels on the induction of miR-10a was further analyzed in detail (Fig. 1B). Cell culture HCV (HCVcc) (HJ3-5/*Gaussia* luciferase [GLuc]2A)⁽¹⁰⁾ infection induced the expression of miR-10a in a dose-dependent manner (up to 26-fold at multiplicity of infection [MOI] = 10). Similarly, lower amino acid levels further induced the expression of miR-10a (up to 47-fold in 1:10 diluted DMEM with ZERO medium) (Fig. 1B). However, the combination of lower amino acid levels (from one-half to 1:10 serially diluted DMEM with ZERO medium) and HCV infection (MOI = 1) did not show further induction of miR-10a (Fig. 1B). In primary human hepatocytes, HCV replication and lower amino acid level significantly induced the expression of miR-10a, although the differences were less apparent in Huh-7.5 cells (Fig. 1C; Supporting Materials and Methods).

We next analyzed the effect of miRNA expressions on HCV replication. Huh-7.5 cells were transfected with H77-derived HCV RNA carrying a GLuc

reporter gene (H77S.3GLuc/2A)⁽¹¹⁾ together with each mimic miRNA, and GLuc activity was then measured to assess HCV replication. Previous reports have revealed that miR-27a and miR-199a-3p inhibit HCV replication,^(7,12) and we found overexpression of miR-10a, miR-195, and miR-214 significantly inhibited HCV replication in this study (Fig. 1D). Conversely, overexpression of miR-19a and miR-218 had no effect on HCV replication (Fig. 1D). The findings were further confirmed by the experiment of HCV infection. Huh-7.5 cells were infected with HCVcc (HJ3-5/GLuc2A),⁽¹⁰⁾ and the effect of miR-10a on HCV infection was analyzed. Overexpression of miR-10a inhibited HCV infection, and the inhibition of miR-10a increased HCV infection. While oleic acid treatment increased HCV infection, overexpression of miR-10a similarly inhibited HCV infection, and inhibitor of miR-10a increased HCV infection (Fig. 1E). Therefore, miR-10a was up-regulated with HCV infection and with poor nutritional status; in turn, elevated miR-10a inhibited HCV replication and infection.

TABLE 3. PATHWAY ANALYSIS OF DIFFERENTIALLY EXPRESSED REPRESENTATIVE MIRNAS WITH PROGRESSION TO LIVER FIBROSIS

	KEGG Pathway	Pathway Description	LS Permutation PValue	KS Permutation PValue	Efron-Tibshirani's GSA Test PValue	Representative Predicted Target Genes
miR-199a-3p						
1	hsa04512	ECM-receptor interaction (17)	0.00001	0.00017	0.01 (-)	FN1, SDC2
2	hsa04810	Regulation of actin cytoskeleton (39)	0.00016	0.00409	0.01 (-)	IQGAP2, CFL2
3	hsa00230	Purine metabolism (16)	0.00028	0.00706	0.035 (-)	POLR3G, AK4
4	hsa04710	Circadian rhythm (9)	0.01744	0.0018	<0.005 (-)	RORA, NR5A2
miR-195						
1	hsa04370	VEGF signaling pathway (27)	0.00033	0.00008	0.005 (-)	NFATC3, PIK3R1
2	hsa00230	Purine metabolism (21)	0.00075	0.01389	0.02 (-)	AK4, PDE3B
3	hsa04510	Focal adhesion (92)	0.00177	0.00657	0.025 (-)	MAP2K1, GSK3B
4	hsa04080	Neuroactive ligand-receptor interaction (16)	0.00433	0.18223	0.185 (-)	THRB, GHR
5	hsa04146	Peroxisome (20)	0.03957	0.00104	0.03 (-)	XDH, ACOX1
6	hsa04960	Aldosterone-regulated sodium reabsorption (19)	0.07635	0.00012	0.02 (-)	IRS1, IGF1
miR-10a						
1	hsa00330	Arginine and proline metabolism (13)	0.00001	0.00076	0.01 (-)	ALDH7A1, ASL
2	hsa01100	Metabolic pathways (38)	0.00001	0.03454	<0.005 (-)	LSS, NDUFA4, TGDS
3	hsa04710	Circadian rhythm (7)	0.00397	0.00002	0.01 (-)	RORA, NR5A2
4	hsa03320	PPAR signaling pathway (9)	0.03678	0.0038	0.025 (-)	PPARA
5	hsa04060	Cytokine-cytokine receptor interaction (11)	0.3413	0.00385	0.245 (-)	PRLR, INHBB
miR-19a						
1	hsa04510	Focal adhesion (71)	0.00001	0.00066	<0.005 (+)	THBS1, COL1A2
2	hsa04512	ECM-receptor interaction (27)	0.00001	0.00002	<0.005 (+)	ITGA6, CD47
3	hsa04974	Protein digestion and absorption (14)	0.00012	0.0021	0.015 (+)	SLC8A1, SLC36A1
4	hsa05414	Dilated cardiomyopathy (36)	0.00026	0.00002	0.085 (+)	PRKX, SGCD
5	hsa05146	Amoebiasis (24)	0.00028	0.00154	<0.005 (+)	ACTN1, RKACB
6	hsa04115	p53 signaling pathway (39)	0.00114	0.00718	0.005 (+)	CCND2, ZMAT3
7	hsa04020	Calcium signaling pathway (36)	0.00118	0.00004	0.105 (+)	ADCY7, EDNRB
8	hsa05219	Bladder cancer (15)	0.00186	0.02397	<0.005 (+)	MAPK1, KRAS
9	hsa04060	Cytokine-cytokine receptor interaction (26)	0.00277	0.02327	0.065 (+)	CXCL12, HGF
10	hsa05412	Arrhythmogenic right ventricular cardiomyopathy (25)	0.00663	0.00478	0.01 (+)	GJA1, SGCD
11	hsa05410	Hypertrophic cardiomyopathy (30)	0.03046	0.00099	0.11 (+)	ITGA2, PELO
12	hsa05200	Pathways in cancer (64)	0.03602	0.00134	0.245 (+)	FZD6, CCND1

Abbreviations: ECM, extracellular matrix; GSA, gene set analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; KS, Kolmogorov-Smirnov; LS, least squares; VEGF, vascular endothelial growth factor.

(); the number of genes consisting the pathway.

(-); generally, down-regulated with progression of liver fibrosis.

(+); generally, up-regulated with progression of liver fibrosis.

miR-10a AND FATTY ACID METABOLISM

Pathway analysis of the gene expression profile regulated by miR-10a revealed that miR-10a was involved in lipid metabolism (Table 3). To assess the functional relevance of miR-10a in lipid metabolism, we evaluated the intracellular levels of triglyceride

(TG) and total cholesterol (T-Chol) in Huh-7 cells in which miR-10a was overexpressed or knocked down. Stained intracellular liquid droplets (LDs) captured by confocal laser microscopy are shown in Fig. 2A. LDs in Huh-7 cells were reduced by miR-10a overexpression. Conversely, LDs increased by inhibiting miR-10a expression and further increased with the addition of oleic acid. Intracellular TG (Fig. 2A, right upper)

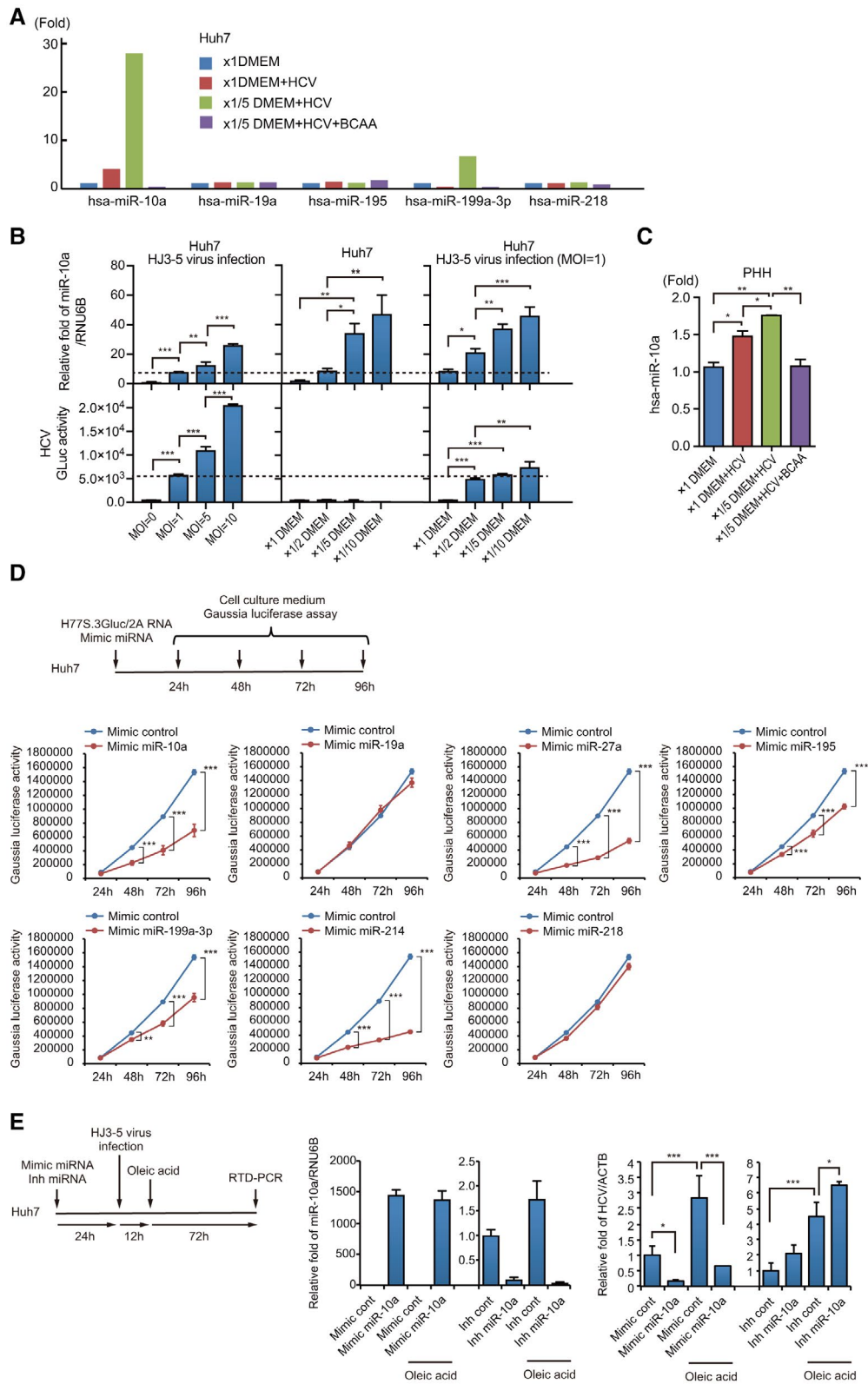


FIG. 1. Relationship of HCV replication and poor nutrition with the induction of miRNAs. (A) In Huh-7.5 cells in a normal culture medium and medium with one-fifth amino acid concentration, JFH-1 infection induced miRNA expression. (B) Infectious dose of HCVcc (HJ3-5/GLuc2A)-dependent induction of miR-10a and amino acid depletion-dependent induction of miR-10a in Huh-7.5 cells. (C) Increased expression of miR-10a in primary human hepatocytes transfected with infectious RNA derived from JFH-1 and in the one-fifth DMEM. (D) Effect of miRNA overexpression on HCV replication. Huh-7.5 cells were transfected with H77-derived HCV RNA carrying a GLuc reporter gene (H77S.3GLuc/2A) and with miRNA, and GLuc activity in the culture medium was measured. (E) Huh-7.5 cells were infected with HCVcc (HJ3-5/GLuc2A), HCV infection was analyzed with excessive or inhibited expression of miR-10a. Values are shown as mean \pm SD. * P < 0.01, ** P < 0.005, *** P < 0.001. Abbreviations: h, hours; PHH, primary human hepatocyte; RNU6B, RNA U6 small nuclear 6, pseudogene.

and T-Chol (Fig. 2C, upper) levels also declined following miR-10a overexpression and increased by the inhibition of miR-10a expression. Alternatively, fatty acid oxidation increased with miR-10a overexpression and decreased with the inhibition of miR-10a expression (Fig. 2A, right lower).

We also assessed the changes in the expression levels of lipid metabolism-related genes in Huh-7 cells induced by miR-10a. Pathway analysis of the gene expression profile suggested a relationship with the PPAR pathway (Table 3); therefore, expressions of the lipid synthesis transcription factors *PPARA*, fatty acid synthase (*FASN*), and sterol regulatory element binding protein 1 (*SREBP1*) were evaluated in Huh-7 cells (Fig. 2B,D-F). miR-10a overexpression suppressed the expression of *SREBP1* and *FASN*, and oleic acid treatment further suppressed their expression. Conversely, in a starved state, expression levels of *SREBP1* and *FASN* were elevated; however, the gene expression suppressive effect remained unchanged by miR-10a overexpression. By contrast, knockdown of miR-10a expression up-regulated the expression of these genes (Fig. 2B,D). *SREBP1* exists in precursor (125 kDa) and nuclear (68 kDa) forms. Western blotting showed that these forms were reduced by overexpression of miR-10a and increased by inhibiting the expression of miR-10a (Fig. 2E). The same experiment was performed using HCV (H77Sv2 GLuc2A)-transfected Huh-7.5 cells, and similar results were obtained (data not shown).

We also found that the expression of *SREBP2*, a cholesterol synthesis-related gene, decreased by the overexpression of miR-10a and increased by inhibiting the expression of miR-10a (Fig. 2C, lower). *PPARA* enhances the expression of *SREBP1c* in the liver.⁽¹³⁾ The overexpression of miR-10a inhibited *PPARA*, and inhibition of miR-10a increased the expression of *PPARA* (Fig. 2F).

The effects of miR-10a on lipid metabolism were not due to apoptosis induced by miR-10a.

Overexpression of miR-10a did not affect cell viability, as evaluated by cell counting and caspase 3/7 release (Supporting Fig. S2).

miR-10a AND CIRCADIAN RHYTHM GENES

Abnormality in the circadian rhythm genes has been reported to be closely associated with metabolic disturbance. RA receptor-related orphan receptor (ROR) alpha (*RORA*) was extracted as a target gene of miR-10a (Table 3). *RORA* is reportedly associated with lipid metabolism⁽¹⁴⁾ and gluconeogenesis⁽¹⁵⁾ in the liver. Brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (*Bmal1*), the core clock gene associated with lipid metabolism, is known to be a tumor suppressor⁽¹⁶⁾ and is a target gene of *RORA*. Therefore, we analyzed the relationships of *RORA* and *Bmal1* with miR-10a. The overexpression of miR-10a down-regulated the expression of *RORA* and *Bmal1*, whereas miR-10a inhibition up-regulated their expression (Fig. 3A). Similar results were observed under oleic acid treatment. By contrast, the expression of *PPARG*, reported to be inhibited by *RORA*,⁽¹⁷⁾ remained relatively unaffected by the overexpression or knockdown of miR-10a (Fig. 3B). The expression levels of miR-10a, *RORA*, and *Bmal1* were enhanced in a starved state, whereas miR-10a inhibition resulted in a more marked increase in the expression levels of *RORA* and *Bmal1* (Fig. 3C,D). Western blotting confirmed that the overexpression of miR-10a considerably inhibited *RORA* and *Bmal1*, whereas the inhibition of miR-10a expression increased the expression levels of these genes at the protein level (Fig. 3E).

We next examined the inhibition of *RORA* expression by miR-10a using a reporter assay. The *RORA* 3'-untranslated region (UTR) had two putative miR-10a binding sites. This involved construction of the expression vectors pmirGLO-*RORA*-1 wild type (WT) and pmirGLO-*RORA*-2 WT with the

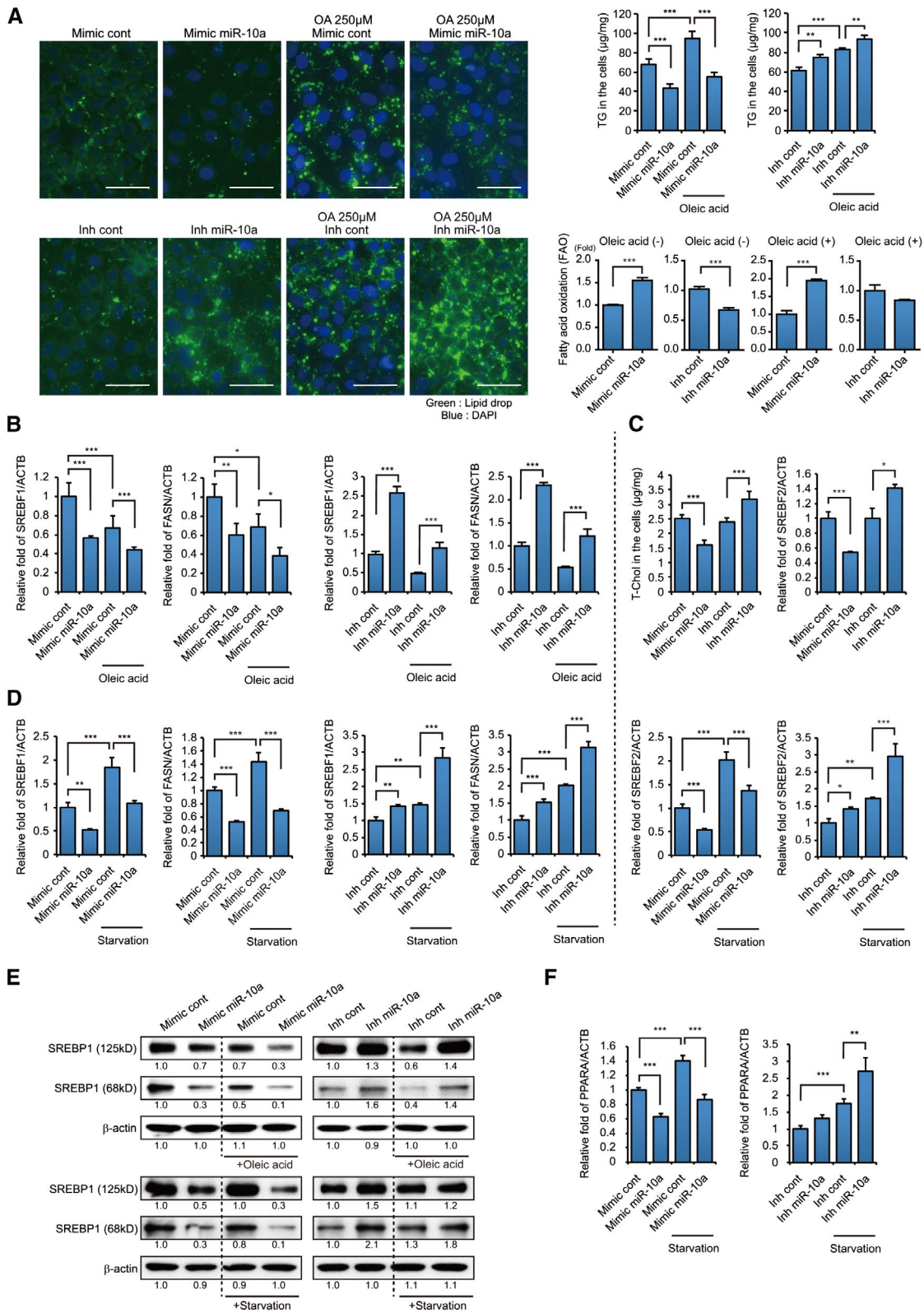


FIG. 2. MiR-10a and lipid metabolism. (A) Changes in the lipid content of Huh-7 cells. Huh-7 cells and Huh-7 cells with added oleic acid (250 μ M) were transfected with mimic-miR-10a or anti-miR-10a. Lipid drops were visualized with boron-dipyrromethene 493/503 staining. Imaging was performed using a CSU-X1 confocal microscope (white scale bar: 50 μ m). Intracellular lipid content is shown on the upper right. Fatty acid oxidation is shown on the lower right. (B-D) Expression of the transcription factors involved in lipid metabolism. Huh-7 cells and Huh-7 cells with added oleic acid (250 μ M) were transfected with mimic-miR-10a or anti-miR-10a. Expression levels of *SREBF1*, *SREBF2*, and *FASN* were quantified with RTD-PCR. (E) Western blotting of SREBP1. Huh-7 cells and Huh-7 cells with added oleic acid (250 μ M) were transfected with mimic-miR-10a or anti-miR-10a. The experiment was repeated 3 times. (F) Expression of the transcription factors involved in lipid metabolism. Huh-7 cells and Huh-7 cells with added oleic acid (250 μ M) were transfected with mimic-miR-10a or anti-miR-10a. Expression levels of PPARA were quantified with RTD-PCR. Values are shown as mean \pm SD. * $P < 0.01$, ** $P < 0.005$, *** $P < 0.001$. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; OA, oleic acid.

3'-UTR sequence, including two candidate binding sites, 3'-UTR (440-640) and 3'-UTR (4,380-4,580), fused to the 3' terminus of a luciferase gene (Fig. 3F). The overexpression of miR-10a considerably reduced luciferase activity in pmirGLO-RORA-1 WT and pmirGLO-RORA-2 WT (Fig. 3F). However, in pmirGLO-RORA-1 mutant (MT) and pmirGLO-RORA-2 MT, with a mutation introduced at the miR-10a binding site, no change was observed in luciferase activity following the overexpression of miR-10a. In contrast, the inhibition of miR-10a expression significantly increased luciferase activity in pmirGLO-RORA-1 WT and pmirGLO-RORA-2 WT, but no change was observed in pmirGLO-RORA-1 MT and pmirGLO-RORA-2 MT. These results suggest that miR-10a directly inhibits the expression of *RORA* by binding to the 3'-UTR of *RORA*.

miR-10a IS A RHYTHMIC GENE AND REGULATES *Bmal1*-DEPENDENT REGULATION OF VARIOUS METABOLIC PROCESSES IN HEPATOCYTES

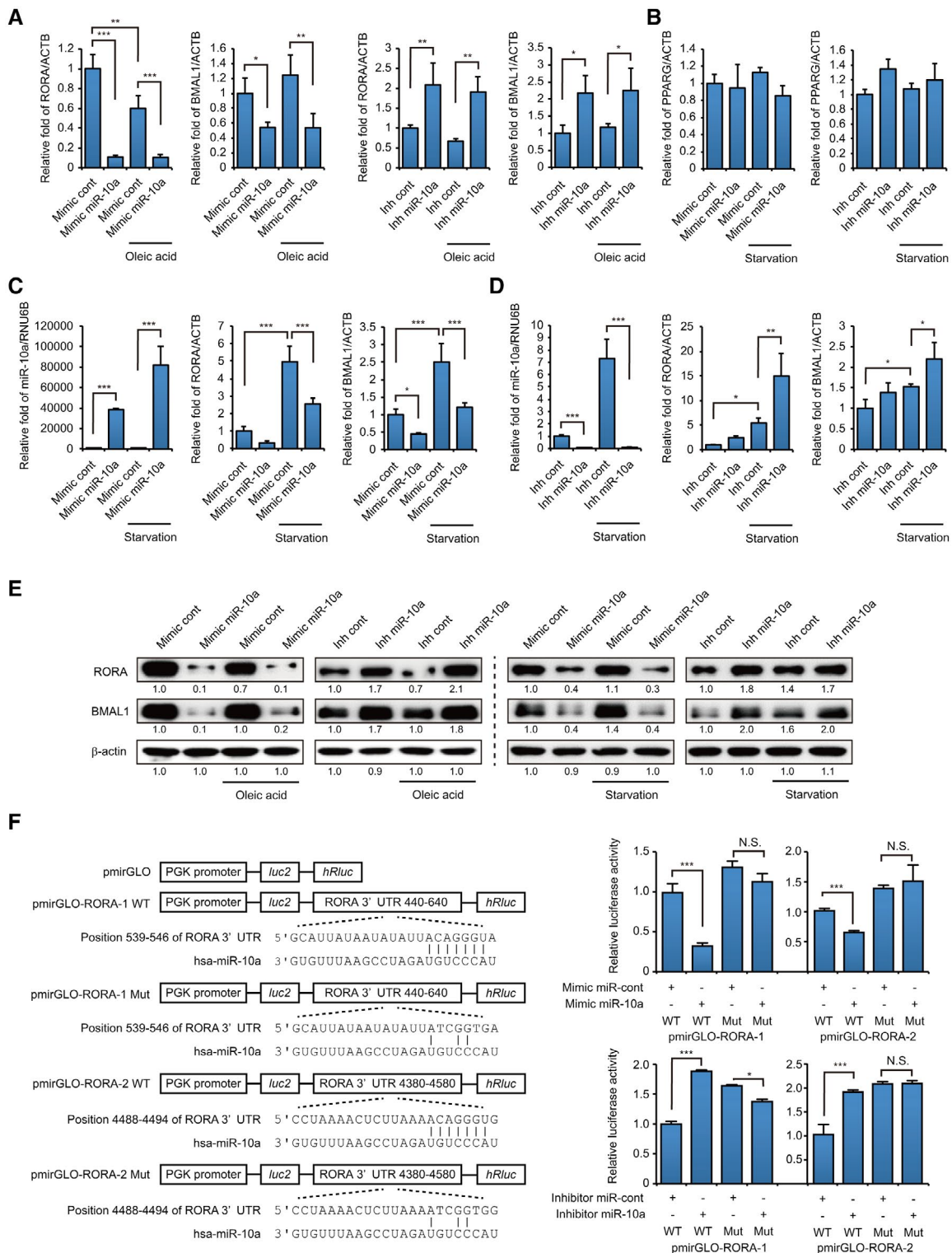
As miR-10a regulates *Bmal1*, we examined whether miR-10a is also related to circadian rhythm. The experiments of synchronization of Huh-7 cells with serum shock (50% FBS) and release revealed the rhythmic expression pattern of miR-10a, similar to the expression pattern of *Bmal1* (Fig. 4A, left). Interestingly, rhythmic expression of *Bmal1* was blunted by the overexpression of miR-10a (Fig. 4A, right). Moreover, up-regulation of *SREBF1*, *SREBF2*, and *FASN*, downstream of *Bmal1* was also hampered by the overexpression of miR-10a (Fig. 4B).

Bmal1 regulates circadian rhythm of the metabolism of lipids, bile acids, and glucose and is important in maintaining metabolic homeostasis.⁽¹⁸⁾ Bile acid is

synthesized by cytochrome P450 family 7 subfamily A member 1 (CYP7A1) from cholesterol, and CYP7A1 is regulated by liver receptor homolog 1 (LRH1). The overexpression of miR-10a reduced the expression of LRH1 (Fig. 4C, left). When chenodeoxycholic acid (CDCA) was added as bile acid in the cell culture, the intracellular cholesterol level was found to be elevated (Fig. 4C, right). CDCA reportedly increases low-density lipoprotein receptor expression in liver cancer cell-line culture,^(19,20) which may be attributed to the increased inflow of cholesterol into cells. The overexpression of miR-10a significantly inhibited the intracellular increase of cholesterol level caused by CDCA. Furthermore, the inhibition of miR-10a expression resulted in a further increase in the elevated intracellular cholesterol level caused by CDCA (Fig. 4C, right).

We next examined the relationship between miR-10a and gluconeogenesis. In the liver, gluconeogenesis is regulated by PPARC coactivator 1 alpha (PGC1 α). PGC1 α interacts with hepatocyte nuclear factor 4 alpha (HNF4 α) and forkhead box O1 (FOXO1) to promote phosphoenolpyruvate carboxykinase (*PCK1*) and *PCK2* transcription, thus enhancing gluconeogenesis.⁽²¹⁻²³⁾ In hepatocyte-specific *Bmal1*-knockout (KO) mice, hypoglycemia has been reported to reduce liver glycogen during fasting and increase blood sugar clearance.⁽²⁴⁾ The overexpression of miR-10a inhibited the expression of *PCK1* and *PGC1 α* involved in gluconeogenesis; however, the expression of these genes was up-regulated by inhibiting the expression of miR-10a (Fig. 4D).

We also examined the relationship between miR-10a and protein synthesis. The overexpression of miR-10a inhibited the phosphorylation of mammalian target of rapamycin (mTOR) and ribosomal protein S6 kinase (S6K) present downstream of mTOR, whereas their expression increased by inhibiting the expression of miR-10a (Fig. 4E, left). Adenosine monophosphate-activated protein kinase (AMPK) acts as a regulatory factor of cellular metabolism whereby it regulates



lipid synthesis, gluconeogenesis, and protein synthesis. AMPK is reported to regulate circadian rhythm genes. We found that the overexpression of miR-10a inhibited AMPK activity, whereas its inhibition enhanced

AMPK activity (Fig. 4E, right). These results demonstrated that the inhibition of lipid synthesis, gluconeogenesis, and protein synthesis by miR-10a was also associated with AMPK inhibition by miR-10a.

FIG. 3. MiR-10a and circadian rhythm genes. (A) Huh-7 cells and Huh-7 cells with added oleic acid (250 μ M) were transfected with mimic-miR-10a or anti-miR-10a. Expression levels of *RORA* and *Bmal1* were measured by RTD-PCR. (B-D) Huh-7 cells in a normal culture medium and Huh-7 cells in a medium with one-fifth amino acid concentration were transfected with mimic-miR-10a or anti-miR-10a. Expression levels of *PPARG*, *RORA*, *Bmal1*, and miR-10a were measured by RTD-PCR. (E) Protein expression levels of *RORA* and *Bmal1* were evaluated by western blotting. (F) Reporter construct carrying *RORA* 3'-UTR. The miR-10a candidate binding site included in 3'-UTR (440-640) of pmirGLO-*RORA*-1 WT and 3'-UTR (4,380-4,580) of pmirGLO-*RORA*-2 WT. A mutation was introduced to the miR-10a binding region of pmirGLO-*RORA*-1 MT and pmirGLO-*RORA*-2 MT. Luciferase activity was measured following transfecting with mimic-miR-10a or anti-miR-10a. All tests were doubled and repeated 3 times. Values are shown as mean \pm SD. ** $P < 0.005$, *** $P < 0.001$. Abbreviations: hRluc, *Renilla* luciferase; luc2, firefly luciferase gene; PGK, phosphoglycerate kinase.

The effects of miR-10a on liver metabolism were not owing to apoptosis induced by miR-10a. Overexpression of miR-10a did not affect cell viability as evaluated using the cell counting kit and the measurement of caspase 3/7 (Supporting Fig. S2). More importantly, miR-10a-induced phenotypes, such as the suppression of TG and T-Chol in Huh-7 cells, and the inhibition of *SREBF1*, *SREBF2*, *FASN*, *PPARA*, *PCK1*, *PPARGC1*, phosphorylated (p)-mTOR, p-S6K, and p-AMPK were all rescued by the overexpression of *Bmal1* or *RORA* (Supporting Fig. S3).

In addition to the 41 patients with CHC (cohort 1), we extended mRNA expression in 50 patients with CHC (cohort 2). We found that the expression levels of *RORA*, *Bmal1*, *PPARA*, *PCK2*, and *LRH1* were significantly decreased in accordance with the progression of liver disease (Supporting Fig. S4). Conversely, expression of programmed cell death 4 (*PDCD4*), which is negatively regulated by mTOR signaling, significantly increased with the progression of liver disease (Supporting Fig. S4). Interestingly, expression of *Bmal1* was negatively correlated with serum levels of ALT, aspartate aminotransferase (AST), and gamma guanosine triphosphate (Supporting Fig. S5A). Moreover, the expression of *Bmal1* was positively correlated with the expression of mitochondrial oxidative phosphorylation (OXPHOS)-related genes (Supporting Fig. S5B). Thus, *Bmal1* is closely associated with liver function and mitochondria biogenesis. Interestingly, patients with sustained virologic response (SVR) by PEG-IFN+RBV therapy expressed higher hepatic *Bmal1* than patients without SVR (Supporting Fig. S4).

EXPRESSION OF miR-10a CAN SERVE AS A USEFUL BIOMARKER FOR HEPATIC RESERVES IN PATIENTS WITH LC

Our results suggested the expression of miR-10a is associated with hepatic reserves and prognosis in

patients with CHC. Therefore, we measured the expression levels of miR-10a in the additional liver tissues of 66 patients with LC (Table 1; Supporting Table S2). Liver tissue miR-10a levels were positively correlated with serum alkaline phosphatase (ALP) levels and negatively correlated with serum albumin levels and high-density lipoprotein cholesterol (HDL-Chol) levels (Fig. 5A). As a marker for fibrosis, miR-10a levels were positively correlated with collagen IV, liver stiffness on FibroScan, the AST to platelet ratio index, and the fibrosis-4 index (Fig. 5B). Expression of miR-10a was also significantly higher in patients with Child-B and Child-C than in those with Child-A and significantly higher in patients receiving BCAAs than in those not receiving BCAAs. In addition, expression of miR-10a was significantly higher in patients with esophageal varices at a high risk of rupture than in those without this risk (Fig. 5C).

The relationship between expression of miR-10a and recurrence-free survival period following radical treatment for HCC was also examined. We found that the recurrence-free survival period was significantly shorter in the group with elevated expression of miR-10a than in the group with low expression of miR-10a (Fig. 5D). Therefore, the expression of miR-10a may serve as a useful biomarker for hepatic reserves in patients with LC.

Discussion

The progression of chronic hepatitis results in various metabolic disorders, such as hypocholesteremia, fasting hypoglycemia, hypoalbuminemia, hyperbilirubinemia, and hyperammonemia. Furthermore, it can be associated with fatal complications, such as esophageal varices and HCC. In recent years, the advent of effective treatment with direct-acting antiviral drugs has markedly improved the SVR rate in patients with CHC. However, in patients with LC, HCC development

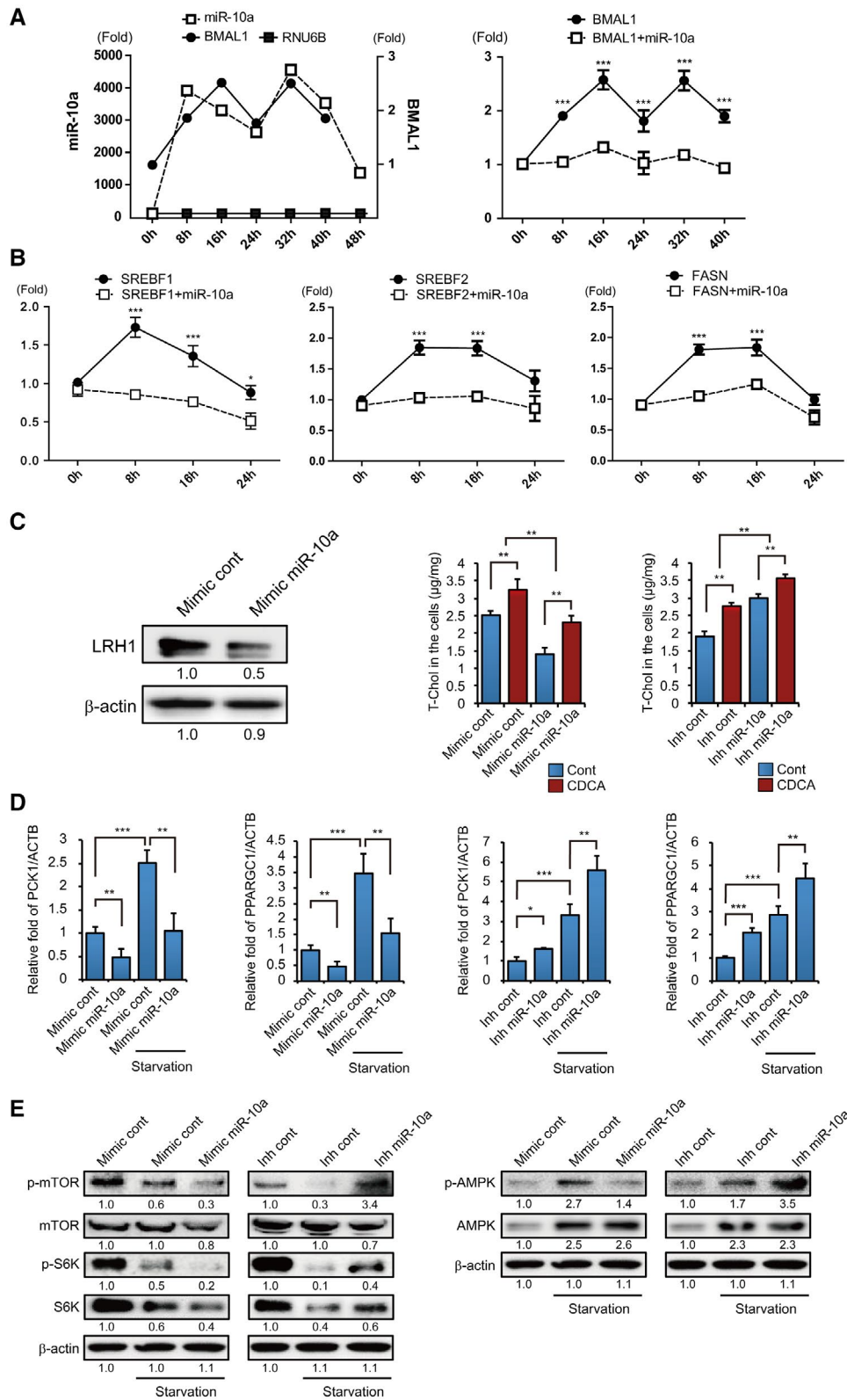


FIG. 4. Rhythmic expression of miR-10a and the relationship with bile acid, gluconeogenesis, and protein synthesis-related genes. (A) Huh-7 cells were synchronized by serum shocked with 50% FBS and then released in serum-free 1X DMEM. Cells were harvested, and the expression of miR-10a, RNU6B, and *Bmal1* was assessed at 8-hour intervals (left). Rhythmic expression of *Bmal1* was evaluated with the overexpression of miR-10a (right). Data were expressed relative to circadian time 0. (B) Rhythmic expression of *Bmal1*-regulating genes, *SREBF1*, *SREBF2*, and *FASN*, under the overexpression of miR-10a. (C) Western blotting of LRH1, a regulator of bile acid synthesis-limiting enzyme CYP7A1 (left). T-Chol contained in Huh-7 cells (right). After transfection with miR-10a, CDCA was added to the cell culture. (D) Expression of gluconeogenesis-related transcription factors. Huh-7 cells were transfected with mimic-miR-10a or anti-miR-10a. Expression levels of *PCK1* and *PGC1 α* were measured by RTD-PCR. (E) Western blotting of mTOR, S6K, and AMPK. Expression levels changed with mimic-miR10a and anti-miR-10a. All tests were doubled and repeated 3 times. Values are shown as mean \pm SD. ** $P < 0.005$, *** $P < 0.001$. Abbreviations: h, hours; RNU6B, RNA U6 small nuclear 6, pseudogene.

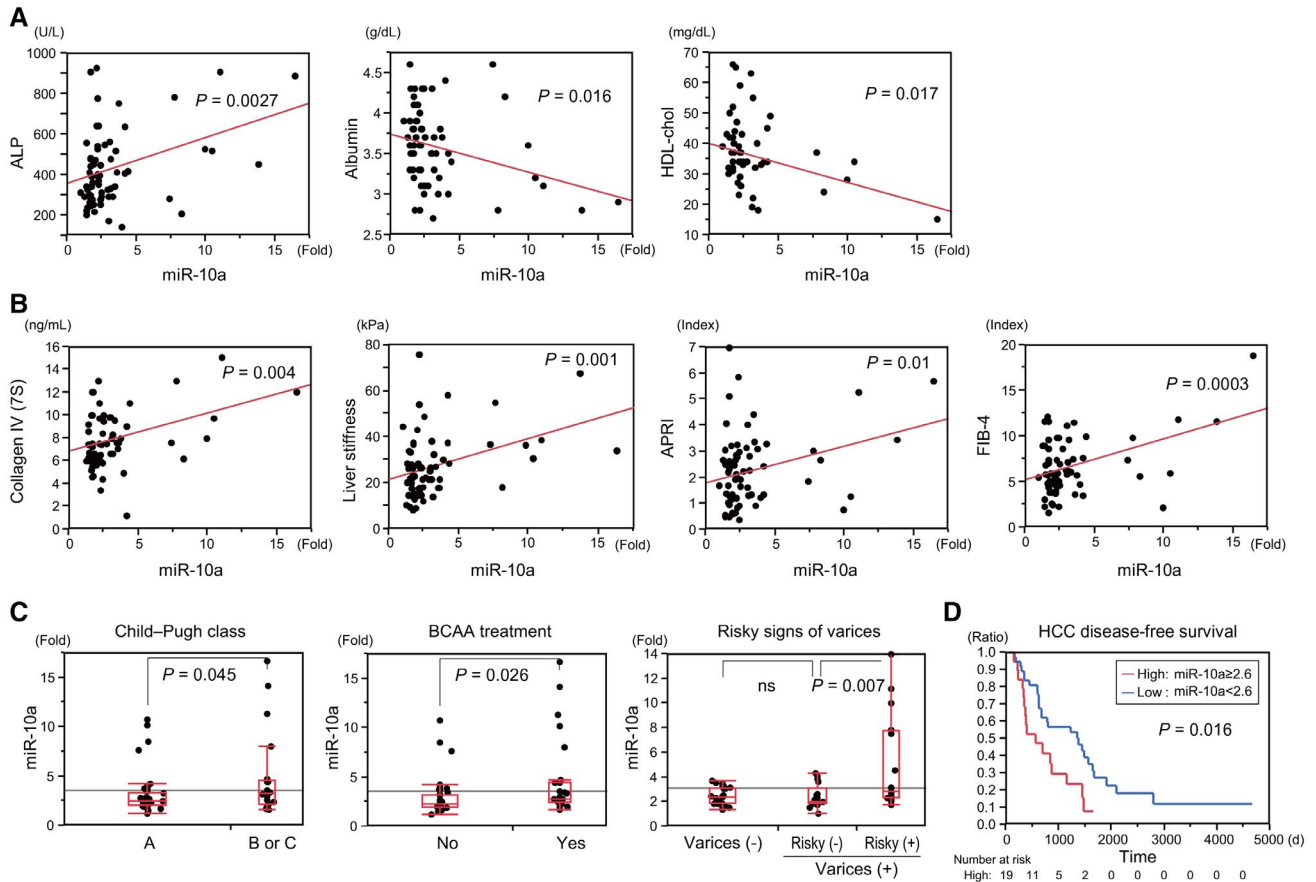


FIG. 5. Expression of miR-10a in liver tissues and clinical data of patients with hepatitis C-related LC. Expression values of miR-10a are shown as relative fold to control RNU6B. (A) Comparison of miR-10a expression in liver tissue and serum markers. Expression of miR-10a was inversely correlated with that of ALP, albumin, and HDL-Chol. (B) Expression of miR-10a in liver tissue correlated with that of fibrosis markers. Expression of miR-10a was significantly correlated with that of collagen IV, liver stiffness measured by FibroScan502, APRI, and FIB-4 index. (C) Relationship among the expression of miR-10a in liver tissue, hepatic reserve, presence or absence of BCAA treatment, and presence or absence of gastroesophageal varices. (D) Expression of miR-10a in liver tissues and the recurrence-free survival period following radical treatment of liver cancer. Expression of miR-10a in the background liver tissues was inversely correlated with disease-free survival. Abbreviations: APRI, AST to platelet ratio index; FIB-4, fibrosis-4; RNU6B, RNA U6 small nuclear 6, pseudogene.

occurs at a rate of 1% per year and liver disease progresses at a rate of 2% per year after achieving SVR.⁽²⁵⁾ Therefore, for patients with LC who achieve SVR, useful biomarkers predicting the complications of LC are warranted for improving its prognosis.

In this study, by comprehensive expression analysis of miRNAs using TaqMan PCR, we identified the miRNAs that are significantly up-regulated in the livers of patients with advanced CHC (Table 2). Among the 20 miRNAs differentially expressed with

the progression of liver fibrosis in this study (Table 2), we focused on miR-10a, miR-195, miR-199a-3p, miR-218, and miR-19a. To identify the genes that could be regulated by these miRNAs, we performed mRNA expression analysis by Affymetrix GeneChip using corresponding liver tissues. Notably, unlike other miRNAs, miR-10a was found to regulate liver metabolism. Therefore, we further focused on miR-10a and investigated the functional relevance of miR-10a to abnormal liver metabolism.

Although we did not identify the promoter region of miR-10a in this study, the expression level of miR-10a was substantially increased by HCV infection and low amino acid levels compared with that of other miRNAs, such as miR-199a-3p (Fig. 1A,B). However, the combination of low amino acid levels (from one-half to 1/10 DMEM) and HCV infection (MOI = 1) did not show further induction of miR-10a (Fig. 1B). This is probably because the amount of HCV infection might not be enough for further induction of miR-10a. Further study could be performed by using a greater amount of HCV. The expression of miR-10a and miR-199a-3p is reportedly induced by transforming growth factor β (TGF- β),^(26,27) and TGF- β signaling was induced by HCV infection and poor nutrition.⁽²⁸⁾ Therefore, TGF- β signaling might be involved in the induction of these miRNAs in this study.

To the best of our knowledge, the present study is the first to demonstrate that miR-10a negatively regulates the expression of the circadian rhythm gene *Bmal1* through the inhibition of the expression of the nuclear receptor *RORA* (Fig. 3). *RORA* binds to ROR-responsive element (*RORE*) in the promoter region of *Bmal1* and positively regulates *Bmal1* transcription.⁽²⁹⁾ We showed that miR-10a is also a rhythmic gene similar to *Bmal1*, and interestingly, rhythmic change of *Bmal1* was blunted by the overexpression of miR-10a (Fig. 4A). Similarly, up-regulation of *Bmal1*-induced expression of *SREBF1*, *SREBF2*, and *FASN* were also hampered by miR-10a (Fig. 4B).

The expression of *Bmal1* fluctuates in an approximately 24-hour cycle in contrast to the period circadian clock 2 (*per2*) gene, which fluctuates in an opposite phase.⁽²⁹⁾ Contrary to humans, in nocturnal mice and rats administered a meal at night, expression levels of *Bmal1* were elevated at night but low during the day.⁽¹⁸⁾ In liver-specific *Bmal1*-KO mice, a considerable reduction of genes associated with lipid neogenesis (*SREBP1c*, *FASN*, acetyl-coenzyme A [CoA]

carboxylase 1 [*ACCI*], and stearyl-CoA desaturase 1 [*SCD1*]) was observed.⁽³⁰⁾ In addition, fasting hypoglycemia and a loss of elevation of *PGC1 α* expression level were observed.⁽³¹⁾ Chromatin immunoprecipitation-on-chip analysis of *Bmal1* in mice livers revealed a significant accumulation of *Bmal1* in the regions of the *mTOR* promoter (8.73E-15) and *LRH1* promoter (7.50E-05), suggesting that *Bmal1* directly induces such gene expressions.⁽³²⁾ Furthermore, it is reported that AMPK activates *RORA*⁽³³⁾ and induces *Bmal1* expression through direct phosphorylation of *PGC1 α* , which plays a role as a coactivator of *RORA* for *Bmal1* transcription.^(34,35) *RORA* indirectly increases *Bmal1* expression by up-regulating *PPARA*.^(36,37)

The present study showed that miR-10a suppressed lipid synthesis, gluconeogenesis, and protein synthesis by inhibiting the expression of various *RORA*-*Bmal1*-related genes, such as *SREBF1*, *SREBF2*, *FASN*, *PPARA*, *PCK1*, *PPARGC1*, *LRH1*, p-mTOR, p-S6K, and p-AMPK (Figs. 2-4). Importantly, miR-10a-induced phenotypes were all rescued by the overexpression of *Bmal1* or *RORA* (Supporting Fig. S3). The results indicated that the regulation of liver metabolism by miR-10a is mainly through the suppression of the *RORA*-*Bmal1* pathway. However, in lipid metabolism, we found that miR-10a stimulated fatty acid oxidation as well as the inhibition of lipogenesis (Fig. 2A). Increased fatty acid oxidation by miR-10a could not be explained simply by the suppression of *RORA*-*Bmal1*; therefore, there might be other miR-10a-mediated pathways independent of *RORA*-*Bmal1*.

A recent study has revealed that *Bmal1* regulates the genes involved in mitochondrial biogenesis and that liver-specific *Bmal1*-KO mice showed loss of metabolic flexibility in response to nutrient influx, causing mitochondrial swelling with increased oxidative stress; when loaded with a high-fat diet, the mice exhibited insulin resistance and fatty liver.⁽³⁸⁾ Therefore, the reduced expression of *Bmal1* in CHC potentially worsened liver dysfunction, causing diabetes and fatty liver. In fact, we found the expression of *Bmal1* was positively correlated with the expression of mitochondrial OXPHOS-related genes (Supporting Fig. S5) and the expression of *Bmal1* decreased in advanced CHC (Supporting Fig. S4).

Expression of miR-10a increases with progression of liver fibrosis; therefore, we examined the direct influence of miR-10a on liver fibrosis using human

hepatic stellate cell-derived Lx-2 cells. We found that the overexpression of miR-10a decreased the expression of phosphorylated mothers against decapentaplegic homolog 2 (pSMAD2)/3 and collagen type I alpha 1 chain (COL1A1). By contrast, the inhibition of miR-10a expression increased the expression of pSMAD2/3 and COL1A1 (Supporting Fig. S6). Therefore, it appears that miR-10a has an inhibitory effect on fibrogenesis in hepatic stellate cells.

Finally, we examined the usefulness of miR-10a as a biomarker for hepatic reserves. The Model for End-Stage Liver Disease (MELD) score serves as a tool to predict prognosis in patients with LC; however, the survival rate of 15%–20% of patients cannot be predicted by the MELD score.⁽¹⁾ To determine the feasibility of miR-10a as a novel biomarker, miR-10a expression was measured in additional liver tissues of 66 patients with LC and compared with clinical data (Supporting Table S2). We found that the expression of miR-10a correlated well with the liver function test (Fig. 5A) and liver fibrosis markers (Fig. 5B) and that the expression of miR-10a increased in patients with poor hepatic reserves and in those with risky varices (Fig. 5C). Furthermore, in 57 patients with HCC, it was observed that an increased expression level of miR-10a led to a shorter recurrence-free survival period (Fig. 5D). It has been reported that the expression level of miR-10a is elevated in pancreatic cancer⁽³⁹⁾ and non-small cell lung cancer⁽⁴⁰⁾ and that it promotes metastasis in uterine cancer.⁽⁴¹⁾ However, the reports on miR-10a expression in HCC are controversial, with certain reports indicating increased expression level⁽⁴²⁾ and others indicating reduced expression level.^(43,44) miR-10a reportedly inhibits HCC metastasis by inhibiting the expression of ephrin type-A receptor 4 (*EphA4*).⁽⁴⁵⁾ However, in the present study, we measured the expression level of miR-10a in the background liver tissues and found that elevated expression level of miR-10a reflects liver damage and that the expression level of miR-10a could be useful for identifying groups at a high risk of HCC.

In conclusion, we revealed miR-10a is a clock gene and involved in abnormal liver metabolism and acceleration of liver dysfunction by inhibiting the expression of *Bmal1*. Furthermore, miR-10a can serve as a useful biomarker to predict prognosis in patients with LC. Further studies are required to

investigate miR-10a in serum and in case of other liver diseases, such as chronic hepatitis B and nonalcoholic steatohepatitis.

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