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ORIGINAL ARTICLE

MicroRNA-500 as a potential diagnostic marker for hepatocellular carcinoma

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Abstract

We identified that microRNA expression changed dynamically during liver development and found that miR-500 is an oncofetal miRNA in liver cancer. miR-500 was abundantly expressed in several human liver cancer cell lines and 45% of human hepatocellular carcinoma (HCC) tissue. Most importantly, an increased amount of miR-500 was found in the sera of the HCC patients. In fact, miR-500 levels in sera of the HCC patients returned to normal after the surgical treatment in three HCC patients. Our findings reveal that diverse changes of miRNAs occur during liver development and, one of these, miR-500 is an oncofetal miRNA relevant to the diagnosis of human HCC.

Keywords: *miRNA; miR-500; hepatocellular carcinoma, liver development, diagnosis*

Introduction

MicroRNAs (miRNAs) are small RNA molecules of 21–25 nt that have the potential to a play central role in physiological and pathological processes, including cell differentiation, apoptosis and oncogenesis (Ambros 2004, Esquela-Kerscher et al. 2006). The biogenesis of miRNAs involves nucleolytic processing of precursor transcripts, which are transcribed from different genomic locations as long primary transcripts (pri-miRNA) by RNA polymerase II in the nucleus (Lee et al. 2004). Pri-miRNAs are processed by the RNase-III family of an enzyme, Drosha, to a ~70 nt precursor called the pre-miRNA. The pre-miRNA is exported to the cytoplasm by Exportin-5 and then cleaved in the cytoplasm by Dicer to ~22 nt double-strand mature miRNA (Han et al. 2006, Lund et al. 2004, Ketting et al. 2001). A single strand of the mature miRNA is assembled into effecter complexes called miRNPs (miRNA-containing ribonucleoprotein particles), which share a considerable amount of similarity with an RNA-induced silencing complex (RISC) (Nelson et al. 2004). They induce gene suppression post-transcriptionally by inducing mRNA degradation or by regulating the translational efficiency of mRNA (Bartel 2004).

Several reports have shown the importance of miRNA functions in tissue development. More recent reports, in particular those regarding comprehensive microRNA profiling analysis, have shown that miRNAs are expressed in a tissue-specific manner and their expression altered

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in the process of development, such as cardiogenesis and haematopoiesis (Chen et al. 2006, 2004). For example, miR-1, which is expressed specifically in cardiac and skeletal muscle, is essential for cardiac morphogenesis and conduction (Zhao et al. 2007). Another study showed that miR-181a regulates intrinsic antigen sensitivity during T-cell development (Li et al. 2007). Another important aspect of miRNA study is the association of its gene targets and disease, which have been investigated by many researchers. Mir-17-92 polycistron has been designated as oncomiR-1 (He et al. 2005), and let-7 family miRNAs and miR-34 function as tumour suppressors (Johnson et al. 2005, Yu F et al. 2007, He et al. 2007); moreover, a number of studies have given evidence that several miRNAs are associated with carcinogenesis and regulate the expression of cancer-related genes.

Although emerging evidence suggests that several miRNAs are involved in the process of liver development (Esau et al. 2006, Fu et al. 2005, Gramantieri et al. 2007), the roles of miRNAs in hepatogenesis and their possible relation to hepatocarcinogenesis have not been thoroughly examined. In this study, to investigate liver development from the biological aspects of microRNA, we performed a mouse miRNA microarray carrying 340 miRNA probes. We report that some of these miRNAs are strongly expressed, and that dynamic changes in their expression profile are observed in the process of liver development. We also show that miR-500 is an oncofetal miRNA, which is highly expressed in fetal liver, more than in adult normal liver, and aberrantly expressed in hepatocellular carcinoma (HCC) tissue. Thus, dynamic miRNA regulation is an important feature as an oncofetal non-coding small RNA relevant to the diagnosis of human liver cancer.

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Materials and methods

RNA extraction

C57BL/6J mice were used in this study. Total RNA from mouse liver tissues (embryo (E) 14, E16, E18, neonate and adult), *in vitro* fetal hepatocyte cultured samples (days 0, 1, 3, 5 and 7), and liver cancer cell lines (HepG2, Huh-7, JHH-7, Alexander, Li-7, and Hep3B) were extracted using the mirVana™ miRNA Isolation Kit (Ambion, Tokyo, Japan). Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

Locked nucleic acid (LNA)-based miRNA microarray

The miRCURY™ LNA array version 8.0, which contains capture probes targeting all human, mouse and rat miRNA listed in the miRBASE version 8.0, was applied to detect the expression of mouse miRNA (Exiqon, Vedbaek, Denmark). Total RNA samples were collected from fetal (E14, 16 and 18), neonate and adult (8-week-old) mice ($n = 7–10$). Total RNA samples (2000 ng) from liver tissue and reference (Universal control, which is made from mouse tissue mixtures) were labelled with the Hy3™ and Hy5™ fluorescent stain, respectively, using the miRCURY™ LNA Array labelling kit according to the procedure described by the manufacturer (Exiqon). Hybridisation and normalisation were performed according to the miRCURY™ LNA array manual, and image analysis of the miRCURY™ LNA array microarray slides was acquired using an Agilent Technologies Microarray Scanner and Agilent Feature Extraction 9.1 (Agilent Technologies, Tokyo, Japan). A hierarchical cluster was produced from microarray data using a Euclidean distance calculation based on Ward's methods by GenMaths software (Applied Maths). All the miRNA microarray data are shown in Supplementary Table 1 (see the online version of this article).

Cell culture

Liver cancer cell lines (HepG2, Huh-7, JHH-7, Alexander, Li-7 and Hep3B) were cultured in liquid culture with Dulbecco's modified eagle medium (DMEM; GIBCO Laboratories, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Equitech-Bio, Kerrville, TX, USA) and a 1% antibiotic antimycotic solution (Invitrogen, Tokyo, Japan). The cells were maintained *in vitro* at 37°C in a humidified atmosphere with 5% CO_2.

Patients and RNA specimens

Liver tissues were obtained surgically with informed consent from patients at the National Cancer Center Hospital (Tokyo, Japan). The study was approved by the Institutional Review Board of the National Cancer Center Research Institute. Liver tissue total RNAs were extracted from 40 HCC patients and their associated non-cancerous tissue. The clinical data and pathological diagnosis are summarized in Supplementary Table 2 (see the online version of this article).

Real-time polymerase chain reaction

Total RNAs of approximately 100 ng were reverse-transcribed using the Taqman miRNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). Real-time quantitative polymerase chain reaction (PCR) amplification of the cDNA template was done using Taqman Universal PCR Master Mix (Applied Biosystems) in

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an ABI PRISM 7300 (Applied Biosystems). The PCR conditions were 50°C for 2 min and 95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Taqman probes for human and mouse miRNA were used to assess the expression levels of miRNA (mmu-miR-101b, ID 4373159; mmu-122a, ID 4373151; mmu-miR-142-5p, ID 4373135; mmu-miR-223, ID 4373075; mmu-miR-451, ID 4373360; has-miR-346, ID 4373038; has-miR-500, ID 4373225; Applied Biosystems). The expression levels were normalised against U6 (RNU6B, ID 4373381; Applied Biosystems) or total RNA volume.

RNA isolation from human serum samples

Whole blood samples were obtained from patients with HCC at the Kyoto University (Kyoto, Japan). All of the donors or their guardians provided written consent and ethics permission was obtained for the use of all samples. Blood samples were taken before and after completion of surgery. Serum samples were stored at -80°C until analysis. For serum RNA isolation, total RNA was isolated using Isogen (Nippon Gene, Japan), according to the manufacturer's instructions.

Measurement of serum miRNA levels by using TaqMan qRT-PCR assays

A fixed volume of 5 µl of RNA solution (14 ng) was used as input into the reverse transcription reaction. Input RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied BioSystems) in a small-scale reverse transcription reaction (comprising 2 µl of H_2O, 1 µl of 10x reverse-transcription buffer, 0.2 µl of RNase inhibitor (20 units ml^{-1}), 0.1 µl of 100 mM dNTPs, 0.7 µl of Multiscribe reverse transcriptase and 5 µl of input RNA), using a Tetrad2 Peltier Thermal Cycler (BioRad, Tokyo, Japan) at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Teverse transcription product (4.75 µl) was combined with 5.25 µl of PCR assay reagents (comprising 5 µl of TaqMan 2x Universal PCR Master Mix, No AmpErase UNG and 0.25 µl of TaqMan miRNA assay) to generate a PCR of 10.0 µl of total volume. Real-time PCR was performed as described above. Serum levels of miR-16 were measured as internal normalisation control as they were not significantly different between controls and patients in prostate cancer and colorectal cancer (Mitchell et al. 2008).

Statistical analysis

The results are given as mean ± SD. The Student's *t*-test was performed for statistical evaluation; $p < 0.05$ or $p < 0.001$ was considered significant.

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Table 1. MicroRNAs (miRNA) abundantly expressed in liver development.

Liver stage	MiRNA name[a]
E14	miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-346, miR-374-5p, miR-451, miR-486, miR-500
E16	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-295, miR-346, miR-367, miR-374-5p, miR-451, miR-464, miR-471, miR-486, miR-500, miR-547
E18	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-324-3p, miR-374-5p, miR-451, miR-486
Neonate	miR-101b, miR-122a, miR-142-3p, miR-142-5p, miR-144, miR-223, miR-463
Adult	miR-21, miR-22, miR-29a, miR-29b, miR-29c, miR-101a, miR-101b, miR-122a, miR-126-5p, miR-192, miR-374-5p

[a]miRNAs are listed in ascending order. E, embryo.

Results

Analysis of the global expression levels of miRNA in the process of liver development by LNA-based miRNA microarray

To examine how the expression profile of miRNA changed in the process of mouse liver development, we performed an LNA-based miRNA microarray at different developmental stages. Total RNAs from E14, 16, 18, neonate and adult liver were isolated and labelled with Hy3, and total RNAs of universal control consisted of several tissue mixtures labelled with Hy5 as a common reference. After normalisation of the miRNA expression, the number of high- and low-expressed miRNAs at different time stages was counted. High-expressed miRNA represents twofold or more upregulated miRNA, and low-expressed miRNA represents twofold or more downregulated miRNA, when compared with an average expression level of all miRNAs (see Supplementary Figure 1 in the online version of this article). Throughout all developmental stages of the liver, most of the miRNA expression levels were classified as low-expressed miRNA; in contrast, the number of high-expressed miRNAs was quite limited and are listed in Table 1. These data indicated that expression levels of the general miRNAs were very low and that a limited number of miRNAs were highly expressed in mouse liver development.

Differential expression patterns of miRNAs in the process of mouse liver development

To determine differentially expressed miRNA and to quantify the expression changes in the process of liver

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development, hierarchical unsupervised clustering analysis was performed using microarray data of E14, 16, 18, neonate and adult mouse liver. The case cluster analysis of the microarray data indicated a similarity of clusters from the viewpoint of the expression pattern between E14 and E16 fetal liver and between neonate and adult liver (Figure 1), indicating that the miRNA expressions changed depending on the developmental stage. These results indicated that expression of most of the miRNAs was regulated precisely in the process of liver development.

The expression pattern of miRNA selected from highly expressed miRNAs (Table 1) was verified by real-time PCR to show the accuracy of miRNA expression acquired from the microarray analysis. The left panels of Figure 2 present the results of microarray analysis for five miRNAs (miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451). Expressions of miR-101b and miR-122a were low at the early stage of liver development and were upregulated during maturation. In contrast, expressions of miR-142-5p, miR-223 and miR-451 were high at the early stage of liver development and were already known as miRNAs expressed in haematopoietic cells (Chen et al. 2004, Zhan et al 2007, Johnnidis et al 2008). The right panels of Figure 2 are the results of real-time PCR for the same set of miRNAs. In comparison to the microarray results and the real-time PCR results, these data obtained from two different methods showed approximately similar expression patterns of miRNAs, confirming the validity of our microarray analysis.

Interestingly, miRNAs (miR-142-5p, miR-451 and miR-223) expressed in haematopoietic cells were highly expressed at the early stages (E14 and E16) and then gradually downregulated in the process of liver development (Figure 2). Because whole fetal liver is a haematopoietic organ and a large number of haematopoietic cells are contained there, this also indicated the accuracy of expression profiling of miRNA in the process of liver development by LNA-based microarray.

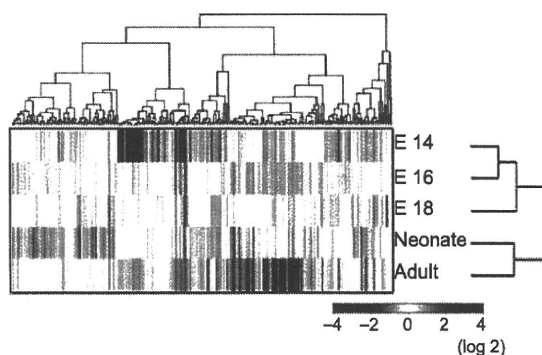

Figure 1. A global expression pattern of miRNA in the process of mouse liver development. The data were subjected to a hierarchical cluster analysis using a Euclidean distance calculation based on Ward's methods. The liver samples are aligned vertically: embryo (E) 14, E16, E18, neonate and adult. Samples were linked by the dendrogram shown on the right to highlight the similarity in their miRNA expression patterns. The expression profile of each miRNA is depicted in the respective row. The expressions of miRNA are linked by the dendrogram shown on the top to highlight the similarity in their expression patterns.

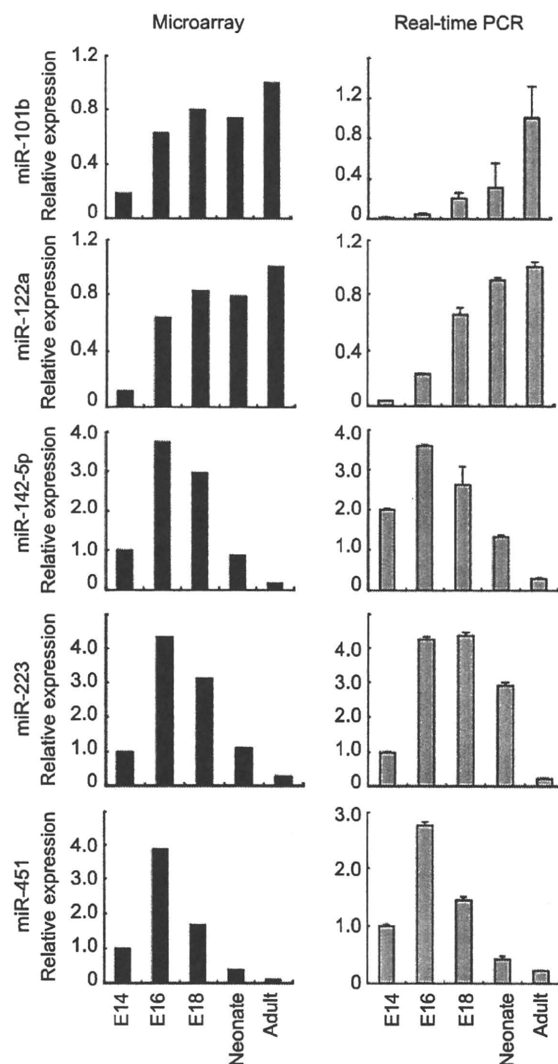

Figure 2. Differential expression of selected miRNA in mouse liver development by microarray and real-time polymerase chain reaction (PCR). miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451 were selected from highly expressed miRNAs to confirm the expression levels of microarray analysis by real-time PCR. The left panels represent the miRNA expression levels by microarray analysis. The right panels represent the miRNA expression levels by real-time PCR. The expression profile is compared for mouse fetal (embryo (E) 14, E16 and E18), neonate and adult liver. In the graphs of miR-142-5p, miR-223 and miR-451, the expression level of E14 fetal liver is set to 1.0. Real-time PCR analyses were performed in triplicate and expression values are normalized with total RNA volume. Data are shown as mean ± SD.

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Differential expression patterns of cancer-related miRNAs in the process of mouse liver development

Interestingly, when analysing the expression patterns of the hierarchical clustering data in detail, we found that the expression of several let-7 miRNA family known as 'tumour suppressor miRNA' was upregulated, and, in contrast, the expression of miRNAs known as 'potential oncogenes' which are involved in cell proliferation, was downregulated in the process of liver development. Therefore, to reveal the expression pattern of cancer-related miRNAs in the process of mouse liver development, the expression profile of 21 selected miRNAs (11 miRNAs as oncogenes and 10 miRNAs as tumour suppressors) is summarized in Figure 3. Many oncogenic miRNA expressions, such as those of miR-17-5p, miR-20, and miR-92, tended to decrease in the process of mouse liver development (Figure 3A). In contrast, except for let-7d* and let-7e, the expression pattern of the let-7 miRNA family was elevated in the process of liver development (Figure 3B). This study provides evidence that the expression of oncogenic miRNA is downregulated and that the expression of tumour suppressor miRNA is upregulated in the process of liver development.

Expression of miR-500 is high in human fetal liver

As reported above, the expression levels of oncogenic miRNAs were downregulated in liver development. We tried to identify new miRNA candidates that act as an oncogenic miRNA in the liver from the microarray data. As a first step toward the elucidation of the role of miRNAs in liver carcinogenesis, we focused on downregulated miRNAs during liver maturation, which are possibly related to cell proliferation; high expressions of miR-140, miR-346, miR-411, miR-470 and miR-500 were detected at an early stage (E14) of liver development and downregulated at the late developmental stages (E16 and E18) (Figure 4A). Among these, miR-500 and miR-346 expressions were remarkably downregulated during development; thus, we concentrated on miR-500 and miR-346, which could be expected to be a potential target relevant to fetal liver development to control the time and spatial expression of sets of mRNA.

In the next step, the occurrence of miR-500 and miR-346 was assessed in human fetal and adult liver. Real-time PCR analysis revealed that the expression of miR-500 in human fetal liver, but not that of miR-346, was significantly higher than that in normal adult liver (Figure 4B and Supplementary Figure 2A (see online version of this article)). Taken together, as miR-500 expression was downregulated in human adult liver, our data suggest that miR-500 is developmentally associated with human fetal hepatocyte specification and functions. The

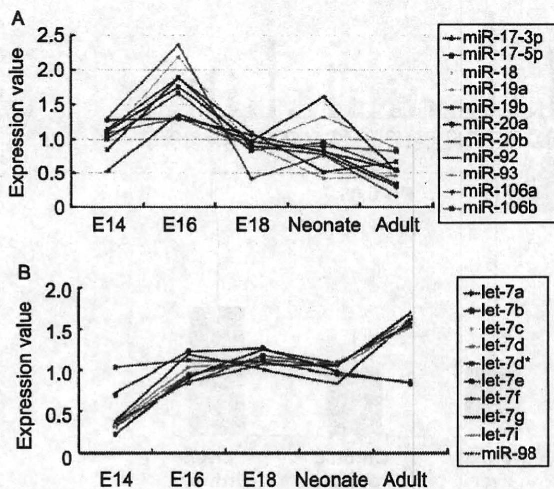

Figure 3. Expression patterns of cancer-related miRNAs in the process of mouse liver development. (A) Expression pattern by a microarray analysis (each sample: $n=7$-10) of miRNA that may act as an oncogene. (B) The expression pattern by the microarray analysis (each sample: $n=7$-10) of the let-7 family miRNAs functioned as a tumour suppressor. Expression levels are normalised by average expression value of each miRNA and shown in the graph.

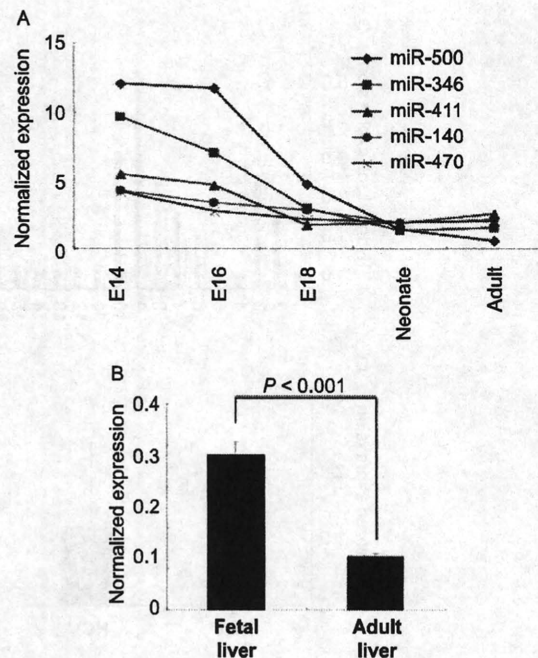

Figure 4. The expression of miR-500 is higher in the fetal stage than in the adult stage (A). The expression profile of miRNA decreased in the process of mouse liver development. Expression values are based on microarray data. (B) Expression of miR-500 in human fetal and adult liver. Real-time polymerase chain reaction analyses were performed in triplicate. Expression values are normalised with U6 snRNA value. The data represent the mean ± SD, $p < 0.001$.

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results of our ongoing knock-down analysis of miRNA in liver cancer cells will be presented in a future work.

Expression of miR-500 is high in human liver cancer

We next examined the expression level of miR-500 in six human liver cancer cell lines (JHH-7, Li-7, Huh-7, HepG2, Hep3B and Alexander) to assess whether miR-500 acts as an oncofetal miRNA and found that it increases 2.4- to 47.6-fold more in Alexander, JHH-7, HepG2, Huh-7 and Hep3B than in normal liver (Figure 5A); in contrast, no detectable amount of miR-500 was found in Li-7. On the other hand, the expression levels of miR-346 in the six liver cancer cell lines were not high (see Supplementary Figure 2B in the online version of this article). To evaluate the potential of miR-500 as an oncofetal miRNA, the expression levels of human miR-500 were analysed by real-time PCR in 40 pairs of malignant neoplasias of hepatocyte lineage (T) and adjacent non-tumorous tissue (NT). Differences in the miR-500 expression level were statistically significant ($p<0.001$) between T and NT (Figure 5B), but miR-346 expression was not significantly changed (see Supplementary Figure 2C in the online version of this article). Some of the samples exhibited remarkably high expression levels of miR-500, and 45% (18/40 patients) of the samples showed 1.2- to 8.6-fold higher upregulation in the cancerous samples than in each non-tumorous sample and

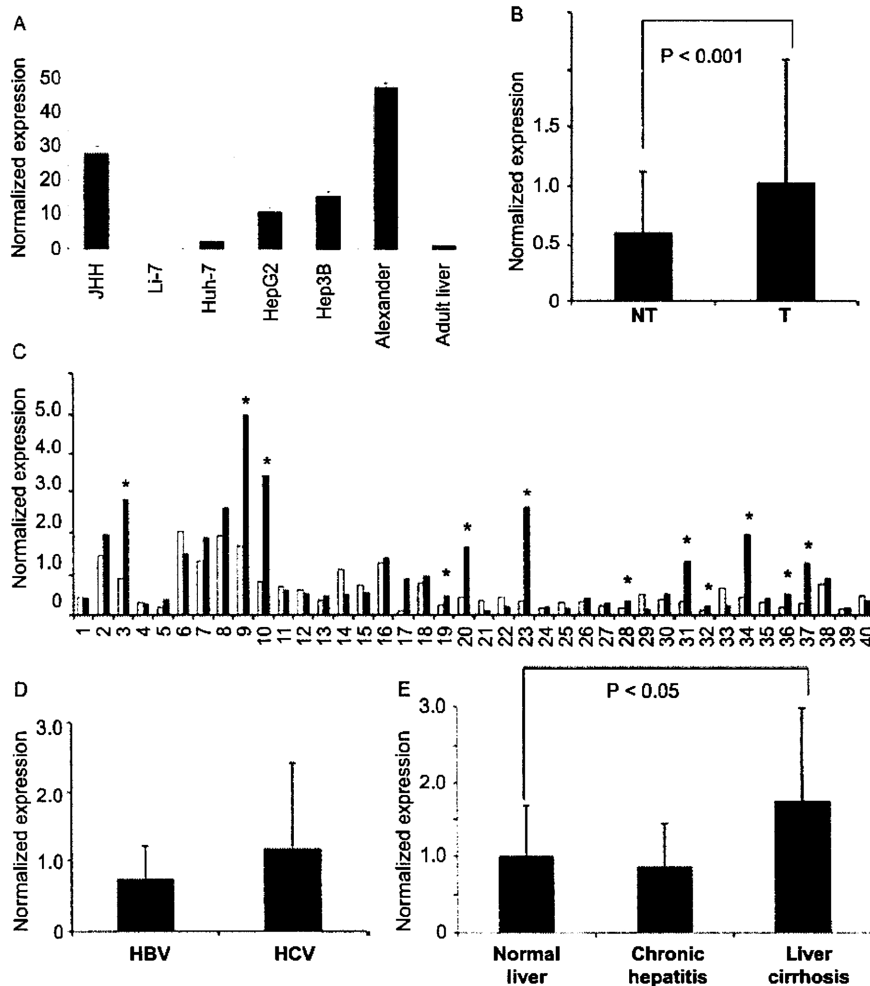

Figure 5. The expression of miR-500 is clearly upregulated in human liver cancer (A) miR-500 expression abundantly detected in liver cancer cell lines (JHH-7, Li-7, Huh-7, HepG2, Hep3B and Alexander). The expression level of normal liver is set to 1.0. The data represent the mean ± SD. (B) Forty pairs of hepatocellular carcinoma (HCC) patients (tumour (T) and non-tumour (NT)) were analysed by real-time polymerase chain reaction of human miR-500. The data represent the mean ± SD, $p<0.001$. (C) Expression levels of miR-500 in each patient (T and NT). Samples of 12 patients (*) showed twofold or more upregulation in HCC. (D) Expression levels of miR-500 in hepatitis B virus (HBV, $n=10$) and hepatitis C virus (HCV, $n=26$). (E) miR-500 expression was upregulated in liver cirrhosis ($n=17$) more than normal liver ($n=11$) and chronic hepatitis samples ($n=19$). The data represent the mean ± SD, $p<0.05$. Expression values are normalised with U6 snRNA value.

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12 patients showed more than 2.0-fold higher expression (30%) (Figure 5C). Based on the clinical data and pathological diagnosis (see Supplementary Table 2 in the online version of this article), there is no significant difference in miR-500 expression between hepatitis virus B and C infection (Figure 5D). Importantly, significant difference in miR-500 expression was found between normal liver and liver cirrhosis samples, but not chronic hepatitis (Figure 5E), suggesting that miR-500 expression was upregulated during cirrhosis development. Thus, although only limited samples expressed miR-500 higher, miR-500 might be useful as a biomarker in the early stage of liver cancer.

Expression profiling of miR-500 in HCC patient serum

Recently, it has been reported that miRNAs are circulating in serum (Chim et al. 2008, Gilad et al. 2008) and tumour-derived miRNAs such as miR-155, miR-21, miR-15b, miR-16 and miR-24 are detected in the plasma and serum of tumour patients (Mitchell et al. 2008, Lawrie et al. 2008). In fact, an increased amount of miR-500 was found in the sera of three out of ten HCC patients, which means that liver cancer-specific miRNA such as miR-500 is circulating in the peripheral blood and can be a novel diagnostic marker. To determine whether or not serum levels of miR-500 truly reflect the presence of cancer in the HCC patients, the presence of miR-500 in the sera of three human HCC patients, post- and presurgical treatment, was also assessed. As can be seen in Figure 6, elevated serum levels of miR-500 in the three HCC patients were significantly reduced after surgery and returned to normal levels. These results expect that the miR-500s abundance profile in serum of the HCC patients might reflect physiological and/or pathological conditions.

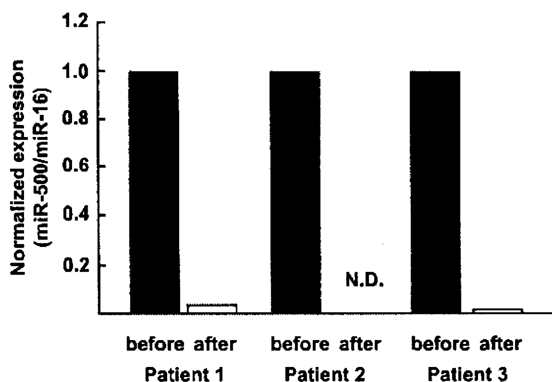

Figure 6. Serum levels of miR-500 in hepatocellular carcinoma (HCC) patients. Changes of serum levels of miR-500 in HCC patients ($n=3$) before (preoperation) and after (postoperation within 6 months) surgical removal of the tumour. Expression levels of the miR-500 are normalised to miR-16. N.D., not detected.

Discussion

Using a global miRNA expression profile in mouse liver development analysed by an LNA-based miRNA microarray, our data indicate that dynamic changes in miRNA expression occur in mouse liver development. However, the number of high-expressed miRNAs was quite limited at all developmental stages of the liver. This finding is also consistent with several reports that dominant miRNA expression is rigidly controlled in a developmental stage-specific and tissue- or cell-type-specific manner (Chen et al 2006, Shan et al. 2007). For example, it has been reported that the expressions of miR-1 and miR-133 are high and specific in adult cardiac and skeletal muscles and modulate skeletal muscle proliferation and differentiation by negatively regulating the histone deacetylase-4 or serum response factor (Chen et al. 2006). On the other hand, expression levels of the general miRNA are low at all stages of liver development. However, our data indicate that the expression pattern of some of the low-expressed miRNAs, including let-7 family, also dramatically change in the process of mouse liver development (Figure 4B). Using this platform, the overall regulation of individual miRNAs of sequential stages of liver development was determined, providing us with a useful baseline for understanding the developmental dynamics of liver miRNA expression.

In this study, we identified a novel cancer biomarker candidate, miR-500, which was designated as an oncofetal miRNA in the early stage of liver cancer, because miR-500 expression is highly expressed in a fetal liver and downregulated in the developmental process and then upregulated in the process of liver cirrhosis. When the expression profile of miR-500 in human tissues was examined, its expression was not specific in the liver and was broadly detected in all tissues (see Supplementary Figure 3 in the online version of this article). However, the expression level of miR-500 is high at the early stages of liver development in mice and humans. Furthermore, miR-500 was abundantly expressed in human liver cancer cell lines (JHH-7, Huh-7, HepG2, Hep3B and Alexander) and liver cancer tissues. Interestingly, six miRNAs (mir-532, 188, 362, 501, 660, 502) in addition to miR-500 make a cluster within a 10-kb distance from miR-500, and their expressions could be modulated by the same transcriptional regulatory unit. However, the levels were not remarkably changed during mouse liver development. Therefore, by analysing these miRNAs together, miR-500 might be a better biomarker in HCC.

We tried to test the effect of miR-500 using liver cancer cell lines. In a knock-down analysis of miR-500 with miR-500 LNA, significant changes in cell proliferation and colony formation were not observed in both Alexander and JHH-7 cells (see Supplementary Figure 5A and B in the online version of this article). Likewise, mature

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miR-500 were transfected into Li-7 cells, which did not express miR-500 and we found there are no significant differences in cell proliferation (see Supplementary Figure 5C in the online version of this article). Although our data indicated that miR-500 did not affect cell proliferation in liver cancer cell lines, there might be a close association between tissue development and carcinogenesis in the fields of miRNA. For detailed analysis of function of miR-500, we await for generation of miR-500 knockout mice.

As several groups have reported that levels of certain circulating miRNA are associated with clinical characteristics in diseases (Gilad et al. 2008, Lawrie et al. 2008), our data suggest that miR-500 was circulating in the sera of the HCC patients and miR-500 levels in sera of the HCC patients returned to normal after the surgery. Although our results are promising for miRNA-based HCC screening, there are several limitations in this study and we suggest: (1) as the sample size is quite small, further validation that miR-500 could be a reliable marker for HCC in a large cohort is necessary; (2) use of better controls to determine whether or not serum miR-500 levels are changed due to the trauma of surgery; (3) it is desirable to examine whether serum miR-500 levels change in patients with chronic hepatitis and liver cirrhosis; (4) it is necessary to compare if serum miR-500 could be better than earlier diagnostic methods such as serum α-fetoprotein.

The differential expression patterns of miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451 were determined by miRNA microarray and real-time PCR analysis. The specific expression of miR-122 in the liver has previously been described by several research groups. Esau et al. (2006) reported that miR-122 was a key regulator of lipid metabolism in the liver, regulating increased hepatic fatty acid oxidation, a decrease in hepatic fatty acid and cholesterol synthesis rates by reductions of several lipogenic genes. Interestingly, two groups demonstrated evidence that the hepatitis C virus genome has predicted binding sites of miR-122 and that miR-122 positively regulated the replication hepatitis C virus in human liver (Jopling et al. 2005, Randall et al. 2007). In addition to miR-122a, we found that miR-101b expression was upregulated in mouse liver development. Furthermore, upregulation of miR-101b and miR-122a expression was observed in the *in vitro* cultured of fetal hepatocytes treated with OsM and Dex (see Supplementary Figure 4A–C in the online version of this article). It has been reported that miR-101 is related to the immune system and megakaryocytopoiesis (Yu D et al. 2007, Garzon et al. 2006); however, the role of miR-101 in the liver has not yet been examined.

During early development in mice, haematopoietic stem cells emerge in the aorta/gonado/mesonephros region and then the stem cells migrate and expand in the fetal liver before haematopoiesis takes place in the bone marrow by the time of birth. Although most of the miRNAs that we observe in the liver developmental process are constitutively expressed, specific miRNAs are enriched at distinct stages of haematopoietic development. We found that the expression of miR-142-5p, miR-223 and miR-451 was downregulated in the process of liver maturation. As it has been reported that miR-142-5p and miR-142-3p are highly expressed in all haematopoietic tissues (Chen et al. 2004), miR-142 may thus play a critical role at the early stage of haematopoiesis. The expression of miR-223 was mainly detected in bone marrow and negatively regulated myeloid progenitor proliferation and granulocytic differentiation and activation (Johnnidis et al. 2008). In addition, miR-451 expression was upregulated during erythroid differentiation, and gain- and loss-of-function studies disclosed that miR-451 was related to erythroid maturation (Zhan et al. 2007).

Recent studies have indicated that a decrease of mature miRNA expression by impaired miRNA processing accelerates tumorigenesis and that a global reduction of miRNAs is observed in human cancers, suggesting that the role of overall miRNAs is to guard against oncogenic transformation (Kumar et al. 2007, Lu et al. 2005). In particular, the let-7 family is broadly known as a tumour suppressor. It has been reported that a decrease of let-7 expression was observed in human lung cancer and that let-7 negatively regulates the expression of H-ras and *HMGA2* oncogenes in breast cancer cells (Johnson et al. 2005, Yu F et al. 2007, Takamizawa et al. 2004). In addition, miR-16 was also reported as a tumour suppressor by inducing apoptosis mediated by Bcl-2 and modulating the cell cycle (Cimmino et al. 2005, Linsley et al. 2007). In a study of liver carcinogenesis, a decrease of miR-122 expression was observed in rat liver tumour (Kutay et al. 2006). Consistent with this report, miR-122a and miR-101b expression levels in 40 pairs of malignant neoplasias of hepatocyte lineage and adjacent non-tumorous tissue were reduced significantly ($p<0.05$, $n=40$) in tumour samples (see Supplementary Figure 4D in the online version of this article). However, in previous studies, it has been revealed that specific miRNAs acted as oncogenes, as their overexpression facilitates cancer progression. For example, miR-17-92 polycistron was overexpressed in lymphomas, lung cancers and colorectal cancers and enhanced cell proliferation (He et al. 2005, Hayashita et al. 2005). Furthermore, the copy number and expression level of miR-155 and its non-coding RNA transcript BIC were greatly increased in B-cell lymphomas (Eis et al. 2005). Our data show that the expression profile of oncogenic miRNAs was downregulated and, vice versa, the expression of tumour-suppressor miRNAs was upregulated in the process of liver development (Figure 4). This suggests that elevated oncogenic miRNAs are important

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at the early developmental stage of the liver because, in this period, cell proliferation is frequent; in contrast, upregulation of tumour suppressor miRNAs is essential for preventing abnormal cell proliferation at the late stage of liver development. Therefore, our data suggest that the tight regulation of expression of cancer-related miRNAs (both oncogenic miRNAs and tumour suppressor miRNAs) occurred during normal liver development.

Finally, we have documented dynamic changes in miRNA expression that were found in the process of mouse liver development and some of them behaved as an oncofetal miRNA in HCC. Although little is known about the expression regulations, targets or roles of miRNAs in the liver, the expression profiles of miRNA in development could be informative with respect to the elucidation of the process of the development and diagnosis of cancer because the expression of some of the cancer-related miRNAs dramatically changed. Further studies on the differential expression of miRNA in liver development could contribute to a better understanding of the process of liver development and embryonic haematopoiesis and could facilitate the discovery of candidate miRNAs for cancer diagnosis and therapeutic targets in liver cancer.

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Regulation of the hepatitis C virus genome replication by miR-199a☆

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Background/Aims: **Hepatitis C virus (HCV) infection causes chronic hepatitis and hepatocellular carcinoma. Current anti-HCV therapies are based on interferon therapy, which is insufficiently effective. microRNAs (miRNAs) are non-coding RNAs that regulate gene expression, and they have recently been shown to play an important role in viral replication.**

Methods: **An algorithm-based search for miRNAs that target the HCV genome yielded one miRNA, miR-199a*, with a sequence similar to the HCV genome that is conserved among HCV genotypes.**

Results: **Overexpression of miR-199a* inhibited HCV genome replication in two cells bearing replicons (replicon cell) HCV-1b or -2a, however, miRNA inhibition by specific antisense oligonucleotide (ASO) accelerated viral replication. Prior transfection of immortalized hepatocytes which were infected with serum of HCV genotype 1b and 2a-infected patients, with miR-199a* reduced HCV RNA replication activity. Mutation in the miR-199a* target site in the replicon reduced the effect of the miR-199a*. HCV replicon RNA is accumulated to the RNA-induced silencing complex (RISC) when miR-199a* was overexpressed to the replicon cell. This antiviral effect by miR-199a* was independent of the interferon pathway.**

Conclusions: **The results of this study suggest that miR-199a* directly regulates HCV replication and may serve as a novel antiviral therapy.**

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Keywords: **HCV; microRNA; Viral replication; Replicon cell; RISC**

1. Introduction

Infection by hepatitis C virus (HCV) infection is a cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. Pegylated interferon (IFN)-α/ribavirin combination therapy is currently the most effective treatment for chronic hepatitis C [2]. However, only 50% of treated HCV-infected patients clear HCV infection [3], and novel approaches are urgently needed.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that control gene expression by degrading or suppressing the translation of target mRNAs [4,5]. miR-122 is an abundant miRNA in the liver, where it comprises 70% of the miRNA in the liver [6]. Both the

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Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; miRNA, microRNA; IRES, the internal ribosomal entry site; RISC, RNA-induced silencing complex; CP, Ceruloplasmin.

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5′- and 3′-UTRs of the HCV genome contain sequences that are partially complementary to miR-122. Administration of miR-122 accelerates HCV replication [7]. Several factors related to the RNAi machinery were recently found to reduce HCV production and HCV RNA level [8]. Targeting multiple viral and cellular elements with RNAi may increase the potency of antiviral gene therapies [9–12]. siRNA-based antiviral strategies are expected to have clinical applications as a means of eradicating persistent infection. It has been hypothesized that several miRNAs play pivotal roles in viral replication and proliferation and it is possible that miRNAs control HCV replication.

In this study, we used a miRNA target search algorithm and succeeded in identifying an miRNA that targets the HCV genome. We demonstrated that specific miRNA effectively acts as an RNA silencing-based antiviral response during HCV replication with miRNA specific machinery. The present study suggests that miRNA-mediated HCV inhibition and it may be possible to apply to novel anti-HCV therapies.

2. Materials and methods

2.1. Cell preparation, plasmid construction, liver tissue and transfection

Huh-7, HepG2, and cured MH14 cells [13] were cultured in D-MEM (Invitrogen) with 10% fetal bovine serum and nonessential amino acids (Invitrogen). SN1a [14] and JFH1 cells [15], which carry the full genome replicon of HCV genotype 1b and 2a, respectively, (Fig. 1A) were cultured in the above medium supplemented with 0.5–1.0 mg/ml G418 (Invitrogen). HuS-E/2 immortalized hepatocytes were cultured as described previously [16]. We obtained normal liver tissue from the Liver Transplantation Unit of Kyoto University after receiving the approval of the Ethical Committee of Kyoto University. To express miRNAs, plasmids containing the miR-199a*, miR-122, miR-19a, or a control sequence were constructed by ligating annealed oligonucleotides encoding each miRNA into the pSilencerH1-puro vector (Ambion) (Table 1). Cells were plated the day before transfection and grown to 50% confluence in 6-well plates. miRNA expression constructs (2 μg) were transfected into cells with FuGENE 6 (Roche). Cells were selected by puromycin (1.25 μg/ml) two days after transfection, and they were harvested more than two or four days later. For miRNA suppression, 50 pmol of 2′-O-methylated antisense oligonucleotide (ASO) (Hokkaido System Science) and Silencer® negative control siRNA (Ambion) were transfected with SilentFect (Bio-Rad).

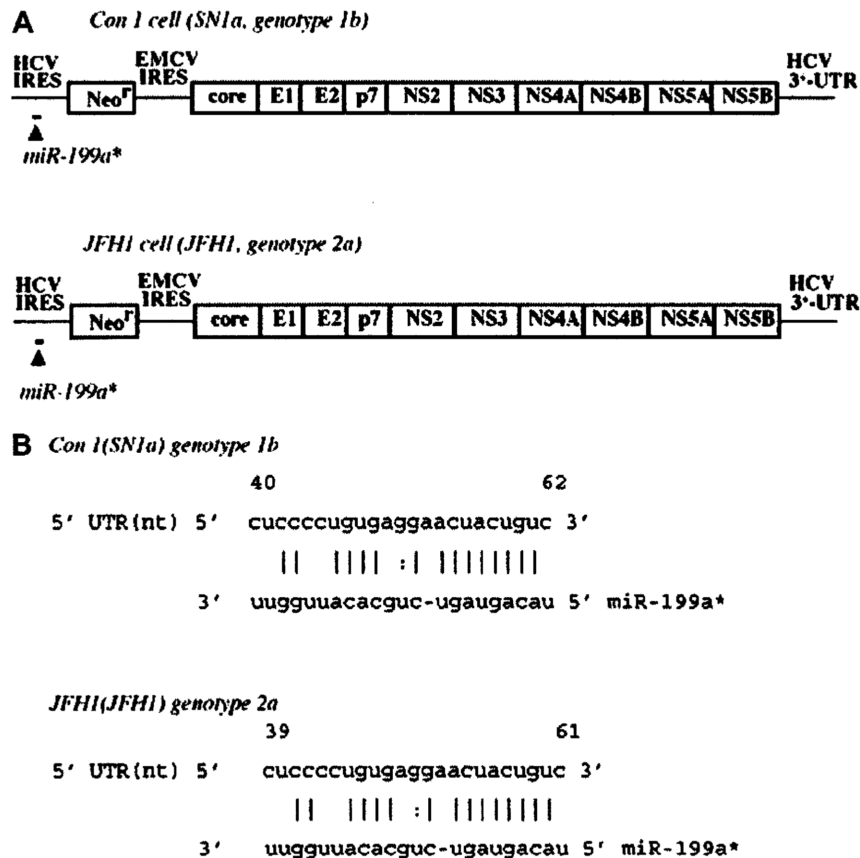

Fig. 1. (A) Schematic representation of the HCV genome-length replicon. The replicon comprised the HCV 5′-UTR, the HCV IRES, neomycin phosphotransferase gene (Neo^r), EMCV IRES, and the coding region from the core to NS5B and 3′-UTR. An arrowhead points to the target site. (B) miRNA target site sequences in the IRES of HCV genotype 1b and 2a. Numbers above sequences of 5′-UTR show nucleotide from 5′ end. Vertical bars indicated the complementary bases between the HCV replicon genome and miRNA. The G:U wobble pair also was shown as colon.

Table 1
Part of list of oligonucleotide of miRNA expression vector from as to miR-199a* as from as to miR-19 as from as to Control as.

(a) Primer sequence	
MxA-s	5′-GCTACACACCGTGACGGATATGG-3′
MxA-as	5′-CGAGCTGGATTGGAAAGCCC-3′
PKR-s	5′-GCCTTTTCATCCAAATGGAATTC-3′
PKR-as	5′-GAAATCTGTTCTGGGCTCATG-3′
2′-5′-OAS-s	5′-TCAGAAGAGAAGCCAACGTGS-3′
2′-5′-OAS-as	5′-CGGAGACAGCGAGGGTAAAT-3′
(b) List of oligonucleotide for miRNA expression vector	
miR-199a* s	5′-GATCCGTACAGTAGTCTGCACATTG GTTTTCAAGAGAAACCAATGTGCAGACT ACTGTATTCAAGAGTTTTTTGGAAA-3′
as	5′-AGCTTTCCAAAAAAATACAGTAGTCTGCA CATTGGTTTCTCTTGAAAACCAATGTGCAGA CTACTGTAGG-3′
miR-19a s	5′-GATCCTGTGCAAATCTATGCAAAACTGAT TCAAGAGATCAGTTTTGCATAGATTTG CACAGGAAA-3′
as	5′-AGCTTTTCCAAAAAAATGTGCAAATCTATGC AAAACTGATCTCTTGAATCAGTTTTGCATAG ATTTGCACAG-3′
Control s	5′-GATCCCCTTTTTTTTGGAAA-3′
as	5′-AGCTTTTCCAAAAAAAAGGG-3′

2.2. Quantitative real-time PCR

The 5′-UTR of HCV genomic RNA was measured by using the ABI PRISM 7700 sequence detector (Applied Biosystems) as described previously [17].

2.3. Immunoblot analysis

Immunoblot analysis was performed as described previously [18], using the following primary antibodies: anti-NS3 antibody (a generous gift from Takamizawa A, Osaka University), anti-core antibody (32-1; kindly provided by Kohara M, The Tokyo Metropolitan Institute of Medical Science), and anti α-tubulin (Oncogene).

2.4. In vitro HCV infection

HuS-E/2 cells were transfected with miRNA expression vector by Effectene transfection reagent (Qiagen) and control siRNA or ASO was electroporated into HuS-E/2 cells 24 h prior to infection (Bio-Rad). *In vitro* HCV infection was performed as described previously [19]. HuS-E/2 cells were inoculated with serum prepared from an HCV positive blood donor or with concentrated JFH1 medium (in a titer equivalent to 10^5 HCVRNA copies). Twenty-four hours post-infection, the cells were washed three times with phosphate buffered saline (PBS) and then maintained in growth medium.

2.5. Quantification of miRNA expression level

On day 4 after transfection the TaqMan® microRNA assay (Applied Biosystems) was used to quantify miR-199a* expression by using U18 as an internal control.

2.6. Transfection type of miRNA in experiments of effects of mutated miRNA sequences

SN1a cells were plated in 6-well plates the day before transfection and grown to 50% confluence. Cell were transfected with 50 pmol of miR-199a* (Hokkaido System Science), two types of mutated miR-199a* (PROLIGO), and control siRNA, by using SilentFect. Cells were harvested at day 4.

2.7. Transient transfection and luciferase assay

A luciferase assay monitored HCV replicon-luciferase as described previously [13]. Briefly, cured MH14 cells (5×10^3 cell/well) were transfected into 48-well dishes with DMRIE-C (Invitrogen). 0.25 μg HCV replicon-luciferase RNA, 0.5 μg miRNA expression vector, and 10 ng Renilla expression vector (internal control) after 96 h later the transfected cells were harvested and lysed, and their luciferase activity was measured with a Dual-Luciferase Reporter Assay System kit (Promega). The experiments were repeated at least three times. An IRES retaining the conserved secondary structure but lacking the miR-199a* recognition site was generated by PCR-based mutagenesis as described elsewhere [20]. HCV replicon-luciferase RNA was transcribed *in vitro* by using a MEGAscript T7 kit (Ambion) according to the manufacturer's instructions.

2.8. Interferon-related-gene response test by luciferase assay and RT-PCR

pISRE (IFNα-stimulated response element)-Luciferase was based on the pGL3-Promoter Vector [13]. After seeding cured MH14, HepG2, and Huh-7 cells (3×10^5each) in a 6-well plate 24 h before transfection then 2 μg of pISRE-Luc, 2 μg of miR-199a* expression plasmid, and 10 ng of Renilla expression plasmid (internal control) were transfected into cells using FuGENE 6. At 90 h post-transfection, these cells were treated with IFNα (Sigma; 500 IU/ml) for 6 h. Whole cell lysates were prepared and assayed with Dual-Luciferase Reporter Assay System kit.

Total RNA was extracted from the cells, and cDNA was synthesized from 5 μg of total RNA. The primer sequences are shown in Table 1 [21].

2.9. Co-immunoprecipitation with Ago2

A cell lysate of RNA-induced silencing complex (RISC) was collected by using microRNA Isolation Kit, Human Ago2 (Wako) according to the manufacturer's instructions. Briefly, 48 h after transfection with miRNA expression plasmid, 5×10^6 cells were washed with PBS and harvested by trypsinization. The cells were suspended in PBS and mixed with beads conjugated human Argonaute2 (hAgo2) monoclonal antibody for 2 h at 4 °C. HCV replicon RNA from the Ago2-IP fraction was quantified by RT-qPCR.

2.10. Statistical analyses

Data were statistically analyzed by the Student's *t*-test; and *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. The HCV genome contains a putative miRNA target sequence

To search for an miRNA with complementarity to the HCV genome, we performed *in silico* analysis with

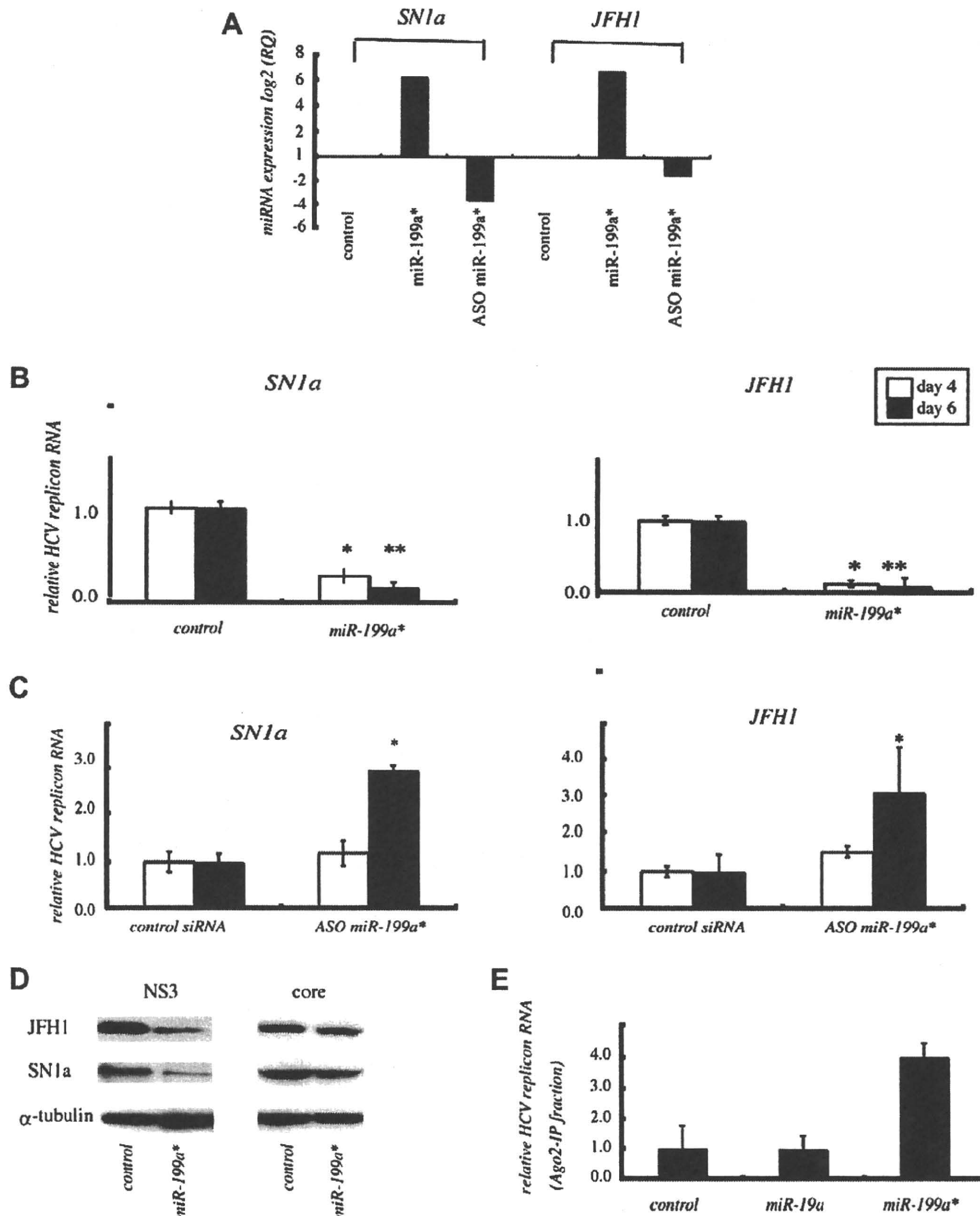

Fig. 2. Overexpression of miR-199a* decreased HCV replicon RNA levels. (A) Expression levels in SN1a and JFH1 cells after inducing overexpression of miR-199a* by miR-199a* expression vector or suppression of expression of miR-199a* by specific ASO were measured by real-time qPCR. (B) Comparison between HCV replicon RNA levels in SN1a (left) and JFH1 cell (right) before and after miRNA expression. The relative amounts of HCV replicon RNA per 50 ng of total RNA were measured by real-time qPCR at 4 and 6 days after transfection of miR-199a* and control vector. The data were normalized by the respective RNA levels in the controls. The data shown are as means ± SD of four independent experiments. *,Significant differences from the control at $p < 0.05$ and $p < 0.01$, respectively. (C) ASO with complementarity to miR-199a* stimulated HCV replicon RNA replication (left: SN1a cells, right: JFH1 cells). Relative amounts of HCV replicon RNA per 50 ng of total RNA were measured at day 4 and 6. (D) Immunoblot analysis of HCV core, NS3, and α-tubulin (internal control) in whole cell lysates from SN1a and JFH1 cells treated with miR-199a* expression or control vector. Fifty micrograms of whole cell lysates harvested 6 days after transfection was analyzed. (E) HCV replicon RNA is accumulated in RISC after miR-199a* expression. HCV replicon RNA was measured by real-time qPCR in 50 ng sample of total RNA from the Ago2-immunoprecipitation fraction.**

ViTa algorithms [22]. miR-199a* was identified as having a target sequence in the internal ribosomal entry site (IRES) of both the genotype 1b replicon (Con 1; GeneBank Accession No. AJ238799) and 2a (JFH1; GeneBank Accession No. AB047639) (Fig. 1). Endogenous expression levels of miR-199a* and 122 in Hela, Huh-7, SN1a (containing Con 1 replicon (genotype 1b)) cells, and normal liver tissue were shown (Supplementary

Fig. 1). Endogenous expression level of miR-122 was abundant in Huh-7, SN1a, and normal liver tissue [6] and endogenous expression level of miR-199a* in Huh-7 and SN1a was more than in normal liver tissue.

3.2. miR-199a* overexpression suppresses the replication of HCV replicon

This study showed that the expression level of miR-199a* was modified by the expression vector and by ASO complementary to miR-199a* in cells bearing either replicon, SN1a or JFH1 (Fig. 2A). Overexpression markedly restricted replicon replication in both cell lines (Fig. 2B). To determine whether miR-199a* has specific anti-viral effects, we inhibited miR-199a* activity with ASO. HCV replicon replication significantly increased in both cell lines upon treatment with ASO on day 4 and 6 (Fig. 2C). Immunoblot analysis showed good concordance with the result of real-time qPCR (Fig. 2D).

3.3. Ago2 co-immunoprecipitates target mRNA

To show that miR-199a* is associated with HCV genome physiologically [23], we performed an Ago2-co-immunoprecipitation (Ago2-IP) analysis to see whether

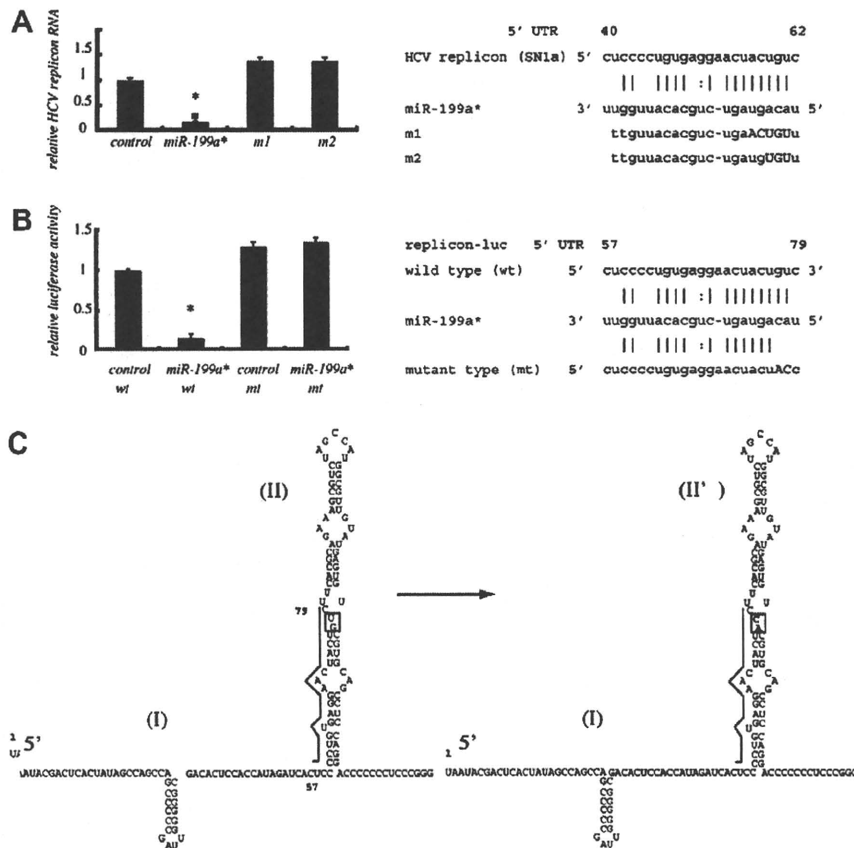

Fig. 3. Suppression of HCV replicon activity by miR-199a* was abolished by mutations in either the miRNA sequence or the miR-199a* target sequence in the HCV-replicon-luciferase construct. (A) Effect of mutated miR-199a* on HCV replicon activity. Comparison of HCV replicon RNA level in SN1a expressing ectopically with wild-type and mutated miRNAs measured by real-time qPCR. Complementarity among HCV replicon genome (upper number corresponds to the 5′-UTR start codon) and miR-199a* and two types of mutated miR-199a*: m1 and m2 are shown. Mutated nucleotides in m1 and m2 sequences are shown in capital letters. Additional nucleotides added to stabilize m1 and m2 are represented by tt. Relative HCV replicon RNA levels were normalized to the control siRNA level and are expressed as means ± SD of four independent experiments. *Significant difference at $p < 0.05$. (B) Effect of the miR-199a* on the wild-type and mutant-type HCV replicon activities. Comparison between the level of replicon activity in cured MH14 cell lysate after co-transfected of either miR-199a* expression vector or control vector, and either wild-type or mutant-type HCV replicon-luciferase construct. Sequences of wild-type (upper) and mutant-type (lower) HCV-replicon-luciferase in the IRES region are shown. Vertical bars and horizontal bars indicate complementary bases and gaps in complementarity between miR-199a* and the HCV-replicon-luciferase [13]. Mutated nucleotides are shown in capital letters. Nucleotide numbers begin at the start codon in the IRES. Mutated HCV-replicon-luciferase (mt) reduced the inhibitory effect of miR-199a*. Each column represents luciferase values standardized to the control vector co-transfected with the wild-type HCV-replicon-luciferase. The data shown are means ± SD of three replicates. (C) The target region of miR-199a* in the stem-loop II in IRES and the upstream stem-loop I together with its flanking region is shown. Small numbers indicate nucleotide positions from the 5′ end [28]. The thick bar indicates the target site. The mutated sequence is shown in II′ with the mutated residues boxed.

RISC could retain HCV genome when miR-199a* was over-produced. SN1a cells were transfected with either miR-199a*, miR-19a expression vector, or a control vector and used to prepare respective Ago2-IP fractionated cell lysates. Since miR-19a* does not recognize either HCV genome 1b or 2a, we used miR-19a as a negative control. The HCV-replicon RNA in the Ago2-IP fraction (IP RNA) was quantified by real-time qPCR. The concentration of HCV-replicon RNA was higher in the IP-RNA obtained by treatment with miR-199a* than by treatment with the control expression vector or miR-19a expression vector (Fig. 2E).

3.4. Effects of mutated miRNA sequences and target site sequences on miRNA suppression

We synthesized two miRNAs, with 5 and 3 bases in the sequence of mutated miR-199a*, yielding m1, and m2. We chose the mutation sites shown in Fig. 3. Transfection of m1 and m2 into SN1a cell did not suppress HCV RNA levels (Fig. 3A).

Mutated HCV-replicon-luciferase construct with two point mutations in the putative binding site of targeted by miR-199a* in the IRES region was synthesized. The luciferase activity of the wild-type replicon on IRES region decreased with treatment of miR-199a* expression vector as compared control expression vector. However, the luciferase activity of the replicon-luciferase with a mutated sequence in the IRES was unaltered by treatment with either miR-199a* or control expression vector (Fig. 3B). Since the recognition site is located in the stem-loop II in the IRES, the mutation was introduced so that the secondary structure of the stem-loop II would be unchanged. Although we did not verify the secondary structure of the mutated stem-loop II by biochemical analysis, "RNA secondary structure prediction" software (http://www.genebee.msu.su/services/rna2_reduced.html) predicted it to be the original structure (Fig. 3C).

3.5. Pretreating immortalized hepatocytes with miRNA reduces HCV replication

Immortalized HuS-E/2 cells ectopically expressing miR-199a* were infected with serum from an HCV-1b or HCV-2a-infected patients. The HCV replication was significantly lower in cells expressing miR-199a*, irrespective of the HCV genotype (Fig. 4A). In contrast, treatment of the cells before HCV infection with miR-199a* ASO caused to accumulate HCV RNA (Fig. 4B). We then performed an *in vitro* infection study with the infectious recombinant HCV-JFH1 virus. HuS-E/2 cells were inoculated with concentrated JFH1 medium. In HuS-E/2 cells after treatment with miR-199a* ASO, we found an increase in JFH1-RNA (Fig. 4C).

3.6. The anti-viral effect of miR-199a is independent of the interferon (IFN) pathway*

Cured MH14, HepG2, and Huh-7 cells co-transfected with the ISRE-luciferase reporter and either miR-199a* expression vector or the control vector were harvested and analyzed for luciferase activity. There was no significant difference in luciferase activity between the control and the miR-199a*-treated group (Fig. 5A) [24]. Moreover, no expression of the IFN-induced genes; MxA, PKR, and 2′-5′-OAS was induced by treatment of miR-199a* (Fig. 5B).

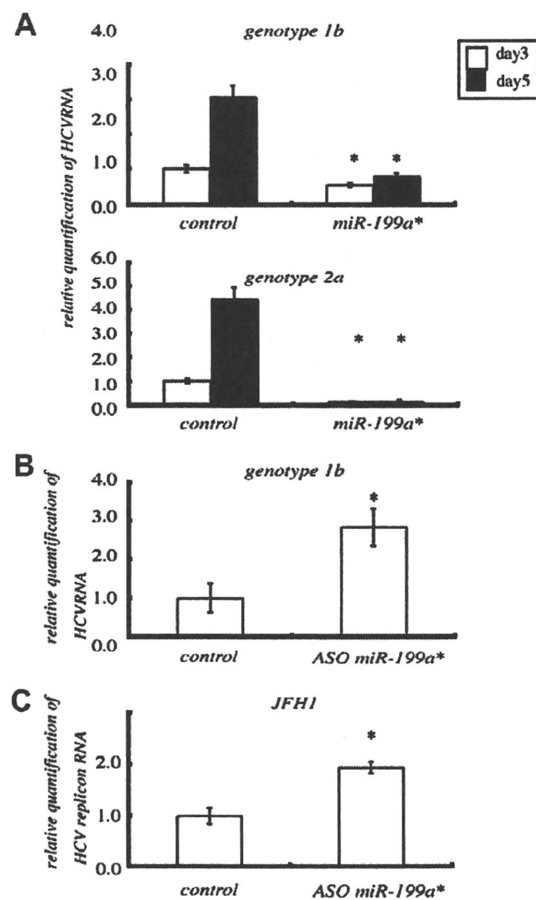

Fig. 4. Overexpression of miR-199a* reduced HCV replication. (A) Overexpression of miR-199a* reduced the amount of HCV RNA in HuS-E/2 cells infected with serum containing HCV-genotype 1b and 2a, in comparison with the control vector. Each column represents the relative amount of HCV RNA normalized to the vector control sample on post-transfection day 3 and 5. The data shown are means ± SD of four independent experiments. *,Significant difference at $p < 0.05$ and $p < 0.01$, respectively. (B) Prior transfection of HuS-E/2 cells with miR-199a* ASO accelerated HCV-1b replication activity on day 3. HCV RNA levels are expressed as means ± SD of four independent experiments. (C) miR-199a* ASO prior to *in vitro* infection with JFH1 replicon RNA accelerated JFH1 replicon RNA. Experimental procedure is described in Section 2.**

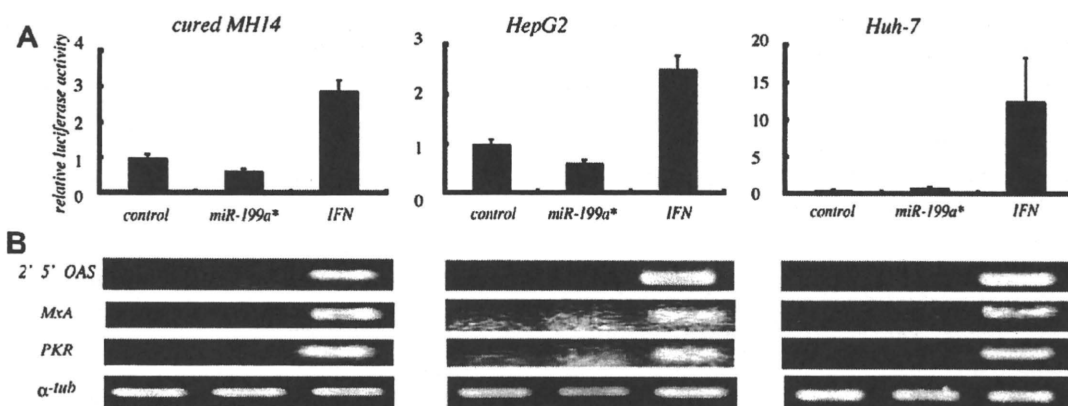

Fig. 5. Treatment of the cells with miR-199a* did not induce the IFN pathway. (A) Cured MH14 (left), HepG2 (middle) and Huh-7 cells (right) were co-transfected with pISRE-Luc and either miR-199a* expression vector or the control vector. In a control experiment to investigate IFN response, one line of cells treated with IFNα (500 IU/ml) for 6 h at 90 h after transfection of the control vector. The cellular luciferase activity was measured 96 h after transfection and normalized to luciferase activity of the cells treated with the control vector treatment. Each column represents the luciferase activity, normalized to the control vector. (B) 2′-5′-OAS, MxA, and PKR expression was measured by semi-quantitative RT-PCR on day 4 after transfection.

4. Discussion

This study demonstrates that HCV replication can be controlled by miRNA. Viruses use non-coding RNAs, such as miRNAs, to inhibit intrinsic anti-viral immunity in mammalian cells. For example, herpesvirus encodes viral miRNAs that dampen host antiviral immunity [25], and many mammalian viruses usurp or divert host mRNA silencing machinery to their advantage. By contrast, some host-encoded miRNAs have anti-viral functions [26]. RNA silencing-based antiviral responses may work in concert with innate and acquired antiviral systems [27].

miR-199a* has a target sequence in domain II of the IRES region in the HCV 5′-UTR, a region that is highly conserved across all HCV genotypes [28] and is crucial for viral replication. Introduction of miR-199a* ASO into replicon cells had the opposite effect of increasing viral replication. Mutagenesis analyses showed that the inhibitory effect of miR-199a* on HCV replication was highly dependent on complementarity between the viral and host sequences. Because HCV-replicon RNA is concentrated in the RISC by overexpression of miR-199a*, miR-199a* and HCV-replicon RNA seem be complexed in cells under certain pathophysiological conditions. Our study indicates that the sequence-dependent interactions contribute to the anti-HCV activities of miR-199a*.

However, miRNAs often recognize target genes with incomplete complementarity, which allows them to recognize many target-candidate genes. To determine whether the anti-viral effect of the miRNA is mediated not only by mechanism that directly targets the HCV genome but by other mechanism, such as modulation of cellular genes, we analyzed the mRNA expression profiles of miR-199a*-transfected cells by microarray. Based on the results of the microarray analysis, as shown in Supplementary Fig. 2, we identified several genes whose expression level changed at least twofold after overexpression. Ceruloplasmin (CP) has already been reported to be involved in HCV replication [29]. Although CP is not contained in a list of miRase targets (http://microrna.sanger.ac.uk/target/v5/) for miR-199a*, it will be necessary to determine whether CP is controlled by miR-199a* as miRNA machinery and whether CP is capable of participating in HCV replication.

Chronic HCV infection causes various liver diseases, from chronic hepatitis to hepatocellular carcinoma. Previously we demonstrated that miRNA expression profiles change with the degree of liver fibrosis and pathological differentiation of HCC [18]. In the present study we demonstrated that miR-199a* can control viral replication. miRNAs may have future application as efficient, safe, and specific means of antiviral therapy.

Acknowledgments

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Appendix A. Supplementary data

Microarray analysis. Fluorescent (cyanine 3-CTP)-labeled cRNA was synthesized from 500 ng of total RNA with a Low RNA Input Fluorescent Linear

Amplification Kit (Agilent Technologies), and hybridized to a Human 1A(v2) Oligo microarray (Agilent Technologies). The signal intensity per spot was analyzed from scanned images using Feature Extraction Software ver8.5 (Agilent Technologies). To compare expression profiles between miRNA-transfected and control cells, median percentile normalization was performed using GeneSpring GX 7.3 (Agilent Technologies).

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2008.06.010.

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Impact of hepatitis B virus (HBV) X gene integration in liver tissue on hepatocellular carcinoma development in serologically HBV-negative chronic hepatitis C patients☆

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Background/Aims: **We analyzed hepatitis B virus (HBV) X gene integration in hepatocytes of HBV-negative, chronic hepatitis C (CH-C) patients with mild fibrosis, and prospectively followed these patients for the development of hepatocellular carcinoma (HCC).**

Methods: **The study included 39 HBV-negative CH-C patients with mild fibrosis. HBV-X integration was determined by Alu-PCR analysis of liver specimens obtained by fine-needle biopsy.**

Results: **Integration of HBV-X gene sequence into liver genome occurred in 9 of the 39 patients. Six of the 39 patients developed HCC during the 12-year follow-up period. No significant difference was found in the incidence of HCC between patients with and without HBV-X integration. However, the two patients with HBV-X integration who developed HCC did not have cirrhosis at the time when HCC was diagnosed, whereas the four patients without HBV-X integration who developed HCC did have cirrhosis.**

Conclusions: **Our findings suggest that HBV-X integration detected at the mild fibrosis stage might not indicate a high risk for HCC. HBV-X integration may be associated with HCC development in the absence of cirrhosis. However, we did not find evidence that HBV-X integration directly plays a role in hepatocarcinogenesis in CH-C patients. Further studies will be needed to clarify this point.**

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Keywords: **HBV-X integration; Chronic hepatitis C; Hepatocellular carcinoma**

1. Introduction

Chronic viral hepatitis is a leading cause of hepatocellular carcinoma (HCC) worldwide [1–4]. Occult hepatitis B virus (HBV) infection, characterized by the absence of circulating HBV surface antigen [HBsAg] but presence of the HBV genome in serum or liver tissue, has been identified in hepatitis C virus (HCV)-infected patients. HBV may affect the clinical course of chronic hepatitis C (CH-C) [5] and increase the risk of hepatocarcinogenesis [6]. Pollicino reported that both integrated and free HBV–DNA sequences were highly prevalent in the liver tissue of CH-C patients with HCC compared to CH-C patients without HCC [7].

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