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Alterations in microRNA expression profile in HCV-infected hepatoma cells: Involvement of miR-491 in regulation of HCV replication via the PI3 kinase/Akt pathway

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ABSTRACT

The aim of this study was to investigate the role of microRNA (miRNA) on hepatitis C virus (HCV) replication in hepatoma cells. Using miRNA array analysis, miR-192/miR-215, miR-194, miR-320, and miR-491 were identified as miRNAs whose expression levels were altered by HCV infection. Among them, miR-192/miR-215 and miR-491 were capable of enhancing replication of the HCV replicon as well as HCV itself. HCV IRES activity or cell proliferation was not increased by forced expression of miR-192/miR-215 or miR-491. Investigation of signaling pathways revealed that miR-491 specifically suppressed the phosphoinositol-3 (PI3) kinase/Akt pathway. Under inhibition of PI3 kinase by LY294002, the suppressive effect of miR-491 on HCV replication was abolished, indicating that suppression of HCV replication by miR-491 was dependent on the PI3 kinase/Akt pathway. miRNAs altered by HCV infection would then affect HCV replication, which implies a complicated mechanism for regulating HCV replication. HCV-induced miRNA may be involved in changes in cellular properties including hepatocarcinogenesis.

1. Introduction

Hepatitis C virus (HCV) is a major causative agent of liver diseases worldwide. Elimination of HCV fails in about 80% of infected patients, which leads to chronic hepatitis, liver cirrhosis, and subsequent development of hepatocellular carcinoma [1]. Combination therapy of pegylated-interferon-α and ribavirin results in sustained clearance of serum HCV-RNA in only ~50% of patients [2,3]. To improve therapeutic efficacy of the virologic response rate, drugs inhibiting the functions of HCV proteins such as NS3, NS5A, and NS5B, are currently under development. Although a number of studies have clarified the mechanisms of the effect of HCV on infected cells or the role of host factors on regulation of HCV replication, there remains much to be investigated.

MicroRNAs (miRNAs) were identified as a population of small RNAs, modulating translation by binding to sites of antisense complementarity in 3′ untranslated regions of target mRNA [4]. With respect to regulation of HCV replication, the relevance of several miRNAs has been recently reported. miR-122, a hepatocyte-specific miRNA, was identified as a positive regulatory factor for HCV replication by binding to two sites in the HCV genome [5]. Each of the interferon-β-induced miRNAs, miR-196, miR-296, miR-351, miR-431, and miR-448, has a partially complementary sequence to HCV, resulting in suppression of HCV replication [6]. Thus, a miRNA with homology to the HCV sequence is likely to have the ability to regulate HCV. Another possible mechanism of miRNA regulation of HCV replication is the targeting of some cellular gene involved in HCV replication. miR-141 was shown to suppress DLC-1 leading to efficient HCV replication [7]. Although some miRNAs were shown to be capable of regulating HCV replication, details of the relationship between miRNAs and HCV replication are still largely unknown.

In the present study, we performed miRNA array analysis to identify miRNA(s) altered by HCV infection in Huh7, a hepatoma cell line. We further investigated whether HCV-regulated miRNA could, in turn, affect HCV replication. As a result, we were able to identify five miRNAs: miR-192 and its homolog miR-215 and miR-194 as upregulated miRNAs and miR-320 and miR-491 as downregulated miRNAs. Among them, miR192/miR-215 and miR-491 enhanced HCV replication in HCV replicon cells as well as in cell culture-infectious HCV (HCVcc)-infected cells. miR-192/miR215 and miR-491 did not increase cell proliferation or HCV internal ribosome entry site (IRES) activity, suggesting that these were not the reasons for increased HCV replication. Further investigation revealed that miR-491 suppressed the PI3 kinase/Akt

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pathway suggesting that this could be responsible for augmentation of HCV replication by miR-491.

2. Materials and methods

2.1. Cells, antibodies

The hepatoma-derived cell line Huh7 was maintained in DMEM supplemented with 10% FCS. The HCV subgenomic cell line Huh-RepSI, harboring HCV-N (genotype 1b), was previously described [8]. Antibodies to phospho-ERK (Thr202/Tyr204), Akt, phospho-Akt (Ser473) were purchased from Cell Signaling Technology. An antibody to β-actin (A-5441) was from Sigma–Aldrich. A mouse monoclonal antibody to HCV core protein (C7-50) was obtained from Affinity BioReagents. A mouse monoclonal antibody to HCV NS5A (clone 388) was from Meridian Life Science, Inc. LY294002, a PI3 kinase inhibitor, was obtained from Calbiochem.

2.2. Immunoblot analysis

Total cellular protein was extracted with lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium vanadate, 50 mM NaF, and protease inhibitor cocktail (Nacalai Tesque, Japan) in phosphate-buffered saline. Protein samples were separated by SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking, the membrane was probed with specific primary antibodies, followed by further incubation with a secondary antibody conjugated with horseradish peroxidase (GE Healthcare). Proteins were visualized using ECL Western blot detection reagents (GE Healthcare) and exposure to film.

2.3. miRNA transfection

Synthesized miRNAs, miR-192, miR-194, miR-215, miR-320, miR-491, and negative control miRNA were purchased from Thermo Fisher Scientific. Cells (2×10^5 per well) were seeded into 6-well plates, transfected with miRNA at a concentration of 10 nM using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instruction. After incubation for 2 days, the cells were harvested and assayed by immunoblot or real-time RT-PCR analysis.

2.4. Dual luciferase assay

We used a dicistronic plasmid, pRLHL, to investigate the effects of miRNAs on HCV IRES (Fig. 2A) [9]. Huh7 cells (1×10^6 cells in a 10-cm dish) were transfected with 10 μg of pRLHL using FuGene6 (Roche). After 24 h, the cells were seeded into 24-well plates (5×10^4 cells per well) and transfected with miRNA or negative control at a concentration of 10 nM as described above. After incubation for 2 days, cells were lysed, and assayed for HCV IRES-dependent firefly luciferase activity and cap-dependent renilla luciferase activity using the Dual Luciferase Reporter Assay System (Promega).

2.5. Cell culture-infectious HCV

HJ3-5(YH/QL) is a chimeric cell culture-infectious virus with a genome consisting of the core to NS2 sequence of genotype 1a (H77) virus placed within the background of the genotype 2a JFH1 virus, and containing compensatory mutations in E1 (Y361H) and NS3 (Q1251L) [10]. Virus stock (10^7 focus-forming units (FFU)/ml) was prepared as described previously [11].

For HCV infection, Huh7 cells (2×10^5 per well) were seeded into 6-well plates. After overnight incubation, the medium was replaced with 1 ml medium containing 4×10^5 FFU virus (the infection was carried out at an m.o.i. of ~2). After 12 h incubation, the cells were washed with PBS and re-fed with normal culture medium. At 5 days after inoculation with the virus, total RNA was obtained from the cells using Trizol (Invitrogen).

2.6. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells with RNAeasy (QIAGEN). The RNA, 1 μg, was reverse transcribed with High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) in a 20 μl reaction, then 1 μl of the reaction was subjected to real-time PCR assay using TaqMan Gene Expression Assays (Applied Biosystems).

2.7. Cell proliferation assay

Cell proliferation was assessed by WST-1 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2,4-disulpho-phenyl]-2H-tetrazolium, monosodium salt) assay according to the manufacturer's suggested protocol (Nacalai Tesque). Briefly, Huh7 cells (1×10^4 per well) were seeded into 96-well flat-bottom plates, transfected with synthesized miRNA or negative control as above, and cultured in DMEM containing 10% FBS. WST-1 reagent, 10 μl, was added to each well, the cells were incubated at 37 °C for 1 h, and absorbance at 450 nm was measured using a spectrophotometer.

2.8. miRNA array analysis

To screen for miRNA affected by HCV infection, we performed microarray analysis using *mir*Vana miRNA Bioarray V9.2 (Ambion), which carries genes for a total 633 kinds of miRNAs containing 471 human genes, 380 mouse genes and 238 rat genes. Using the flash PAGE system (Ambion), miRNA was purified from 22 μg total RNA extracted from HCVcc-infected cells or mock-infected cells. The purified miRNA samples from HCVcc-infected cells and mock-infected cells were labeled with Cy3 and Cy5, respectively, using mirVana miRNA Labeling kit (Ambion) and CyeDye Mono-Reactive Dye Pack (GE Healthcare Biosciences). The labeled miRNA was hybridized to the array for ~16 h at 42 °C. After hybridization, the array was washed with Low Stringency Wash (Ambion) once and High Stringency Wash (Ambion) twice. Next, the array was dried with centrifugation at 600*g* for 3 min and scanned with GenePix 4000B scanner (Axon Instruments, CA, USA). The signal data were calculated with an Array-Pro Analyzer ver. 4.5 (Media Cybernetics, Inc.). The array data were normalized by global normalization using the Microarray Data Analysis Tool (Filgen, Inc.).

3. Results

3.1. Identification of miRNAs regulated by HCV infection

Huh7 cells were infected with HCVcc at ~2 m.o.i. After incubation for 5 days, total RNA was extracted from the cells followed by purification with small RNA and miRNA array analysis. A portion of the cells was subjected to immunofluorescence analysis for staining of HCV core protein to verify that more than 90% of the cells were infected with HCV. The ratio of Cy3 intensity to Cy5 intensity was calculated and alteration of the miRNA expression profile was analyzed. A ratio of more than 1.5-fold increase/decrease was considered to be altered. To exclude miRNAs with low expression levels, those with a net intensity of Cy3 and Cy5 of more than 1000 were picked out. As a result, the miRNAs of miR-192, miR-194, miR-320, and miR-491 were identified as altered miRNAs (Table 1). miR-192 and miR-194 were up-regulated by HCV infection,

Table 1
miRNAs altered by HCV infection.

miRNA	Intensity				Sequence
	Cy3 (HCV)	Cy5 (mock)	Net	Cy3/Cy5	
miR-192	987.90	607.05	1594.95	1.63	CUGACCUAUGAAUUGACAGCC
miR-194	793.48	498.00	1291.48	1.59	UGUAACAGCAACUCCAUGUGGA
miR-215	156.21	69.39	225.60	2.25	AUGACCUAUGAAUUGACAGAC
miR-320	897.44	1401.93	2299.37	0.64	AAAAGCUGGGUUGAGAGGGCGAA
miR-491	925.38	2495.47	3420.85	0.37	AGUGGGGAACCCUUCCAUGAGGA

and miR-320 and miR-491 were down-regulated. In addition, miR-215, whose net expression while relatively low, was also studied in the subsequent investigation as an upregulated miRNA because it is considered to be a cousin of miR-192 (see their homologous sequences in Table 1). miR-215 showed a high induction level, and miR-192 and miR-215 were reported to have common induction mechanisms and target genes [12,13].

3.2. Regulation of HCV replication by miRNAs

Next, we checked whether the miRNAs were capable of regulating HCV replication. To assess this, we transfected Huh-RepSI, a HCV subgenomic replicon cell line, with synthesized miRNAs, and then monitored HCV RNA abundance and NS5A protein abundance using real-time RT-PCR and immunoblot analysis, respectively. Among the five miRNAs tested, miR-192/miR-215 and miR-491 significantly increased replicon abundance (Fig. 1A and B), while miR-194 and miR-320 did not show any significant change. HCV subgenomic replicon RNA contains the NS3 through NS5B region, which is required for genome RNA replication, but not for virus particle production. To confirm that the effect of the miRNAs was reproducible in a system equipped with the entire HCV life cycle, we used Huh7 cells infected with HCVcc. As expected, HCV abundance was upregulated by the three miRNAs in the HCVcc-infected cells similarly to HCV replicon cells (Fig. 1C and D). In addition, the HCV strain used in the experiment was a chimera of genotype 1a (H77, core to NS2) and genotype 2 (JFH-1, NS3 to NS5B) [10]. In particular, the genotype of the replication machinery of the virus (namely, NS3 to NS5B) was JFH-1. This differed from that of Huh-RepSI (HCV-N, genotype 1b) [8], which suggests that the enhancing effect of miR-192/miR-215 and miR-491 on HCV genome replication was not genotype-specific.

3.3. Effect of miRNAs on HCV IRES, cell proliferation

Since miR-192/miR-215 and miR-491 were shown to be capable of enhancing HCV replication, we next tried to elucidate how they regulate it. First, we examined whether the miRNAs can regulate HCV IRES activity. In this experiment, we transfected replicon cells with a dicistronic vector, pRLHL [9], which contained the firefly luciferase gene driven by HCV IRES and the renilla luciferase gene translated in a cap-dependent manner which was used as a control of general translational activity (Fig. 2A). After 24 h, the miRNAs were transfected, then luciferase activities induced by HCV IRES and cap translation were measured at 2 days after

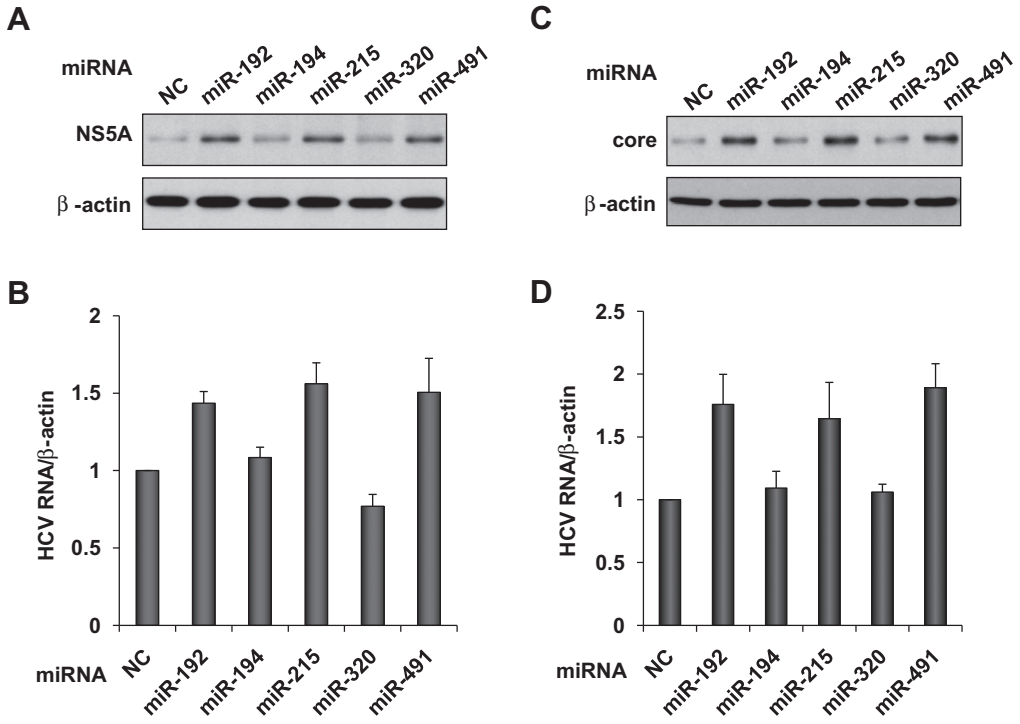

Fig. 1. Regulation of HCV replicon or HCVcc abundance by miRNAs. Cells of Huh-RepSI, a HCV subgenomic replicon, were transfected with synthesized miRNAs and assayed for NS5A protein expression (A) or HCV RNA abundance (B). HCVcc-infected Huh7 cells were transfected with synthesized miRNAs and assayed for core protein expression (C) or HCV RNA abundance (D). NC: negative control miRNA.

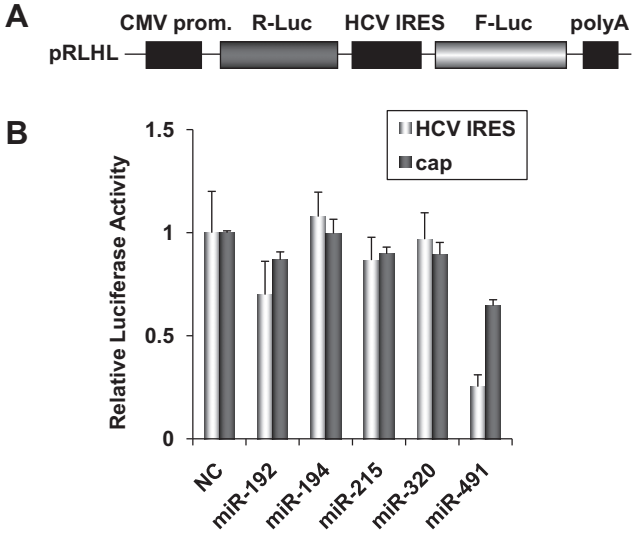

Fig. 2. Regulation of HCV IRES and cap-dependent translation by miRNAs. Huh-RepSI cells were transfected with a dicistronic vector, pRLHL (A), incubated for 24 h. The cells were seeded to 24-well plates and transfected with the miRNAs. After further incubation for 2 days, the cells were harvested and assayed for dual luciferase activity (B).

transfection (Fig. 2B). In this assay, activation of IRES was determined by the ratio of IRES-dependent luciferase activity to cap-dependent luciferase activity. Interestingly, none of the miRNAs could increase the HCV IRES activity. miR-491 suppressed cap-dependent translation and showed more suppression of HCV IRES activity. Thus, these results indicated that there was some mechanism upregulating HCV replication other than regulation of IRES activity.

Previous work demonstrated that HCV replication was affected by cell proliferation [14]. This led us to access the effects of the miRNAs on cell proliferation. Compared to negative control miRNA-transfected cells, however, none of the transfectants of the miRNAs, including those which increased HCV replication, revealed upregulation of cell proliferation, and miR-491 even suppressed it (Fig. 3). Therefore, regulation of cell proliferation was not the reason for the increase of HCV replication. The effect of miR-491 of suppressing cell growth was likely to be caused by inhibition of general translation as shown in Fig. 2B.

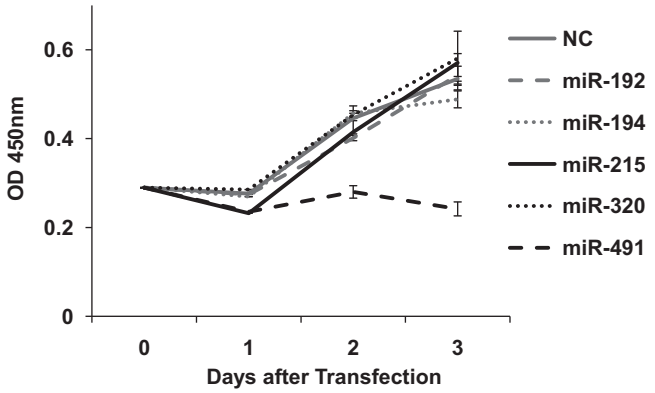

Fig. 3. Regulation of cell proliferation by miRNAs. Huh7 cells were seeded into 96-well plates, transfected with the miRNAs. At day 0, 1, 2, and 3 after transfection, the cells were subjected to WST-1 assay as described in Section 2.

3.4. Effect of miRNAs on intracellular signaling

To clarify the mechanism of the regulation of HCV replication, we next focused our investigation on intracellular signaling pathways. Previous studies have reported that HCV replication is regulated by intracellular signaling pathways, such as ERK [15], p38 [8], PI3 kinase/Akt [11], and smad [16], in addition to JAK/STAT. Since transfection of the miRNAs had no effect on the JAK/STAT signaling pathway (data not shown), we examined the phosphorylation of ERK and Akt. Because both showed a suppressing effect on HCV replication, suppression of the pathway was anticipated in cells in which HCV replication was enhanced. As shown in Fig. 4A, phosphorylation of Akt at Ser-473 was markedly suppressed in the cells transfected with miR-491, while no significant inhibition of ERK activity was observed. To further investigate the relevance of the PI3 kinase/Akt pathway to miR-491-induced upregulation of HCV replication, we used LY294002, a PI3 kinase inhibitor. When the PI3 kinase pathway was blocked by this reagent, the HCV RNA level was enhanced up to 2-fold. miR-491 transfection also resulted in an increase of HCV abundance, though the effect was less

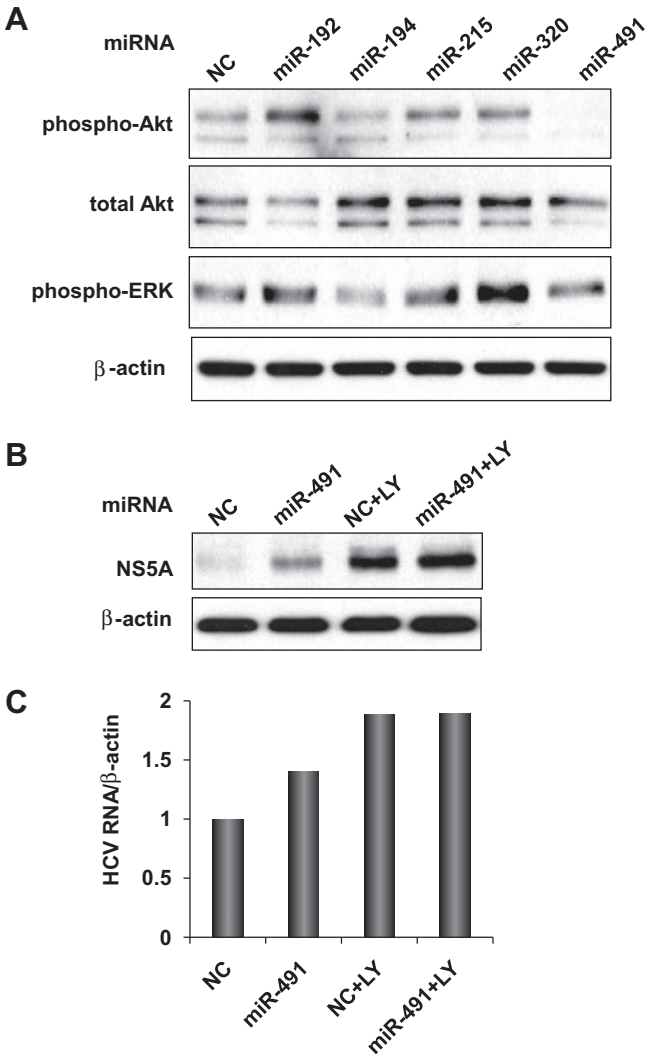

Fig. 4. Involvement of Akt suppression in miR-491-mediated upregulation of HCV replication. (A) Immunoblot analysis of miRNA-transfected HCV replicon cells using antibodies to Akt, phospho-Akt, phospho-ERK and β-actin. (B and C) HCV replicon cells were transfected with miR-491 or treated with Akt inhibitor, and assayed for NS5A protein abundance (B) or HCV RNA abundance (C). LY: LY294002.

than that of LY294002, presumably because of incomplete inhibition of Akt. When miR-491 transfected cells were cultured in the presence of LY294002, the HCV replication level was enhanced to the same extent as that in the LY294002-treated cells with negative control miRNA. Since no additive effect of miR-491 was observed under strong blockade of the PI3 kinase/Akt pathway, inhibition of this pathway was likely to be responsible for the miR-491-induced upregulation of HCV replication.

4. Discussion

In the present study, we tried to identify the miRNA(s) affected by HCV infection and establish how they influence HCV replication. Five miRNAs, miR-192, miR-194, miR-215, miR-320, and miR-491, were identified as HCV-regulated miRNAs by miRNA array analysis. Three upregulated miRNAs, miR-192, miR-194, and miR-215, were previously identified as p53-inducible miRNAs [12,13]. Two miRNA clusters which encode identical miR-194 sequences (i.e., the miR-194-2/miR-192 cluster on chromosome 11 and the miR-194-1/miR-215 cluster on chromosome 1) contain two closely related miRNAs, miR-192 and miR-215, suggesting that their expressions are regulated similarly which led to their simultaneous identification. miR-192/miR-194/miR-215 are known to act as tumor-suppressing miRNAs by inducing cell cycle arrest [12]. In Huh7 cells, however, the p53 function is believed to be abolished by a point mutation at codon 220. Therefore, the upregulation of miR-192/miR-194/miR-215 was likely to be exerted in a p53-independent manner. Since miR-192 and miR-194 are considered to be substantially expressed in human liver tissue [17] and there are several reports about the suppression of p53 function by HCV (reviewed in Ref. [18]), the result may not necessarily be the same if the investigation is conducted in human hepatocytes or in cells with intact p53 activity.

The downregulated miRNAs, miR-320 and miR-491, are considered to be relevant to carcinogenesis. miR-320 induces G1 arrest and suppresses cell proliferation by targeting CDK6 [19], CD71 [20], IGF1 [21] and induces apoptosis by suppressing Bcl-2 and Mcl-1 [22]. miR-491 is also capable of inducing apoptosis by targeting Bcl-xL [23], which is often upregulated in HCC tissues [24]. In this study, we showed that miR-491 inhibited the PI3 kinase/Akt pathway, which is one of the important pathways leading to cancerous properties. Importantly, miR-320 was identified as one of the significantly repressed miRNAs in CH-B, CH-C, and HCC compared with normal liver tissue [25]. Although the details of the relevance of miR320 and miR-491 to hepatocarcinogenesis have not yet been clarified, as these two miRNAs have a tendency to suppress genes related to carcinogenesis, their downregulation in HCV-infected cells may play some role in hepatocarcinogenesis.

Thus far, several miRNAs have been reported to regulate HCV replication. miR-122 was shown to be a direct activating factor for HCV replication [5], but alteration of this miRNA was not observed in response to HCV infection in this study. IFN-β-induced miRNAs, miR-196, miR-296, miR-351, miR-431 and miR-448, have been identified as anti-HCV miRNAs [6]. These miRNAs are able to regulate HCV replication by direct interaction with HCV genome RNA. In the case of miR-192/miR-215, there are several sites in the HCV genome sequence which show weak homology to the miRNAs (data not shown). Although the possibility of miR-192/miR-215 binding to the HCV genome and regulating replication cannot completely be excluded, this seems unlikely because the homologous sequence to miR-192/miR-215 cannot be found in the UTR region like miR-122 and direct binding to RNA usually suppresses the RNA function for protein synthesis. There is, however, a very rare case of miR-122-mediated facilitation of HCV replication by binding to two sites within the HCV genome.

Although the mechanism of miR-491-mediated suppression of the PI3 kinase pathway is not clear, it was speculated that some gene involved in Akt activation was the target of miR-491. However, the candidate of the target gene was not clearly found in the list of putative target genes of miR-491 revealed by in silico analysis. We tried to evaluate the mRNA levels of upstream genes of Akt, such as the genes which belong to the family of PI3 kinase, PTEN, growth factor receptors, using the RT-PCR method, but none of them was affected by miR-491 (data not shown). Nevertheless, investigation of target genes of miR-491 should be of interest for the field of oncology because here we have shown that miR-491 suppresses Akt, which is a factor closely related to various types of cancer via cell survival. Also, it has been demonstrated that miR-491 can induce apoptosis by ablating Bcl-xL [23]. Indeed, our observation that cell viability was significantly suppressed by forced expression of miR-491 presumably via decrease of Akt signaling suggests the anti-oncogenic feature of miR-491. Further study of the mechanism of miR-491, its target genes, and expression pattern in cancer tissue remain to be performed.

In conclusion, we showed altered expression profiles of miRNAs by HCV infection, and some of them were capable of regulating HCV replication, which may represent a complicated mechanism of HCV replication. A number of studies have demonstrated regulation of many cellular factors by miRNAs, which results in modulation of cellular functions including cell growth, apoptosis, cellular stresses, metabolism, and carcinogenesis. The miRNAs identified in this study may also be involved in changes in the phenotype of HCV-infected cells.

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