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Specific delivery of microRNA93 into HBV-replicating hepatocytes downregulates protein expression of liver cancer susceptible gene MICA

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ABSTRACT

Chronic hepatitis B virus (HBV) infection is a major cause of hepatocellular carcinoma (HCC). To date, the lack of efficient in vitro systems supporting HBV infection and replication has been a major limitation of HBV research. Although primary human hepatocytes support the complete HBV life cycle, their limited availability and difficulties with gene transduction remain problematic. Here, we used human primary hepatocytes isolated from humanized chimeric uPA/SCID mice as efficient sources. These hepatocytes supported HBV replication in vitro. Based on analyses of mRNA and microRNA (miRNA) expression levels in HBV-infected hepatocytes, miRNA93 was significantly downregulated during HBV infection. MiRNA93 is critical for regulating the expression levels of MICA protein, which is a determinant for HBV-induced HCC susceptibility. Exogenous addition of miRNA93 in HBV-infected hepatocytes using bionanocapsules consisted of HBV envelope L proteins restored MICA protein expression levels in the supernatant. These results suggest that the rescued suppression of soluble MICA protein levels by miRNA93 targeted to HBV-infected hepatocytes using bionanocapsules may be useful for the prevention of HBV-induced HCC by altering deregulated miRNA93 expression.

INTRODUCTION

Hepatitis B virus (HBV) infection is a major global health problem, and more than 350 million people globally are chronic carriers of the virus [1]. A significant number of these carriers suffer from either liver failure or hepatocellular carcinoma (HCC) during the late stages of the disease [2]. In fact, chronic infection with HBV is responsible for 60% of HCC cases in Asia and Africa and at least 20% those in Europe, Japan, and the United States [3].

While nucleoside and nucleotide analogs have been applied in the attempts to suppress HBV replication [4, 5], complete elimination of HBV (including cccDNA) remains difficult [6, 7], and an increased understanding of HBV replication and pathogenesis at the molecular level is essential for clinical management of chronic HBV infection. However, the lack of appropriate cell culture systems supporting stable and efficient HBV infection has been a major limitation. Although transient transfection or viral transfer of HBV genes or genomes are used in the study of specific steps of the HBV cell cycle [8-12], they do not accurately reflect the biology of HBV infection and replication. Thus, humanized mice are used for hepatitis virus research [13-18]. Although these mice are useful, immune deficient, chimeric mice are difficult to handle

and maintain. Therefore, a more convenient *in vitro* system is required for HBV research.

Primary human hepatocytes can support the complete HBV life cycle *in vitro* [7, 19], but a major drawback is their limited availability. To overcome difficulties regarding availability, we used chimeric mice as sources of primary human hepatocytes, which grow robustly during the establishment of chimeric mice, due to continual liver damage induced by urokinase-type plasminogen activator (uPA) [14, 15].

Another shortcoming of utilizing primary human hepatocytes is their difficulty with gene transduction due to the low transfection efficiency of their primary cell-like nature. Efficient gene delivery methods will significantly improve studies on primary hepatocytes for HBV replication. In addition, cell-specific targeting is required for efficient drug delivery *in vivo*. As a specific gene delivery method to liver-derived cells, bionanocapsules (BNCs) consisted of HBV envelope L particles have been tested for the selective delivery of genes, drugs, or siRNAs into liver-derived cells [20, 21]. Because these BNCs are consisted of HBV L protein, they may be applicable for drug delivery to HBV-infected primary human hepatocytes.

MicroRNAs (miRNAs) are endogenous ~22-nucleotide RNAs that mediate important gene-regulatory events by base-pairing with mRNAs and activating their repression [22]. We previously reported that modifying the expression of miRNAs in liver cells can efficiently regulate the expression levels of the MHC class I polypeptide-related sequence A (MICA) protein [23], which we previously identified as a crucial factor for the susceptibility of hepatitis virus-induced HCC and possibly hepatitis virus clearance [24, 25]. While emerging evidence suggests that miRNAs play crucial roles in chronic HBV infection [26], the comprehensive changes in miRNA expression levels induced by HBV infection in human hepatocytes or in alternative systems reflecting HBV-infected hepatocytes have not been explored.

In this study, we infected primary human hepatocytes isolated from chimeric mice with HBV and identified the transcripts and miRNAs whose expression levels changed. We explored whether BNCs carrying synthesized miRNAs could successfully deliver miRNAs into primary hepatocytes and rescue the modulated miRNA expression due to HBV replication. We found that BNCs carrying synthesized miRNA93 could efficiently restore deregulated soluble MICA protein levels in the supernatant of HBV-replicating primary hepatocytes. These results suggest that miRNA93 delivery into HBV-replicating hepatocytes using BNC methods may enhance HBV immune clearance or suppress HCC by altering miRNA93 levels in HBV-infected cells.

RESULTS

Changes in expression levels of transcripts and miRNAs during HBV replication in human primary hepatocytes

We examined changes in transcript and miRNA expression levels during HBV infection and replication in hepatocytes. Primary human hepatocytes were used for maintainng HBV replication *in vitro*. We first isolated primary hepatocytes from humanized chimeric mice. To examine the infectivity of HBV into the primary hepatocytes *in vitro*, HBsAg and HBV-DNA levels in the cell culture supernatant were measured after the cells were infected with approximately 1.5×10^7 copies of HBV/well in a 24-well plate at day 0. Although both HBsAg and HBV-DNA levels transiently decreased at approximately day 3, levels of both started to increase and were maintained until after day 23 post-infection (Figure 1a and b). These results suggested that human primary hepatocytes isolated from chimeric mice can efficiently support HBV replication *in vitro*, which can be used as an efficient *in vitro* HBV replication system.

To examine comprehensive changes in mRNA and miRNA expression levels in HBV-infected hepatocytes, cells at day 7 post-infection were collected and subjected to cDNA as well as miRNA microarrays. Among 24,460 genes examined, 65 were significantly upregulated by more than 4-fold, and 29 were downregulated to less than 25% (Supplementary Table 1 and 2); however, more than 800 total genes were upregulated or downregulated if the thresholds of the changes were set at 2-fold and 1.5-fold, respectively (Figure 1c; complete datasets have been deposited as GEO accession number: GSE55928). Among the upregulated genes, those associated with the cytochrome family, such as CYP2A7, CYP2C8, CYP2A6, CYP3A4, changed significantly, which was consistent with previous reports [27, 28]. However, few inflammatory cytokines or genes associated with cell growth changed significantly. Based on these results, host factors related to innate immunity may not sense HBV (at least under these replicating conditions), suggesting that this system may mimic the status of hepatitis B patients before seroconversion, in whom inflammation seldom occurs regardless of the high viral load.

Regarding changes in miRNA expression levels during HBV replication, among 2,019 mature miRNAs, 35 were upregulated and 14 downregulated by an increase or decrease of more than two-fold (Figure 1d and Supplementary Tables 3 and 4; complete datasets have been deposited as GEO accession number: GSE55929). Among these miRNAs, miR93-5p was significantly downregulated during HBV replication by more than 50%. Since miRNA93 regulates the expression levels

of the MICA protein [23, 29], which is involved in the susceptibility to hepatocellular carcinoma in chronic hepatitis patients [24, 25], we focused on this miRNA in further analyses.

Efficient delivery of miRNAs into liver cell lines using bionanocapsules

Efficient delivery methods of genes or compounds into targeted tissues or cells are essential to translate the *in vitro* results into clinical settings. Here, we utilized BNCs [21, 30, 31], which were originally developed to deliver genes and drugs with high efficiency and specificity to human liver-derived cells, as an efficient delivery method for miRNAs into human liver cells, including primary hepatocytes. Since BNCs are composed of HBV L proteins, the distribution of these BNCs and infected HBV should be similar. To confirm the efficiency of delivery of miRNAs into liver-derived cells by BNCs, we delivered BNCs carrying let-7g or miRNA93 to the human hepatocellular carcinoma cell lines, Huh7 and HepG2 cells, and to human normal hepatocytes immortalized with SV40 large T antigen, Fa2N4 cells [28]. The day after delivery of the BNCs, cells were collected and subjected to Northern blotting against let-7g, miRNA93, and U6, the loading control, and the results showed successful delivery of miRNAs into all cell lines tested (Figure 2a). The biological function of the delivered miRNAs was confirmed using luciferase-based reporters, which measured let-7g and miRNA93 functions [23]. Huh7 and Hep3B cells transfected with reporter constructs were delivered with let-7g or miRNA93 using BNCs, followed by a luciferase assay at the next day. Delivered miRNAs significantly decreased the corresponding luciferase activity, suggesting that the delivered miRNAs were functioning within the cells (Figure 2b).

We next examined the delivery of miRNAs into 293T cells (human embryonic kidney cell lines) to explore cell-specificity. Only a small increase in miRNA93 expression levels was observed 24 hours after transfer into 293T cells, based on Northern blots (Figure 2c), indicating that the BNCs had high specificity for hepatocyte-derived cells. The expression of transferred miRNA into Huh7 cells could be observed even 3 days after delivery (Figure 2d), suggesting that the delivered miRNAs are expressed

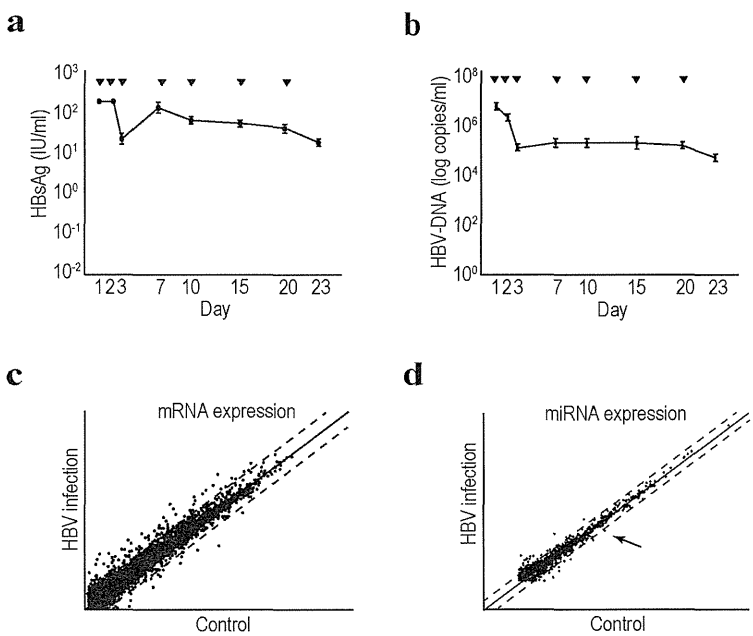

Figure 1: Comprehensive transcriptome and miRNA analyses in HBV-replicating human primary hepatocytes. a, b, Efficient HBV replication in human primary hepatocytes isolated from chimeric mice. Primary human hepatocytes isolated from chimeric mice were seeded into the wells of a 24-well plate. Serum from HBV-infected patients was added to infect the cells with HBV. Media was changed at the indicated days (▼). The supernatant was collected when the media was changed for the analyses of HBsAg levels (a) and HBV-DNA levels (b). Data represent the means ± s.d. of three independent experiments. c, Scatter plot reflecting the transcriptomic results comparing the control and HBV-replicating primary human hepatocytes. Cells at day 7 after HBV infection were used for the analyses. Intensity normalization was performed using global normalization based on the expression levels of all genes analyzed. Dashed lines indicate the thresholds: two-fold increase or 50% decrease in expression levels. The full data are deposited in NCBI GEO database accession: GSE55928. d, A scatter plot of the miRNA microarray results was used to determine the expression levels of comprehensive mature miRNAs. Total RNA from control and HBV-replicating primary hepatocytes at day 7 after infection was used. Dashed lines indicate the thresholds: two-fold increase or 50% decrease in expression levels. Intensity normalization was performed using global normalization based on the expression levels of all miRNAs. The arrow indicates the result for miRNA93. The full data are deposited in NCBI GEO database accession: GSE55929.

for several days.

miRNA delivery into human primary hepatocytes using bionanocapsules

Based on the efficient delivery of miRNA via BNCs into human liver-derived cell lines, we examined the BNC-mediated delivery of miRNAs into non-dividing human primary hepatocytes isolated from chimeric mice, as described above. BNCs could deliver miRNAs efficiently, even into non-dividing human primary hepatocytes, based on Northern blots (Figure 3a), irrespectively of the use of Polybren (Figure 3a).

Since the expression levels of miRNA93 were downregulated by HBV replication (Figure 1d and Supplementary Table 4), we delivered miRNA93 via BNCs into HBV replicating human hepatocytes to rescue the downregulation of miRNA93 levels and examine the effects of decreased miRNA93 on transcript levels (Figure 3b). The rescue of miRNA93 expression, recovered the baseline-level expression of some genes, such as 17-beta-hydroxysteroid dehydrogenase 14 (HSD17B14) and tripartite motif-containing protein 31 (TRIM 31), which

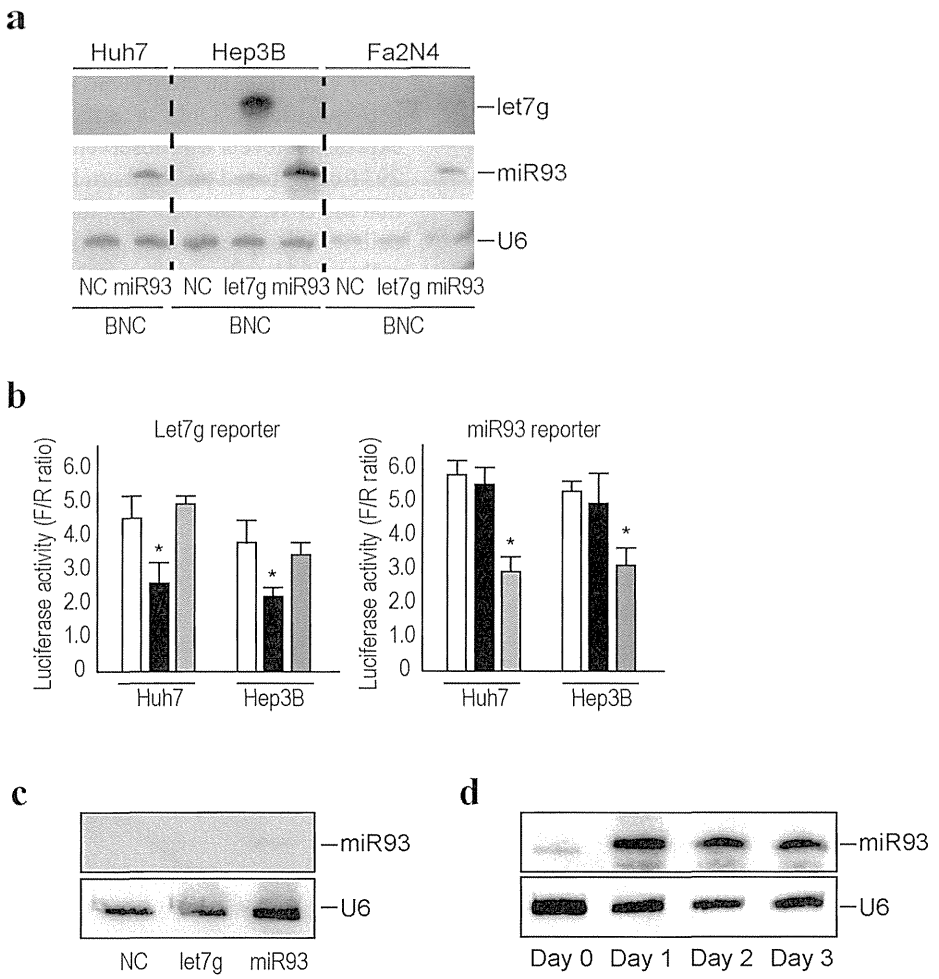

Figure 2: Efficient delivery of miRNAs into liver cell lines using BNCs. a, Northern blotting of miRNAs delivered into liver cells by BNCs. Liver cancer cell lines, Huh7 and Hep3B, and primary hepatocytes immortalized by SV40, Fa2N4, were incubated with BNCs harboring the indicated miRNAs (miRNA93 or let7g) or BNCs without miRNAs (NC). After 24 hours, cells were harvested and subjected to analysis. Membranes were re-probed for let7g, miRNA93, and U6 as a loading control. The results shown are representative of three independent experiments. b, miRNAs delivered using BNCs were biologically functional. Huh7 and Hep3B cells were transfected with the indicated reporter constructs, which indicate the activity of each miRNA function. Twenty-four hours after transfection, cells were mixed with BNCs containing let7g (black bar), miRNA93 (gray bar), or negative control (white bar). Forty-eight hours after transfection, cells were subjected to a dual luciferase assay. Data shown represent the means ± s.d. of the raw ratios (FL/RL), obtained by dividing the firefly luciferase values by the renilla luciferase values, of three independent experiments. $*p < 0.05$. c, Delivery of miRNAs via BNCs were liver cell-specific. The 293T cells (human embryonic kidney cells) were incubated with BNCs containing let7g, miRNA93, or negative control (NC). After 24 hours, cells were subjected to Northern blotting for miRNA93. U6 was used as a loading control. The results shown are representative of two independent experiments. d, miRNA93 expression in Huh7 cells after the delivery of miRNA93 via BNCs. Cells were sequentially collected after incubation with BNCs containing miRNA93 and subjected to Northern blotting. U6 was used as a loading control. The results shown are representative of three independent experiments.

were increased by HBV replication (Supplementary Table 1), suggesting that the mRNA levels of these genes may be directly or indirectly regulated by miRNA93. Although the enhanced decay of target transcripts by miRNAs has been reported [22, 32], miRNAs generally function as translational repressors [33]. However, these miRNA93 delivery results may not be accurate due to direct or indirect effects of miRNA93. In addition, changes in protein levels may differ from our transcript expression results.

Modulation of MICA protein expression levels by delivery of miRNA93 using BNCs

We previously identified miRNA93 as a critical regulator of MICA protein expression [23], which plays a role in the susceptibility to HBV-induced HCC [25]. MiRNA93 regulates MICA protein levels, but not transcript levels [23, 29]. Although it was found that miRNA93 expression levels decreased during HBV replication in primary hepatocytes (Figure 1d and Supplementary Table 4), MICA transcript levels were not affected (GEO accession number: GSE55928), suggesting that the effects of miRNA93 on MICA may be mediated by translational repression and not by mRNA decay, as we reported previously [23]. To confirm changes in the expression level of the MICA protein on the cell surface of primary hepatocytes induced by HBV infection, cells were subjected to FACS analyses. However, the protein expression levels on the cell surface did not change significantly (Figure 4a). MICA is a soluble protein released into the supernatant after shedding by ADAM10 and ADAM17[34]. Our results suggested that the

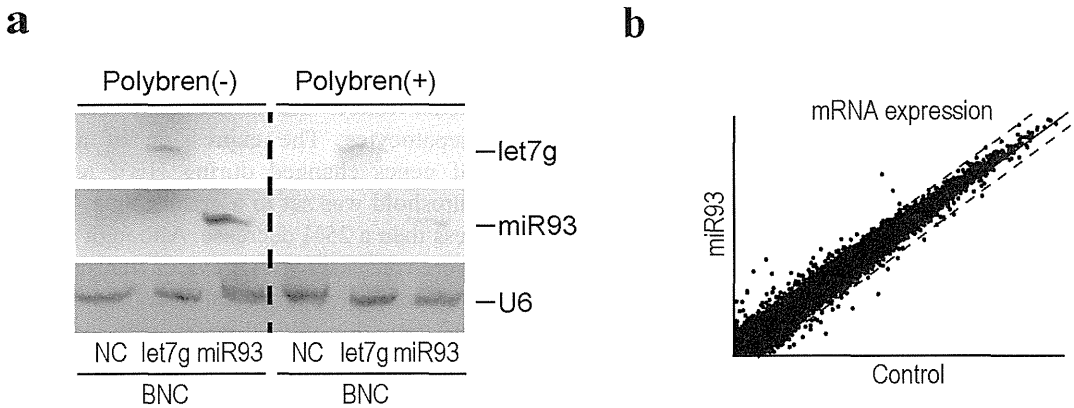

Figure 3: Efficient delivery of functional miRNAs into human primary hepatocytes using BNCs. a, Northern blotting for miRNAs delivered into cells using BNCs. Human primary hepatocytes isolated from chimeric mice were incubated with BNCs containing the indicated miRNAs (miRNA93 or let7g) or BNCs without miRNAs (NC), with or without Polybren. After 24 hours, cells were harvested and subjected to analysis. Membranes were re-probed for let7g, miRNA93, and U6 as a loading control. The results shown are representative of three independent experiments. b, A scatter plot reflecting the transcriptome results between the control and primary human hepatocytes treated with BNCs containing miRNA93. Cells were harvested 24 hours after BNC treatment. Intensity normalization was performed using global normalization based on the expression levels of all genes analyzed. Dashed lines indicate the thresholds: a two-fold increase or 50% decrease in expression levels. The full data are deposited in GEO database accession: GSE55928.

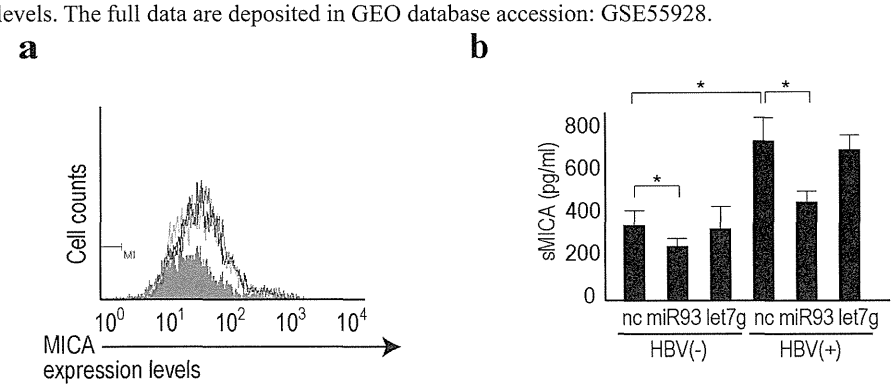

Figure 4: Soluble MICA protein levels were regulated by miRNA93 in human primary hepatocytes. a, Membrane-bound MICA protein expression was not affected by miRNA delivery into human primary hepatocytes. Flow cytometric analysis of membrane-bound MICA protein expression in cells delivered BNC-mediated control (green line), let7g (blue line), or miRNA93 (red line). Gray-shaded histograms represent background staining, assessed using isotype IgG. Representative results from three independent experiments are shown. b, Soluble MICA protein levels in the supernatants of primary hepatocytes after delivery of the indicated miRNAs (let7g or miRNA93) or negative control (NC) with or without HBV replication. Data represent the means ± s.d. of three independent experiments. *$p<0.05$.

modulated expression of MICA in primary hepatocytes during HBV replication affects this shedding process. To explore this possibility, we examined MICA protein levels in the supernatant using ELISA. As predicted, HBV infection significantly increased the protein concentration of MICA in the supernatant (Figure 4b).

Because an increase in soluble MICA levels in the serum of chronic hepatitis B patients is significantly associated with increased susceptibility to HCC [25], this increase during HBV replication needs to be prevented. Thus, we examined the effects of delivery of BNCs carrying miRNA93 into HBV-infected hepatocytes. Even though the MICA mRNA levels were not significantly affected by miRNA93 delivery based on microarray results (GEO accession: GSE55928), soluble MICA protein in the supernatant significantly decreased according to ELISA (Figure 4b). These results suggested that miRNA93 delivery into the liver decreases soluble MICA levels in the serum, which may be used to prevent HCC in chronic hepatitis B patients.

DISCUSSION

We report that HBV replication in human hepatocytes decreases miRNA93 expression and increases soluble MICA levels. Increased soluble MICA levels in the serum are strongly associated with HBV-related HCC [25], and the increased soluble MICA levels could be antagonized by the delivery of miRNA93 into hepatocytes using BNCs. Thus, BNCs carrying miRNA93 may be used to prevent HCC in patients with chronic HBV infection.

Methods of efficient long-term HBV replication *in vitro* are not commonly available. Although transient transfection assays using fragments or tandem-units of the HBV genome or the full-length HBV genome without vector backbone have been applied [8-12], these models can be analyzed only for short-term replication after transfection. Although stable cell lines carrying HBV genomes are also used, HBV particles are derived from the HBV genome and integrate into the host genome, which differs from natural infection, in which HBV replication mainly relies on HBV cccDNA [6, 7]. Although the most ideal system for HBV infection and replication studies *in vitro* are primary human hepatocytes, they are difficult to obtain. Freshly isolated human hepatocytes from chimeric mice used in this report are relatively easily to obtain, since they proliferate under immune-deficient and liver-damaging conditions. These cells could support HBV replication for a substantial period and are valuable resources for studies on HBV infection and replication.

Another essential tool used in this study is that of BNCs. Primary hepatocytes are generally difficult to transduce with exogenous genes via transfection. Although viral-mediated gene transfer is useful even for primary cells, we chose BNCs as the miRNA delivery method for several reasons. First, since BNCs are composed of HBV L particles, these BNCs preferentially target primary hepatocytes and theoretically target similar cells as does HBV. Second, since we want to develop future therapeutics based on our experimental results, we avoided using viral materials such as lentiviruses or retroviruses to improve biosafety. Third, although BNCs have been established to transfer genes or drugs [21, 31, 35], transfer of miRNAs has not yet been examined, which prompted us to investigate delivery of miRNAs. We found that BNCs could efficiently deliver miRNAs into primary hepatocytes. Although further studies are required, delivery of miRNAs into hepatocytes via BNCs may be a promising approach to target hepatocytes *in vivo*, as BNCs are efficient delivery vehicles in xenograft models using human liver-derived cells [21].

The present results regarding comprehensive transcriptome analyses using HBV replicating hepatocytes may be applicable for future HBV research. While similar experiments are typically performed using transfection in HBV protein-expressing cells, or other relatively artificial experimental settings, the results here may better reflect the *in vivo* situation for HBV-infected hepatocytes. The expression of approximately 0.3% of genes changed during HBV replication when the threshold was set to a greater than 4-fold increase or to less than a 25% decrease. Although some of these genes were consistent with previous transcriptomic studies [36-38], we observed several novel characteristics. First, few inflammation-related genes were included among genes whose expression levels were significantly changed. The reason for this discrepancy remains unclear, but the results were considered accurate, since inflammation is rare when HBV replicates prior to seroconversion in chronic HBV-infected patients. Thus, HBV may be able to evade the sensing system related to innate immunity [39-41]. It should be explored whether changes in HBV sequences or the presence of host cells other than hepatocytes affect gene expression in hepatocytes *in vivo*. Second, based on comprehensive analysis of transcript changes, many CYP-related genes were upregulated during HBV replication, which is consistent with previous reports [27, 28]. Since the biological significance of these changes remain unclear, further studies are required to explore the biological significance during HBV replication.

Microarray analyses of changes in miRNA expression levels in HBV-replicating cells revealed that miRNA expression levels were not affected by HBV replication (2.4% among 2,000 miRNAs when the threshold was set to more than a two-fold increase or less than a 50% decrease). However, the miRNAs whose expression levels changed may play crucial roles in the regulation of target gene expression without affecting transcript expression levels, for example, targeting of the MICA protein by miRNA93, whose expression levels were downregulated by HBV replication. The results of comprehensive miRNA expression level analysis in

HBV-replicating cells may increase our understanding of deregulated gene expression induced by HBV replication in hepatocytes.

MiRNA93 is a critical regulator of MICA protein expression [23, 29]. Thus, the decreased expression of miRNA93 by HBV suggested that the regulation of MICA expression by miRNA93 has biological significance. Polymorphisms in the MICA gene are associated with HBV and HCV-induced HCC [25, 42], and the increase in soluble MICA in the serum can be used as a susceptibility marker for HBV-induced HCC [25]. The increased levels of MICA protein expression agreed with the decreased miRNA93 expression. However, this increase was observed for soluble MICA protein levels and not membrane-bound MICA. While MICA is post-translationally dependent on the cell context or the status of viral infection [34], MICA may be readily processed from the cell surface in HBV-replicating primary hepatocytes and mainly released as soluble protein. Soluble MICA protein may function as a decoy for the NKG2D receptor in immune cells and as an evasion or immune surveillance system during chronic HBV infection. It may also be associated with HBV-induced HCC since HBV-infected hepatocytes may evade from the immune surveillance. Based on these results, BNCs carrying miRNA93 can be used to eliminate HBV-infected hepatocytes, which may be a novel approach for the prevention of subsequent virus-induced HCC.

MATERIALS AND METHODS

Cells

Primary human hepatocytes isolated fresh using the collagenase perfusion method from chimeric uPA/SCID mice with humanized livers [14, 17] were obtained from Phoenix Bio (Hiroshima, Japan). The purity of human hepatocytes was greater than 95%. A total of 3.0×10^5 cells/well were seeded on a type I collagen coated-24-well plate and maintained in DMEM with 10% FBS, 5 ng/ml EGF, 0.25 μg/ml insulin, 0.1 mM ascorbic acid, and 2% DMSO [43]. These cells were able be maintained at a high density for more than 3 weeks, supporting the long-term replication of HBV infection *in vitro*.

HBV infection *in vitro*

Serum from chronically HBV-infected patients with no HBe antibody before seroconversion was used for *in vitro* infection. Serum containing 9.0 log IU/ml of HBV genotype C in a volume of 3 μl, which is approximately 1.5×10^7 copies of HBV, was added to the 3.0×10^5 cells/well, followed by the addition of 4% PEG 8000 at day 0. Cells were washed, and the media was changed at days 1 and 2 and every 5 days thereafter. The media was collected to measure HBsAg and HBV-DNA at days 1, 2, 3, 7, 10, 15, 20 and 23 to confirm HBV replication. Measurements were performed at the clinical laboratory testing company SRL. Inc. (Tokyo, Japan).

cDNA array and miRNA microarray

Human 25K cDNA microarray and human 2K miRNA microarray analyses were performed using miRNA oligo chips according to the standard protocols (Toray Industries, Tokyo, Japan). The data and the experimental conditions were deposited in a public database (GEO: accession numbers: GSE55928 and GSE55929).

Bionanocapsules for miRNA delivery

Hollow particles consisting of HBV L proteins (pre-S1, pre-S2, and S regions) were used as the BNCs, as described previously [20, 21, 30]. The incorporation of miRNAs (miRNA93 or let-7g) into the hollow space and the delivery of miRNAs into human liver cells were performed as described previously [31]. Briefly, 32 μl BNC was added to 1 ml culture media at a final concentration of 50 nM miRNA 24 h before the indicated assays (unless otherwise specified).

Northern blotting of miRNAs

Northern blotting of miRNAs was performed as described previously. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (10 μg) was resolved on denaturing 15% polyacrylamide gels containing 7 M urea in 1× TBE and then transferred to a Hybond N+ membrane (GE Healthcare) in 0.25× TBE. Membranes were UV-crosslinked and prehybridized in hybridization buffer. Hybridization was performed overnight at 42°C in ULTRAhyb-Oligo Buffer (Ambion) containing a biotinylated probe specific for miRNA93 (CTA CCT GCA CGA ACA GCA CTT TG) and let-7g (AAC TGT ACA AAC TACT ACC TCA), which was heated to 95°C for 2 min prior to hybridization. Membranes were washed at 42°C in 2× SSC containing 0.1% SDS, and the bound probe was visualized using the BrightStar BioDetect Kit (Ambion). Blots were stripped by boiling in a 0.1% SDS, 5 mM EDTA solution for 10 min prior to rehybridization using a U6 probe (CAC GAA TTT GCG TGT CAT CCT T).

Reporter plasmids, transient transfection, and dual luciferase assays

The firefly luciferase reporter plasmid was used to examine let7g and miRNA93 function. pGL4-TK, a renilla luciferase reporter, was used as an internal control [44]. Transfection and dual luciferase assays were performed as described previously [45].

Flow cytometry

The expression levels of MICA on the cell surface were determined using flow cytometry, as described previously [23]. Briefly, cells were hybridized with anti-MICA (1:500; R&D Systems, Minneapolis, MN, USA) and isotype control IgG (1:500; R&D Systems) in 5% BSA/1% sodium azide/PBS for 1 h at 4°C. After washing, cells were incubated with goat anti-mouse Alexa 488 (1:1,000; Molecular Probes, Eugene, OR, USA) for 30 min. Flow cytometry was performed and the data analyzed using Guava Easy Cyte Plus (GE Healthcare, Little Chalfont, UK).

ELISA for MICA

The concentration of MICA in the cell culture supernatant was measured using a sandwich ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Significant differences between groups were determined using the Student's *t*-test when variances were equal and using Welch's *t*-test when variances were unequal. *P*-values less than 0.05 were considered statistically significant.

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Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget

AUTHOR CONTRIBUTIONS

M.Ohno and M.Otsuka planned the research and wrote the manuscript. M.Ohno, T.K., C.S., T.Y., and A.T. performed the majority of the experiments. R.M., N.K., M.S. and N.K. measured performed ELISA. S.K. provided materials and wrote the manuscript. K.K. supervised the entire project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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The flavonoid apigenin inhibits hepatitis C virus replication by decreasing mature microRNA122 levels

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ABSTRACT

Despite recent progress in the development of direct-acting antivirals against hepatitis C virus (HCV), chronic HCV infection remains an important health burden worldwide. MicroRNA122 (miR122), a liver-specific microRNA (miRNA), positively regulates HCV replication, and systemic application of antisense oligonucleotides against miR122 led to the long-lasting suppression of HCV viremia in human clinical trials. Here, we report that apigenin, a flavonoid and an inhibitor of maturation of a subset of miRNAs, inhibits HCV replication in vitro. Apigenin decreased the expression levels of mature miR122 without significantly affecting cell growth. Because supplementation of synthesized miR122 oligonucleotides or overexpression of constitutively active TRBP blocked these effects, the inhibitory effects of apigenin on HCV replication seemed to be dependent on the reduction of mature miR122 expression levels through inhibition of TRBP phosphorylation. Thus, apigenin intake, either through regular diet or supplements, may decrease HCV replication in chronically infected patients.

Introduction

Hepatitis C virus (HCV) constitutes a significant health problem worldwide, with an estimated 130–170 million people chronically infected (Scheel and Rice, 2013). Chronic HCV infection leads to severe liver diseases, including advanced liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). A recent HCV therapy consisting of a triple combination of pegylated interferon α (peg IFN-α), ribavirin, and protease inhibitors increased cure rates (Jacobson et al., 2011; Poordad et al., 2011; Bacon et al., 2011; Sherman et al., 2011). However, substantial side effects, resistance, and drug-drug interactions are concerns with this therapy. Although an IFN-free regimen with direct-acting antivirals (DAAs) is beginning to reach patients and increase cure rates (Manns and von Hahn, 2013; Deuffic-Burban et al., 2014), several issues remain, including treatment failure, resistant clones, and economic burden.

MicroRNA122 (miR122) is a highly abundant microRNA (miRNA) expressed in the liver and essential for the stability and propagation of HCV RNA (Jopling, 2012; Pfeffer and Baumert, 2010; Jopling et al., 2005). It binds to two closely spaced target sites in the highly conserved 5′-untranslated region (5′-UTR) of the HCV genome. These sites are conserved across all HCV genotypes and subtypes (Li et al., 2011). This positive regulation through 5′-UTR sites is a unique process, as compared to the usual function of miRNAs in the repression of gene expression via 3′-UTRs in target mRNAs. Although the precise mechanisms by which miR122 positively regulates HCV replication through its binding to the 5′-UTR of the HCV genome are not yet fully elucidated (Jopling, 2012), it was demonstrated that LNA-based anti-miR122 oligonucleotides led to the long-lasting suppression of HCV viremia and improvement of HCV-induced liver pathology in chimpanzees (Lanford et al., 2010). Based on experimental results, human clinical trials using miravirsen, an LNA-modified DNA phosphorothioate antisense oligonucleotide against miR122, have been conducted, and in Phase 2a studies miravirsen resulted in a dose-dependent reduction in HCV levels, without major adverse events and with no escape mutations in the miR122 binding sites of the HCV genome (Janssen et al., 2013). The miR122 binding sites are conserved across all HCV genotypes and subtypes, and miR122 could represent a host target for antiviral therapy.

We previously demonstrated that the flavonoid apigenin (4′,5,7-trihydroxyflavone) has inhibitory effects on the maturation of a subset of miRNAs and on subsequent miRNA function (Ohno et al., 2013). These effects were mediated by the inhibition of TRBP

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phosphorylation through inhibition of ERK activation (Ohno et al., 2013). We reported that the administration of apigenin to mice improved glucose intolerance induced by overexpression of miR103 in the liver, likely through suppression of mature miR103 expression. Moreover, we found that miR122 was also affected by apigenin (Ohno et al., 2013).

We hypothesized that apigenin may exert inhibitory effects on HCV replication by decreasing mature miR122 expression levels. In this study, we assessed the effects of apigenin on HCV replication and the possible molecular mechanisms by using an in vitro HCV replicon reporter system. Based on our findings, we propose potential novel management methods for chronic HCV infection and possibly other pathological states mediated by miR122.

Results

Apigenin inhibits the biogenesis of miR122

We recently showed that apigenin inhibits the biogenesis of a subset of miRNAs (Ohno et al., 2013). Because previous screening of the comprehensive miRNA expression changes revealed that miR122 was one of the miRNAs affected by apigenin (NCBI Gene Expression Omnibus (GEO) accession number: GSE46526) (Ohno et al., 2013), we here measured mature miR122 levels in Huh7 cells by quantitative RT-PCR, after apigenin treatment for 5 days. While the expression levels of let-7g, an unrelated miRNA, were not affected, apigenin significantly reduced mature miR122 expression levels in a dose-dependent manner (Fig. 1a). Northern blotting confirmed the reduced expression levels of miR122 after apigenin treatment (Fig. 1b). By contrast, expression of miR122 precursor, but not let-7g precursor, was increased after apigenin treatment (Fig. 1c), as we described previously (Ohno et al., 2013). Consistent with the reduced levels, miR122 function was inhibited by apigenin, as determined by the increased reporter values from a transiently transfected reporter construct with two miR122 target sites in tandem in its 3′-UTR (Fig. 1d). The effect was miR122 function-specific because no effects were observed with use of a reporter construct with mutations in the miR122 target sites (Fig. 1d). Apigenin had no significant effects on Huh7 cell growth at a final concentration of up to 5 μM (50 μM apigenin slightly reduced the cell number) (Fig. 1e). These results suggest that up to 5 μM of apigenin inhibits the expression levels of mature miR122 in Huh7 cells without affecting cell viability.

Apigenin inhibits HCV replication

Because miR122 positively regulates HCV replication (Jopling et al., 2005), we hypothesized that apigenin might inhibit HCV replication by decreasing the expression levels of mature miR122. To test this hypothesis, we used Huh7 cells harboring an HCV replicon reporter construct (HCV-Feo), referred to as Huh7-Feo cells (Yokota et al., 2003). These cells continuously carry a replicon expressing a chimeric protein consisting of firefly luciferase and neomycin phosphotransferase under the HCV 5′ IRES and can be used to monitor intracellular HCV replication by measuring luciferase activity (Fig. 2a) (Yokota et al., 2003). Treatment of Huh7-Feo cells with apigenin (final concentration 5 or 50 μM) for 5 days significantly reduced HCV-Feo replication (Fig. 2b). The pattern of luciferase values closely matched the pattern of luciferase protein expression levels (Fig. 2c). The decrease in replication observed with 5 μM apigenin treatment was only slightly less than that obtained following the transfection of anti-miR122 oligonucleotides at 10 pM. Although 50 μM apigenin may have adverse effects on cell viability, as described above, these results suggest that 5 μM apigenin can significantly inhibit HCV replication without affecting cell viability, possibly through downregulation of mature miR122 expression levels.

Apigenin inhibits HCV replication through downregulation of miR122 levels

To further investigate the mechanisms of the observed negative effects of apigenin on HCV replication, we applied synthesized mature miRNAs to Huh7-Feo cells to determine whether miRNA supplementation could antagonize the effects of apigenin. Overexpression of miR122 after the transfection of synthesized miR122, compared with the expression levels of miR122 in cells with no treatment or with let-7g transfection, was confirmed by Northern blotting (Fig. 2d). As expected, overexpression of miR122 efficiently antagonized the negative effects of apigenin on HCV replication, while supplementation of an unrelated miRNA, let-7g, had no effect on HCV replication (Fig. 2e). These results suggest that apigenin inhibits HCV replication by downregulating mature miR122 expression.

Phosphorylation mimic TRBP blocks the effects of apigenin

Because we previously demonstrated that apigenin inhibits the maturation of a subset of miRNAs by inhibiting the phosphorylation of TRBP, which contributes to the maturation of a subset of miRNAs by binding to Dicer (Paroo et al., 2009). We constructed a flag-tagged TRBP-expressing construct and a phosphorylation mimics in which serine was substituted with aspartic acid (TRBP (SD)) (Paroo et al., 2009). The expression levels of the wild-type TRBP and TRBP(SD) constructs were comparable (Fig. 3a). After stably expressing these constructs in Huh7-Feo cells by lentiviral transduction, we determined the expression levels of miR122. While overexpression of wild-type TRBP slightly enhanced the expression levels of miR122, apigenin significantly reduced its expression levels (Fig. 3b). However, in TRBP(SD)-expressing cells, miR122 expression levels were significantly increased and were not affected by apigenin treatment (Fig. 3b), probably due to the phosphorylation mimic TRBP(SD) being constitutively active. Consistent with the changes in miR122 expression levels, replication of the HCV replicon, as determined by luciferase values, was inhibited by apigenin in cells expressing wild-type TRBP but not in cells expressing TRBP(SD), which showed a slight increase in replication (Fig. 3c). These results suggest that apigenin inhibits HCV replication through the inhibition of mature miR122 expression levels, probably by modulating TRBP phosphorylation, consistent with our previous report (Ohno et al., 2013).

Discussion

In this study, we demonstrate that apigenin inhibits HCV replication by decreasing the expression levels of mature miR122, possibly through inhibition of the phosphorylation of TRBP, an important factor for the maturation of a subset of miRNAs (Paroo et al., 2009).

Our study revealed that apigenin inhibits HCV replication. A liver-specific miRNA, miR122, has been reported to be linked with pleiotropic physiological functions (Jopling, 2012; Otsuka et al., 2014), such as liver development, cholesterol metabolism, iron metabolism, and fatty acid metabolism (Takata et al., 2013a). A particularly intriguing function of miR122 is its role in promoting HCV replication (Jopling et al., 2005). The success of miravirsen, an LNA-modified DNA phosphorothioate antisense oligonucleotide against miR122, against HCV in a Phase 2a study (Janssen et al., 2013) shows its promise as a novel anti-HCV drug and as the first miRNA-targeting therapy to be trialed. While miravirsen hybridizes

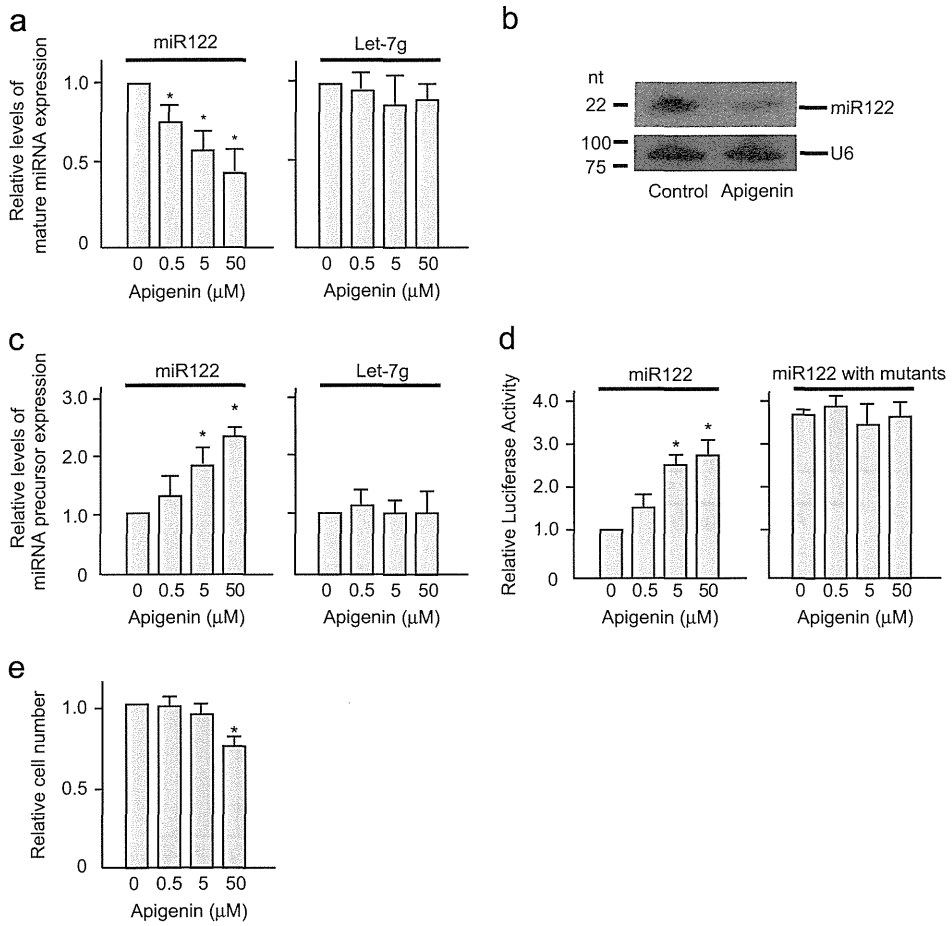

Fig. 1. Apigenin decreases mature miR122 expression levels. (a) The expression levels of mature miRNAs in Huh7 cells were determined by quantitative RT-PCR. Cells were treated with apigenin at the indicated doses for 5 days. Expression levels of mature miR122 and let-7g were determined. Relative expression levels are indicated as the means ± s.d. of three independent experiments. * $p < 0.05$. (b) The expression levels of mature miR122 were determined by Northern blotting. Huh7 cells were treated with 5 μM apigenin for 5 days. Expression levels of mature miR122 were determined by Northern blotting. U6 levels were determined as a loading control by reprobing the same membrane. Representative results from three independent experiments are shown. (c) The expression levels of miRNA precursors in Huh7 cells were determined by quantitative RT-PCR. Cells were treated with apigenin at the indicated doses for 5 days. Expression levels of miR122 and let-7g precursors were determined. Relative expression levels are indicated as the means ± s.d. of three independent experiments. * $p < 0.05$. (d) Endogenous miR122 function was determined by reporter assay. Huh7 cells were transiently transfected with reporter constructs containing miR122 binding sites or mutants. Cells were treated with apigenin at the indicated doses for 36 h. Data represent the mean ± s.d. of three independent experiments. * $p < 0.05$. (e) The number of cells was counted after treatment with apigenin at the indicated doses for 5 days. Experiments were performed in duplicate in a single test and the data represent the means ± s.d. of three independent tests. * $p < 0.05$.

to the 5′ region of mature miR122, resulting in sequestration and inhibition of miR122 (Janssen et al., 2013), it also binds to the stem-loop structure of pri- and pre-miR-122 and inhibits both Dicer- and Drosha-mediated processing of miR122 precursors (Gebert et al., 2014). Therefore, the importance of miR122 in HCV replication appears to depend on its expression level as well as its binding capacity. Because apigenin reduced the expression levels of mature miR122, and supplementation of synthesized mature miR122 blocked the effects of apigenin on HCV replication, the inhibitory effects of apigenin on HCV replication seem to be dependent on reduced levels of mature miR122. However, other potential molecular mechanisms for the effects of apigenin on HCV replication may exist.

Apigenin decreased the expression levels of a subset of mature miRNAs, including miR122, similar to the case of miR103, as we observed previously using microarray analyses (GEO accession number: GSE46526) (Ohno et al., 2013). We reported that the decreased maturation of miR103 was due to decreased phosphorylation of TRBP resulting from inhibition of ERK activities (Paroo et al., 2009). Because overexpression of the TRBP(SD) increased the levels of mature miR122, it appears that the reduced maturation of miR122 was also dependent on the activity of TRBP, which was inhibited by apigenin (Ohno et al., 2013). However, mature miRNA levels might be regulated not only by their synthesis but also, potentially, by their degradation, although this has not yet been established definitively (Jopling, 2012), and the effects of apigenin on the levels of mature miRNAs may be more diverse than expected. These effects should be explored in future studies involving the identification of the specific molecular target with which apigenin directly interacts and the development of an apigenin synthesis method, as has been achieved for resveratrol, another pleiotropic polyphenol (Snyder et al., 2011).

miR122 levels are frequently reduced in HCC compared with background liver tissues (Hou et al., 2011; Kutay et al., 2006; Gramantieri et al., 2007; Tsai et al., 2009), and lower miR122 expression levels in HCC tissues are correlated with a poor prognosis (Kojima et al., 2011). Because mice lacking miR122 in the liver showed spontaneous inflammation and liver tumors (Hsu et al., 2012; Tsai et al., 2012), miR122 may function as a tumor suppressor. However, to date no detectable liver toxicity has been reported with antisense oligonucleotide inhibition of miR122 in mice, primates, or humans (Lanford et al., 2010; Janssen et al., 2013; Elmén et al., 2008; Krützfeldt et al., 2005). We reported previously that transgenic mice expressing an antisense oligonucleotide specific for

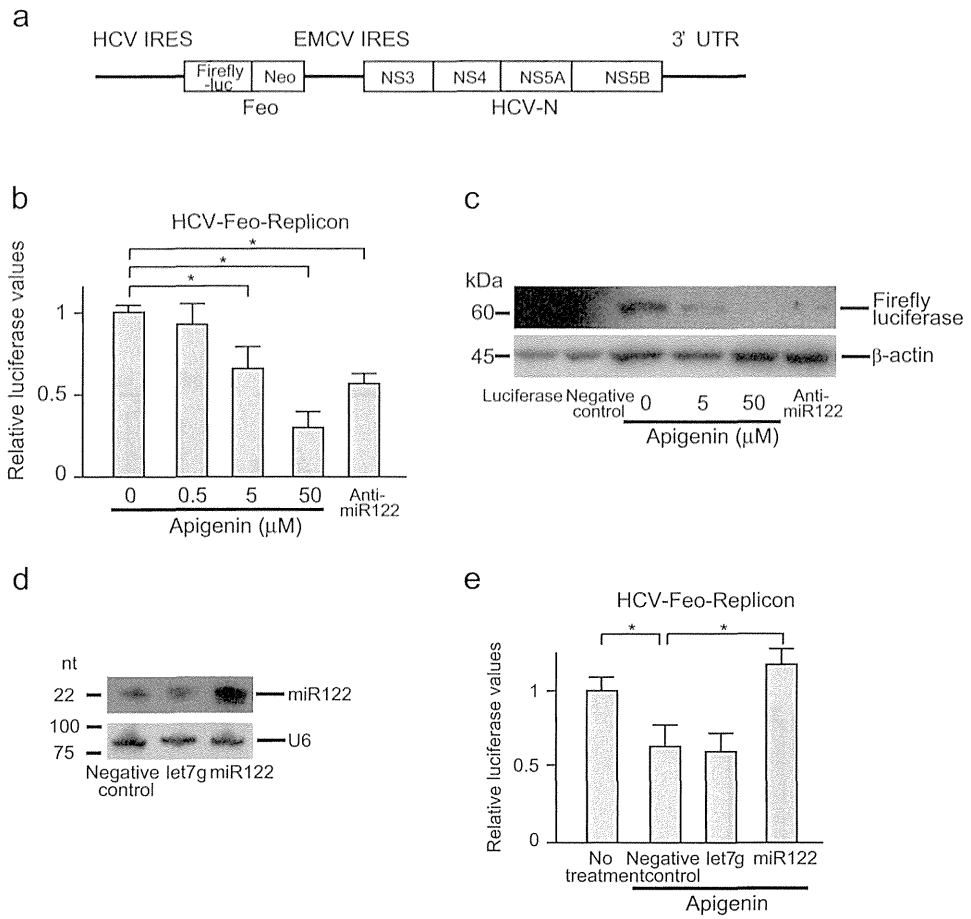

Fig. 2. Apigenin inhibits HCV replication in vitro by modulating miR122 levels. (a) Structure of the HCV replicon reporter. IRES, internal ribosomal entry site; firefly-luc, firefly luciferase gene; neo, neomycin resistance gene. NS3, NS4, NS5A, NS5B, and HCV are nonstructural proteins. Fusion genes consisting of the firefly luciferase gene and neomycin resistance gene are referred to as Feo. Huh7-Feo cells harbor an HCV replicon reporter construct (HCV-Feo) and expresses a chimeric protein consisting of firefly luciferase and neomycin phosphotransferase under the HCV 5′ IRES. (b) Apigenin inhibited replication of the HCV replicon reporter construct. Huh7-Feo cells were treated with apigenin for 5 days and the luciferase values were determined. Synthesized anti-miR122 oligonucleotides were transfected as a reference. Data represent the means ± s. d. of three independent experiments. * $p < 0.05$. (c) Apigenin inhibits the expression of luciferase protein under the HCV 5′ IRES of the reporter construct. Huh7-Feo cells were treated as described in (b) and luciferase protein was quantified by Western blotting. 293T cell lysates obtained after transient transfection with luciferase-expressing plasmid or control plasmid were used as controls. Representative results from three independent experiments are shown. (d) and (e) Synthesized miR122 blocks the inhibitory effects of apigenin on HCV replication. Synthesized mature miR122 and let-7g were transfected into Huh7-Feo cells. (d) Cells were harvested 48 h after transfection and mature miR122 levels were determined by Northern blotting. U6 levels were used as a loading control. Representative results from three independent experiments are shown. Synthesized miR122 overexpression blocked the inhibitory effects of apigenin on HCV replication (e) Huh7-Feo cells were treated or not treated with 5 μM apigenin for 5 days. Synthesized miRNAs were transfected 48 h before measurement. Luciferase values for HCV-Feo were determined by reporter assay. Data represent the means ± s.d. of the absolute luciferase values of three independent experiments. * $p < 0.05$.

miR122 showed no spontaneous pathological features (Kojima et al., 2011). In addition, mice treated with apigenin for 2 weeks suffered no detectable harmful events in our previous studies (Ohno et al., 2013). Therefore, while several miRNAs other than miR103 and miR122 are affected by apigenin (Ohno et al., 2013), apigenin treatment in vivo seems to be a safe and convenient method of reducing the expression levels of a subset of miRNAs. One of the reasons for this is that the effects of apigenin are relatively mild; it reduces but does not completely abolish target miRNA expression levels. The effects of apigenin as well as its long-term safety need to be determined in a small animal model.

The appropriate dose of apigenin in this in vitro study was 5 μM. The fasting plasma concentration of flavonoids, including apigenin, is proportional to their intake. Intake of 100 mg flavonoids results in a plasma concentration of ~410 nM (Cao et al., 2010). Apigenin is abundant in parsley, celery, and other herbs, according to the USDA Database for the Flavonoid Content of Selected Foods (Release 3.1). For example, fresh parsley contains 215.46 mg apigenin per 100 g edible portion, dried parsley contains 4303.50 mg, and celery seeds contain 78.65 mg. The plasma concentration of apigenin theoretically reaches 1.7 μM, assuming one eats 10 g of dried parsley per day. Thus, although it is not impossible to reach a plasma apigenin concentration of 5 μM through normal dietary intake, apigenin supplementation may be required to obtain an appropriate dose. However, the apigenin concentration in the liver may be higher than the plasma due to direct blood flow from the intestine, which absorbs nutrients, and eating foods rich in apigenin may be sufficient to reach the appropriate liver concentration. The concentration of apigenin in liver tissues should thus be determined in future studies.

In HCV therapy, treatment has become more effective with the advent of DAAs (Scheel and Rice, 2013; Manns and von Hahn, 2013). Thus, it is uncertain the extent to which our finding, that apigenin may inhibit HCV replication, contributes to patient care in the DAA era. However, worldwide access to drugs and the implementation of economical therapy are major challenges (Scheel and Rice, 2013; Manns and von Hahn, 2013), in addition to cases of non-responders and patients with clones resistant to DAAs. Our findings may provide novel insights into HCV management. The combination of apigenin with other agents, including

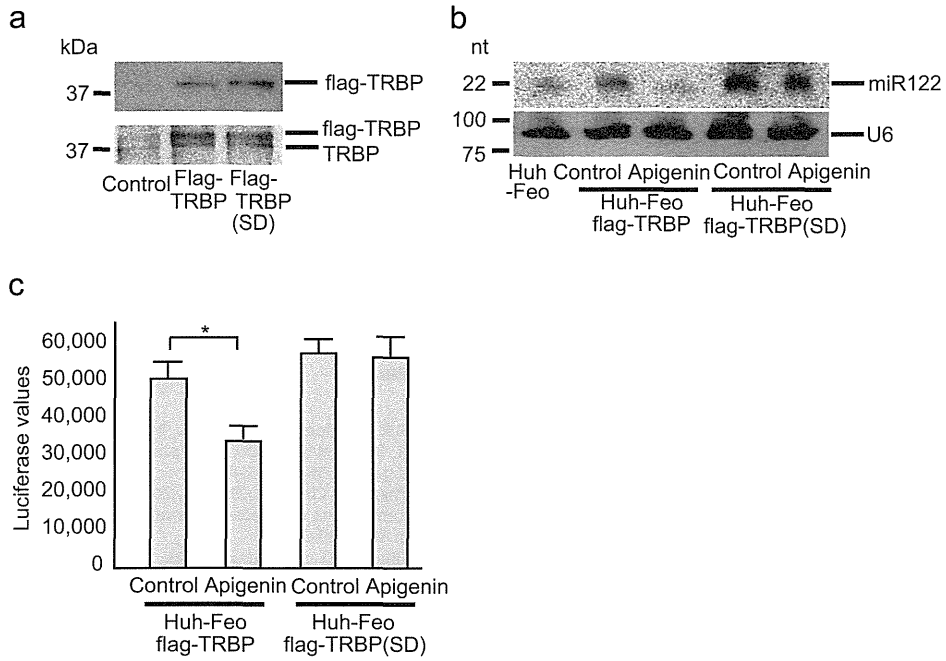

Fig. 3. Phosphorylation mimic TRBP blocks the inhibitory effects of apigenin on HCV replication. (a) Confirmation of flag-tagged TRBP-expressing construct expression. The expression of flag-tagged TRBP or phosphorylation mimic TRBP (TRBP(SD)) was confirmed by Western blotting using anti-flag and anti-TRBP antibodies. 293T cells were transiently transfected with constructs expressing the indicated proteins. Representative results from three independent experiments are shown. (b) TRBP (SD) blocked the inhibitory effects of apigenin on mature miR122 expression. Huh7-Feo cells were transduced with TRBP- or TRBP(SD)-expressing lentiviruses and selected. The expression levels of mature miR122 in Huh7-Feo cells, Huh7-Feo cells with TRBP expression, and Huh7-Feo cells with TRBP(SD) expression were determined by Northern blotting after 5 days of 5 μM apigenin treatment. U6 levels were used as a loading control. Representative results from three independent experiments are shown. (c) TRBP(SD) blocked the inhibitory effects of apigenin on HCV replication. Huh7-Feo cells with TRBP or TRBP(SD) expression were treated with 5 μM apigenin for 5 days and then subjected to luciferase assay. Data represent the means ± s.d. of the absolute luciferase values of three independent experiments. * $p < 0.05$.

conventional interferon, to eliminate HCV remains important. In addition, reducing miR122 expression may benefit patients with high cholesterol levels, because targeting miR122 with antisense oligonucleotides in vivo, including miravirsen in humans, decreases elevated cholesterol levels (Janssen et al., 2013). The effects of taking apigenin or eating foods rich in apigenin are worth considering.

Materials and methods

Cell culture

Huh7 and 293T cells were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan). Huh7-Feo cells that harbor an HCV replicon reporter construct (HCV-Feo) were kindly provided by Yokota et al. (2003). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Reagents

Apigenin was purchased from Wako Chemicals (Osaka, Japan) and was dissolved in dimethyl sulfoxide (DMSO). Apigenin was added to the cell culture media at a 1:1000 dilution to reach the final working concentration. An equal volume of DMSO was used as a negative control.

Antibodies

Anti-luciferase antibody (#PM016) was purchased from MBL (Nagoya, Japan). Anti-β-actin antibody (#A5441), anti-flag M2 antibody (#F3165), and anti-TRBP antibody (SAB4200111) were purchased from Sigma (St. Louis, MO).

Western blotting, transfection, and dual luciferase assays

Western blotting, transfection, and dual luciferase assays were performed as we previously described (Kojima et al., 2011).

Quantitative RT-PCR analysis of miRNA expression

To determine miR122 and let-7g expression levels, cDNA was first synthesized from RNA, and quantitative PCR was then performed using the Mir-X miRNA First-Strand Synthesis and SYBR qRT-PCR Kit (Clontech, Mountain View, CA). miRNA precursor expression levels were determined according to a previous report (Suzuki et al., 2009) using the reported primers. Relative expression values were calculated by the CT-based calibrated standard curve method. These calculated values were then normalized to the expression of U6 snRNA. The reverse primer was provided in the kit.

Plasmids, viral production, and transduction

The firefly luciferase-based reporter carrying miR122-responsive elements in its 3′-UTR, which was used to examine miR122 function, and the internal control Renilla luciferase-based plasmids have been described previously (Kojima et al., 2011). A reporter with mutations in the miR122-responsive elements was constructed by inserting annealed oligonucleotides containing mutant miR122 binding sites downstream of the luciferase gene of pGL4. The oligonucleotide sequence used was CAA ACA CCA TTG TCA CAG CAG T. An HCV replicon expressing a chimeric protein consisting of firefly luciferase and neomycin phosphotransferase under the HCV 5′ IRES was used to monitor intracellular HCV replication levels (Yokota et al., 2003). To construct plasmids expressing wild-type TRBP or phosphorylation mimic TRBP (TRBP(SD)), flag-tagged TRBP cDNAs, amplified by PCR using plasmids containing cDNAs provided

by Paroo et al. (2009) as templates, were cloned into the pLVSIN vector (Takara, Shiga, Japan) at the *Not*1 site by a standard infusion method (Clontech).

Lentiviral production and transduction

Lentiviral particles carrying the flag-tagged TRBP-expressing constructs were produced using a pPACKH1 lentivector packaging plasmid mix according to the manufacturers' recommendations (System Biosciences). Huh7-Feo cells were transduced with lentiviruses using polybrene (EMD Millipore, Billerica, MA, USA), and were then selected with puromycin (6 μg/ml).

Northern blotting of miRNAs

Northern blotting of miRNAs was performed as described previously (Takata et al., 2013b). Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 10 mg of RNA were resolved in denaturing 15% polyacrylamide gels containing 7 M urea in 1 × TBE and then transferred to a Hybond N+ membrane (GE Healthcare) in 0.25 × TBE. Membranes were UV-crosslinked and prehybridized in hybridization buffer. Hybridization was performed overnight at 42 °C in ULTRAhyb-Oligo Buffer (Ambion) containing a biotinylated probe specific for miR122 (CAA ACA CCA TTG TCA CAC TCC A) that had been heated to 95 °C for 2 min. Membranes were washed at 42 °C in 2 × SSC containing 0.1% SDS, and the bound probe was visualized using a BrightStar BioDetect Kit (Ambion). Blots were stripped by boiling in a solution containing 0.1% SDS and 5 mM EDTA for 10 min prior to rehybridization with a U6 probe (CAC GAA TTT GCG TGT CAT CCT T).

Cell counting

Relative cell proliferation was assessed using Cell Counting Kit-8 (Dojindo Laboratories), as we previously described (Kojima et al., 2011).

Synthesized miRNAs and transfection

Synthesized anti-miR122 oligonucleotides, mature miRNA122, and let7g mimics were purchased from Sigma and were transiently transfected at a final concentration of 10 pM using RNAi Max Reagent according to the manufacturer's instructions (Sigma).

Statistical analysis

When the variances were equal, statistically significant differences between groups were identified using Student's *t*-test. When the variances were unequal, Welch's *t*-test was used instead. *P* values <0.05 were considered to indicate statistical significance in in vitro experiments.

Author contributions

C.S. and M.Otsuka planned the research and wrote the manuscript. C.S., M.Ohno, T.K., T.Y. and A.T. performed the majority of the experiments. K.G., R.M., and N.K. provided materials and supported some parts of experiments. C.S., M.Ohno and M.Otsuka analyzed the data. K.K. supervised the entire project.

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Increased serum autotaxin levels in hepatocellular carcinoma patients were caused by background liver fibrosis but not by carcinoma

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ABSTRACT

Background: Controversy exists as to whether autotaxin (ATX) may be importantly associated with pathophysiology of hepatocellular carcinoma (HCC).
Methods: We evaluated serum ATX levels and its mRNA expression in consecutive 148 HCC patients treated with radiofrequency ablation (RFA) and 30 patients with hepatic resection.
Results: Although increased serum ATX levels were observed in almost all the patients treated with RFA, they were not reduced after RFA. Furthermore, serum ATX levels were associated not with tumor burden but with the parameters predicting for liver fibrosis, such as liver stiffness values. Then, in surgically-treated patients, there was no significant correlation between serum ATX levels and ATX mRNA expression levels in HCC tissues. Notably, ATX mRNA expression levels in HCC tissues were not higher than those in peri-tumorous tissues. Finally, serum ATX levels in surgically-treated HCC patients were rather correlated with ATX mRNA expression levels in peri-tumorous tissues as well as with liver fibrosis stage.
Conclusion: The increase in serum ATX levels in HCC patients may not be caused by abundant ATX production in HCC tissues but by fibrosis in the background livers.

1. Introduction

Because ATX was originally discovered in a conditioned medium from human melanoma cell cultures as a stimulator of cell migration [1], ATX has long been speculated to play a role in cancer invasion or metastasis as an autocrine motility factor [2]. Indeed, a potential role of ATX in cancer pathophysiology has been intensively studied; enhanced ATX expression has been reported in Hodgkin lymphoma [3], glioblastoma [4], non-small cell lung cancer [5], renal cell carcinoma [6], breast cancer [7], thyroid carcinoma [8] as well as melanoma [2].

The pathophysiological significance of ATX has been unveiled by the finding that ATX possesses lysophospholipase D activity [9,10], hydrolyzing lysophosphatidylcholine to produce lysophosphatidic acid (LPA), a lipid mediator, eliciting a wide variety of biological responses including cell migration, neurogenesis, angiogenesis, smooth-muscle contractions, platelet aggregation, and wound healing [11]. The pathophysiological functions of ATX are now assumed to be largely attributable to the ability of ATX to produce LPA. Of note, ATX is responsible for producing LPA in the blood by hydrolysis of lysophospholipids (mainly lysophosphatidylcholine) [12]. In fact, in the plasma from heterozygous ATX-null mice, LPA levels were about half those in the plasma from wild-type mice [13], and a strong correlation between serum ATX activity and plasma LPA levels has also been observed in humans [14] and rats [15], suggesting that serum ATX activity may be one of the key determinants of plasma LPA levels.

There has been evidence showing the overexpression of ATX in HCC [16–19]. Furthermore, this increased ATX expression in HCC reportedly led to abundant tumor-secreted LPA, and as a result serum LPA levels were higher in HCC patients with more tumor burden, i.e., with metastasis than in those without [18]. In contrast, there has been a report, showing no correlation between plasma LPA levels and the tumor burden of HCC [20]. Thus, controversy exists as to whether ATX and LPA are importantly associated with HCC pathophysiology. To clarify this, serum ATX levels were analyzed in HCC patients who underwent radiofrequency ablation (RFA) or hepatic resection in consideration of tumor burden, treatment effects and ATX mRNA expression in HCC tissues.

Abbreviations: ATX, autotaxin; HCC, hepatocellular carcinoma; RFA, radiofrequency ablation; LPA, lysophosphatidic acid; APRI, aspartate aminotransferase-to-platelet ratio index.

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2. Patients and methods

2.1. Patients

We enrolled 148 consecutive patients with HCC who underwent RFA between November 2012 and March 2013 at the Department of Gastroenterology, and 30 consecutive patients with HCC who underwent hepatic resection between January 2013 and June 2013 at the Hepato–Biliary–Pancreatic Surgery Division, the Department of Surgery, the University of Tokyo Hospital, Tokyo, Japan. HCC was diagnosed as previously described [21]. Patients with HCC recurrence as well as those with the first occurrence were included in this study.

This study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Research Ethics Committee of the Faculty of Medicine of the University of Tokyo. Informed consent from the patients was obtained for the use of all the samples.

2.2. RFA

The detailed procedure of RFA using a cooled-tip electrode (Covidien) was meticulously described elsewhere [22,23]. In general, we performed RFA on Child-Pugh class A or B patients, a single tumor ≤5 cm in diameter, or ≤3 tumors ≤3 cm in diameter. Treatment efficacy was evaluated by dynamic CT performed 1 to 3 days post-ablation. In the case of incomplete ablation, patients underwent additional ablation sessions until complete tumor coverage was achieved.

2.3. Evaluation of tumor volume

We estimated the volume of each HCC lesion as the sphere taking the greatest axial dimension as the diameter in our present analyses.

2.4. Measurement of ATX antigen

Serum ATX antigen level was determined in all enrolled patients 1 day to 1 week prior to the treatment using a specific 2-site enzyme immunoassay, as previously described, in which the within-run and between-run CVs were 3.1–4.6% and 2.8–4.6%, respectively [24]. In 90 patients, serum ATX antigen level was also measured 2 to 4 weeks after RFA.

2.5. Measurement of liver stiffness

Liver stiffness was measured using transient elastography (FibroScan 502; EchoSens), as described previously [25]. Patients were without active liver damage with >100 U/l of alanine aminotransferase (ALT) and cardiac insufficiency, because liver stiffness value has been shown to be unreliable to predict the degree of liver fibrosis in those conditions [26,27].

2.6. Quantitative real time PCR

Total RNA of HCC and peri-tumorous tissues was extracted using TRIZOL reagent (Invitrogen). One microgram of purified total RNA was transcribed using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative real time PCR was performed with SYBR green PCR Master Mix (Applied Biosystems Inc.) with primers specific for ATX and beta-actin designed by NCBI Primer Designing Tool. The primer pairs were used as follows: human ATX (NM_006209.4): 5′-CGTGGCTGGGAGTGTACTAA-3′ and 5′-AGAGTGTGTGCCACAAGACC-3′, human beta-actin (NM_001101.3): 5′-GGGTCAGAAGGATTCCTATG-3′ and 5′-CCTTAATGTCACGCACGATTT-3′. The PCR reaction was performed in a volume of 25 μl containing 1 μl of cDNA, 12.5 μl of SYBR Green Master Mix. PCR amplifications were run using the following conditions: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The target gene mRNA expression level was relatively quantified to beta-actin using $2^{-\Delta\Delta Ct}$ method (Applied Biosystems, User Bulletin No. 2).

2.7. Histological staging

Fibrosis stage, determined according to the METAVIR group scoring system, was classified as F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; or F4, cirrhosis.

2.8. Statistical analysis

The Wilcoxon signed-rank test was used when comparing 2 matched samples (before and after RFA) to assess whether their population mean ranks differ. The Wilcoxon rank-sum test was used to determine whether 2 groups of data are different. Correlations were determined using the Spearman's rank correlation coefficient. The association between ATX mRNA expression in peri-tumorous tissues and fibrosis stage was assessed with the Jonckeere–Terpstra test. A 2-sided $P < 0.05$ was considered statistically significant. All statistical analyses were performed using R 2.13.0 (http://www.R-project.org).

3. Results

3.1. Characteristics of patients who underwent RFA

The characteristics of the enrolled patients who underwent RFA are shown in Table 1. The median age was 72.4 y, in agreement with the notion that HCC patients in Japan have been getting old [28]. Male and

Table 1
Characteristics of patients who underwent RFA.

Variable	$n = 148$
Age (y)	72.4 (65.6–78.9)
Sex, *n* (%)	
Male	105 (70.9)
Female	43 (29.1)
Viral markers, *n* (%)	
HBsAg, positive	19 (12.8)
Anti-HCVAb, positive	107 (72.3)
Both positive	1 (0.7)
Both negative	21 (14.2)
AST (U/l)	44.0 (31.8–66.3)
ALT (U/l)	35.0 (20.8–54.0)
Albumin (g/dl)	3.5 (3.1–3.9)
Platelet count ($\times 10^9$/l)	112 (74–152)
Child Pugh classification	
Class A	119 (80.4)
Class B	28 (18.9)
Class C	1 (0.7)
Size of main tumor (cm)	1.5 (1.1–2.0)
No. of tumors	
1	97 (65.5)
2	31 (20.9)
3	14 (9.5)
4	2 (1.4)
5	4 (2.7)
Total volume of tumors (cm^3)	2.5 (0.9–4.2)
Serum ATX level (mg/l) in total	2.21 ± 1.03
In male	1.94 ± 1.01
In female	2.87 ± 0.76
AFP (ng/ml)	11.9 (4.9–35.1)
	$n = 101$
Fibroscan (kPa)	22.3 (12.9–35.8)
Serum hyaluronic acid level (mg/l)	272.8 (129.1–507.7)
Serum type IV collagen 7S (ng/ml)	6.4 (4.7–8.3)

Values are expressed as median and range (25th–75th percentiles), mean ± SD, or number (percent). Abbreviations: HBsAg, hepatitis B surface antigen; anti-HCVAb, antihepatitis C virus antibody.