

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 play a 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 effector 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.

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% CO₂.

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

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 H₂O, 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. Reverse 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.

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

^amiRNAs 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

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

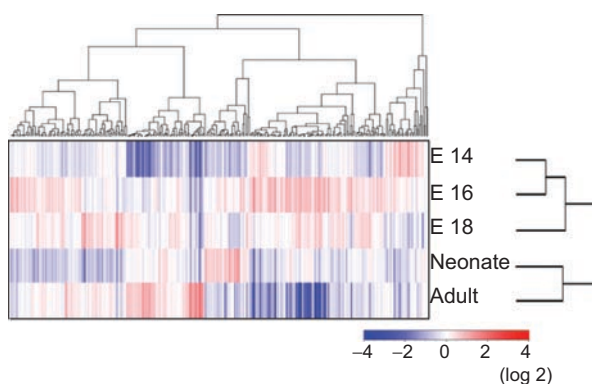


Figure 1. A global expression pattern of miRNA in the process of mouse liver development. The data were subjected to a hierarchical cluster analysis using a Euclidean distance calculation based on Ward's methods. The liver samples are aligned vertically: embryo (E) 14, E16, E18, neonate and adult. Samples 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.

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.

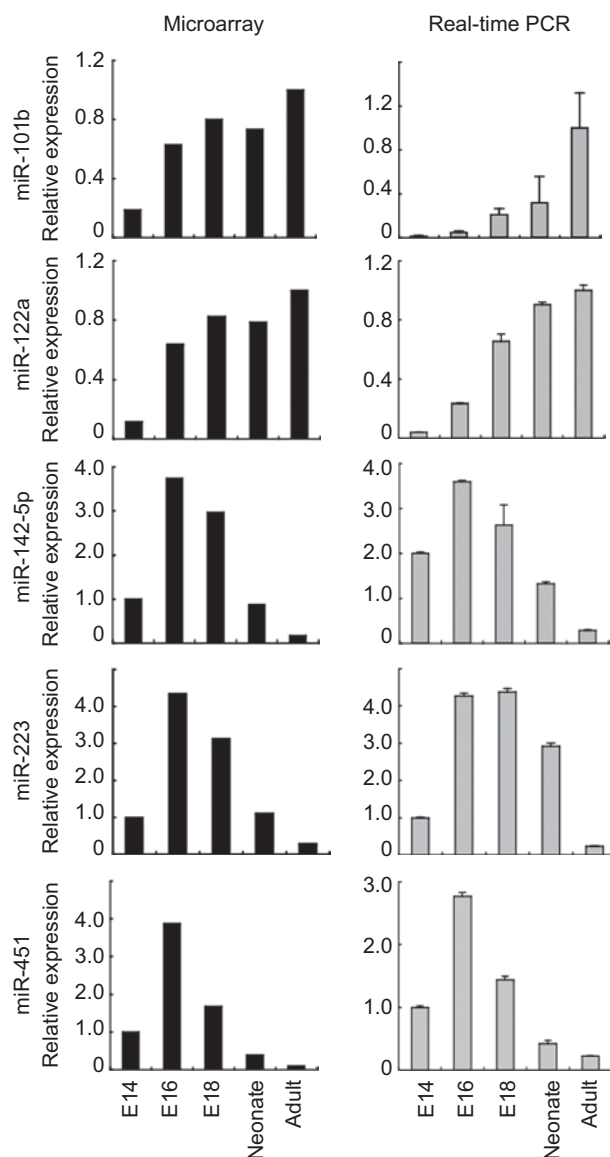


Figure 2. Differential expression of selected miRNA in mouse liver development by microarray and real-time polymerase chain reaction (PCR). miR-101b, miR-122a, miR-142-5p, miR-223 and miR-451 were selected from highly expressed miRNAs to confirm the expression levels of microarray analysis by real-time 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 \pm SD.

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

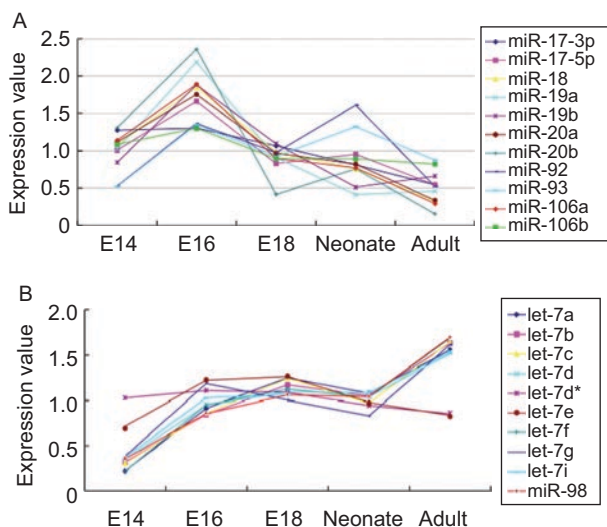


Figure 3. Expression patterns of cancer-related miRNAs in the process of mouse liver development. (A) Expression pattern by a microarray analysis (each sample: $n=7-10$) of miRNA that may act as an oncogene. (B) The expression pattern by the microarray analysis (each sample: $n=7-10$) of the let-7 family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

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 down-regulated 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

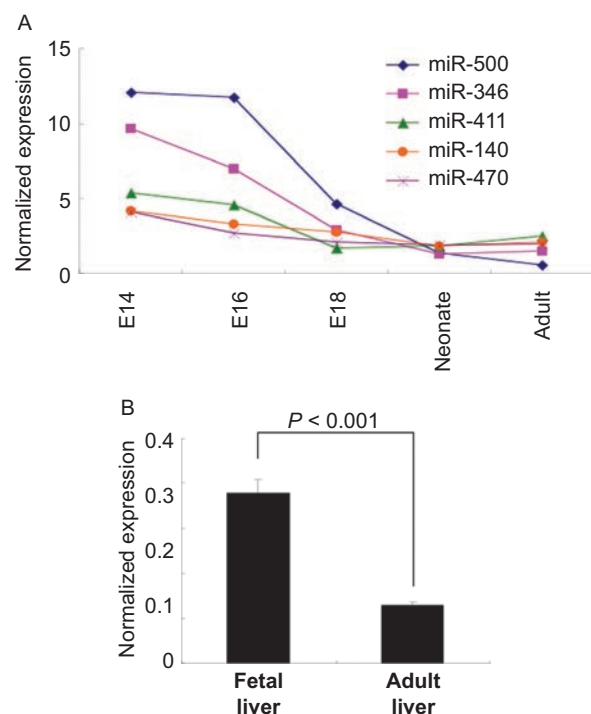


Figure 4. The expression of miR-500 is higher in the fetal stage than in the adult stage (A). The expression profile of miRNA decreased in the process of mouse liver development. Expression values are based on microarray data. (B) Expression of miR-500 in human fetal and adult liver. Real-time polymerase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

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

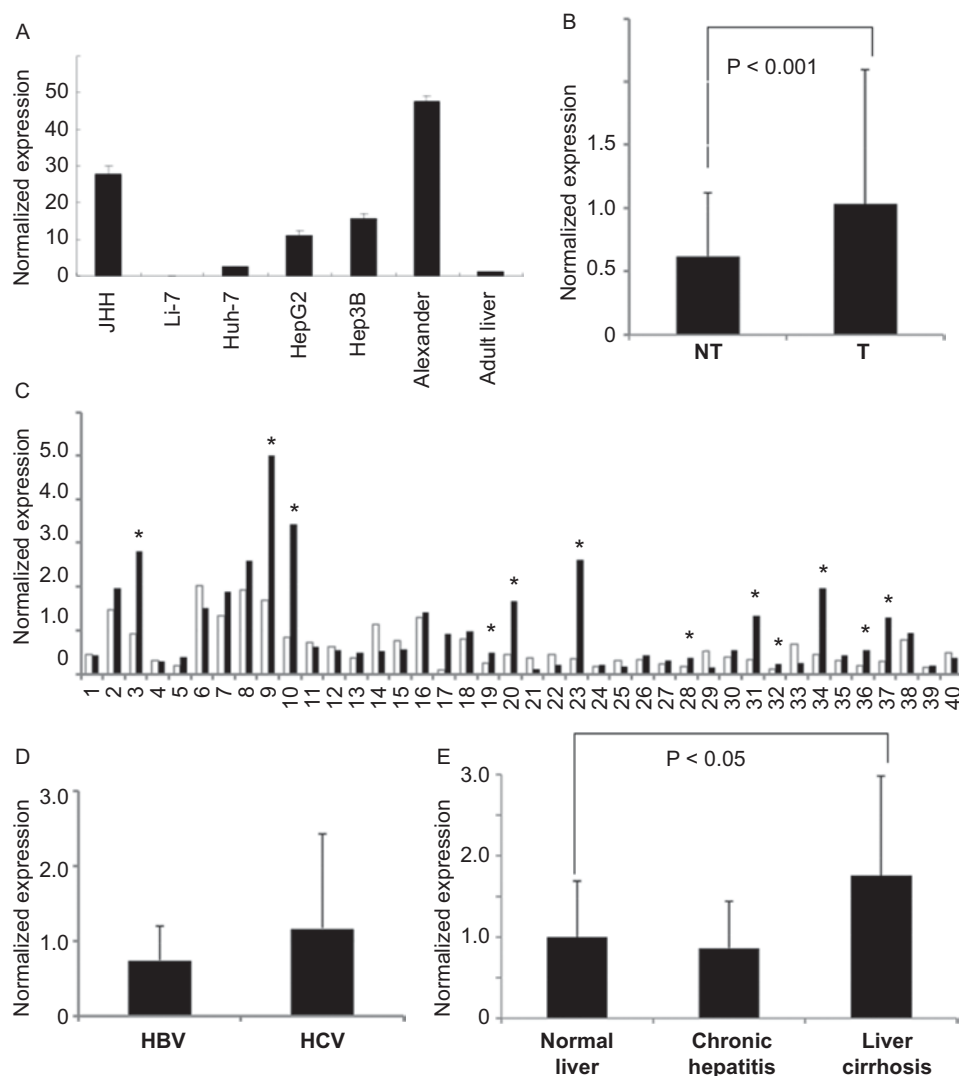


Figure 5. The expression of miR-500 is clearly upregulated in human liver cancer (A) miR-500 expression abundantly detected in liver cancer cell lines (JHH-7, Li-7, Huh-7, HepG2, Hep3B and Alexander). The expression level of normal liver is set to 1.0. The data represent the mean \pm SD. (B) Forty pairs 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 \pm 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 \pm SD, $p < 0.05$. Expression values are normalised with U6 snRNA value.

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.

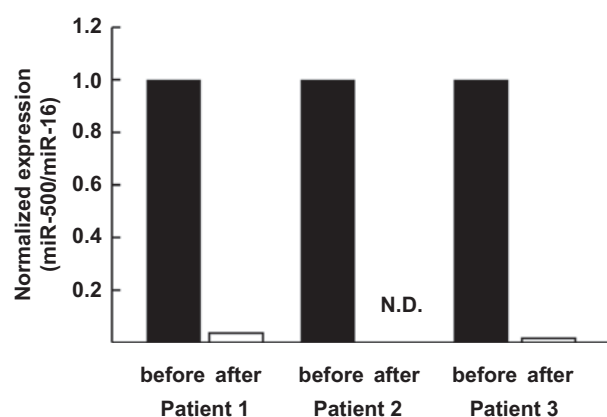


Figure 6. Serum levels of miR-500 in hepatocellular carcinoma (HCC) patients. Changes of serum levels of miR-500 in HCC patients ($n=3$) before (preoperation) and after (postoperation within 6 months) surgical removal of the tumour. Expression levels of the miR-500 are normalised to miR-16. N.D., not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

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

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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