Serum microRNA-125a-5p as a potential biomarker of HCV-associated hepatocellular carcinoma

KYOKO OURA[1], KOJI FUJITA[1], ASAHIRO MORISHITA[1], HISAKAZU IWAMA[2],
MAI NAKAHARA[1], TOMOKO TADOKORO[1], TEPPEI SAKAMOTO[1], TAKAKO NOMURA[1],
HIROHITO YONEYAMA[1], SHIMA MIMURA[1], JOJI TANI[1], HIDEKI KOBARA[1],
KEIICHI OKANO[3], YASUYUKI SUZUKI[3] and TSUTOMU MASAKI[1]

[1]Department of Gastroenterology and Neurology; [2]Life Science Research Center;
[3]Department of Gastroenterological Surgery, Kagawa University, Miki 761-0793, Japan

Abstract. During diagnosis of early stage hepatocellular carcinoma (HCC), single or small lesions are difficult to identify using screening ultrasonography, and conventional tumor markers are frequently negative. MicroRNAs (miRNAs) are small non-coding RNAs that suppress the translation of target mRNAs and exert significance as biomarkers. The aim of the present study was to use samples of patients with HCC and those with other liver diseases caused by hepatitis C virus (HCV) infection to investigate the expression profile of serum miRNAs, and identify a miRNA that can serve as a HCC biomarker. Initially, changes in 2,555 miRNAs between pre- and post-curative treatment serum from 12 patients with early stage HCC were examined using microarray analysis. The serum levels of miR-125a-5p in 40 individuals with HCV-associated chronic hepatitis (CH), liver cirrhosis (LC) or HCC were measured using reverse transcription-quantitative polymerase chain reaction, and 5 miRNAs, including miR-125a-5p, miR-423-5p, miR-1247, miR-1304 and miR-3648, were identified to be downregulated following curative treatment in patients with HCC. Among these, miR-125a-5p was identified to be similarly decreased following treatment in all patients. Additionally, the expression levels of miR-125a-5p were significantly upregulated in patients with HCC in the early and advanced stages of disease, compared with patients with CH or LC (P<0.05). Serum miR-125a-5p fluctuates depending on the presence of HCC, and may serve as a noninvasive biomarker to aid in diagnosing early carcinogenesis in HCV-associated chronic liver diseases.

Introduction

Hepatocellular carcinoma (HCC) is a common cancer worldwide, particularly in East Asian countries, including Japan (1). HCC is the sixth most commonly occurring cancer and the third most common cause of cancer-associated mortality worldwide in 2012 (2). Multiple risk factors have been associated with the occurrence of HCC, including chronic liver injury due to hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, autoimmune liver disease, drug-induced liver injury, alcohol and aflatoxin B exposure (2-5). HCC had one of the worst prognoses among any cancer with a 5-year survival rate of 15-25% in United States and East Asian countries from 2007 to 2010, partially due to the resistance to chemotherapy and a high recurrence rate (6,7). One of the most prevalent reasons for a poor prognosis is the difficulty in early detection, and as a result, curative therapy is no longer feasible at the time of detection, due to intrahepatic and extrahepatic metastases (2).

To assist the diagnosis of HCC, imaging techniques used in screening, including ultrasonography, computed tomography (CT) and/or magnetic resonance imaging (MRI), are notably beneficial (2). However, in the case of early HCC, the diagnosis of small lesions is relatively inaccurate (8), and repeated examination is costly. Other common approaches used in screening for HCC in high-risk patients are serum tumor markers, including α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonists-II (PIVKA-II), which can be measured simultaneously in blood samples obtained for other liver function tests (9). However, the sensitivity and specificity of high serum AFP and PIVKA-II levels for HCC are reported to range from 39-64 and 76-91%, and 41-77 and 72-98%, respectively (10). Therefore, additional biomarkers that can be used complementarily are required, particularly those associated with early HCC.

Correspondence to: Dr Tsutomu Masaki, Department of Gastroenterology and Neurology, Kagawa University, 1750-1 Ikenobe, Miki 761-0793, Japan
E-mail: tmasaki@med.kagawa-u.ac.jp

MicroRNAs (miRNAs) are small, non-coding RNAs 18-25 nucleotides in length that suppress the translation of the target mRNAs by binding to their 3' untranslated region (11,12). miRNAs control a number of important biological processes, including cell proliferation, differentiation and development (13-15), and specific miRNAs function as oncogenes or tumor suppressors (16). The expression profiles of human miRNAs indicate that specific miRNAs such as miR-15 and miR-16, let-7, miR-34 are deregulated in cancer, and are differentially expressed in various carcinoma types, including gastrointestinal, urological, gynecological and lung cancer (17). Additionally, with respect to HCC, it has been reported that the expression levels of a number of miRNAs differ between cancerous and noncancerous specimens from radical resection of patients with HCC (18). In our previous investigation, we reported that the miRNA profile is different between HCC and normal liver cell lines (19), and it is hypothesized to exert significance as biomarkers (20).

It has previously been identified that circulating miRNAs can exist stably in numerous body fluids, including the peripheral blood (21), which can be used for the diagnosis, evaluation and prognosis of colorectal, esophageal, gastric and pancreatic cancer (22). When released from cells and tissues, miRNA exists in exosome-encapsulated form or bound to protein or lipid in the serum (23). The research demonstrated that miRNAs are stable and detectable in the serum and are not degraded by RNase. Since serum can be obtained noninvasively and the miRNAs exhibit specificity to the disordered tissue, application of circulating miRNA in diagnosis is expected; however, its biological significance is unknown. Therefore, the present study investigated the expression profile of circulating miRNAs using serum samples from patients with HCV-associated HCC, and analyzed whether a specific circulating miRNA could help in the detection of early HCC.

Materials and methods

Patients and samples. In order to identify biomarkers of HCV-associated HCC from among the candidate miRNAs, the present study examined miRNA changes between the pre- and post-treatment serum of patients with early stage (stage I or II) HCC according to the Tumor-Node-Metastasis classification based on the criteria of the Liver Cancer Study Group of Japan (24). Paired samples were obtained from a total of 12 patients with HCC, who underwent curative treatment, such as radiofrequency ablation or hepatectomy, in Kagawa University Hospital (Kagawa, Japan), from April 2013 to April 2015. The characteristics of the patients are summarized in Table I. All patients had HCC with chronic hepatitis (CH) or liver cirrhosis (LC) due to HCV infection without any other liver diseases, such as HBV infection and alcoholic, autoimmune or metabolic liver diseases.

The present study examined candidate biomarkers using paired serum samples from 12 patients with HCC pre- and post-curative treatment. The pre-treatment samples were collected prior to the first curative treatment, and the post-treatment samples were collected following confirmation that there was no long-term recurrence for at least 1 year following treatment. Tumor marker measurement was performed every three months in all cases to ensure there was no recurrence. Prior to treatment, 9 patients were positive for at least one tumor maker (AFP, AFP-L3 and/or PIVKA-II), yet were negative for all markers for at least 6 months following treatment (Fig. 1A-C). Additionally, imaging tests, including ultrasonography, dynamic CT and/or MRI examination, were performed every three months and it was confirmed that there was no recurrence. Dynamic CT images prior to and 1 year following treatment in cases 1 and 2 are presented in Fig. 2.

In the second experiment, the expression of the specific miRNAs was examined using multiple serum samples from individual patients with various liver diseases. A total of 40 individuals were enrolled including 10 age and sex matched patients with CH, 10 patients with LC, 10 patients with early stage (stage I or II) HCC and 10 patients with advanced stage (stage IV) HCC. Characteristics of the patients are summarized in Table II. All subjects were patients with liver disease associated with HCV infection, and patients with other liver diseases were excluded. Serum samples were collected from patients with HCC from the time of first diagnosis with HCC, and patients with CH or LC prior to receiving antiviral therapy for HCV.

Written informed consent was obtained from all participants, and the present study was approved by the Ethics Committee of Kagawa University Hospital (Kagawa, Japan) (Ethics approval Heisei 22-063).

Plasma preparation. Whole blood samples (5 ml) were collected from each individual directly into RNase free tubes, followed by centrifugation at 1,500 x g for 15 min at 4˚C. The samples with signs of hemolysis or chyle were excluded from the present study. Each serum sample was immediately transferred to a RNase free tube and stored at -80˚C until subsequent analysis.

Total RNA extraction. RNA from total serum was extracted with a miRNeasy Serum/Plasma kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. To ensure RNA quality, only RNA sample that exhibited $A_{260/280}$ ratios between 1.9-2.1 were selected. The $A_{260/280}$ ratios were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). RNA concentrations were measured using a NanoDrop 2000 spectrofluorometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and each sample was diluted with RNase free water.

miRNA microarray analysis. The RNA quantity was measured using a RNA 6000 Nano kit (Agilent Technologies, Inc.), and the samples were labeled using a miRCURY Hy3 Power Labeling kit (Exiqon; Qiagen GmbH) and hybridized to the human miRNA Oligo Chip (v.21; Toray Industries, Tokyo, Japan), which can analyze 2,555 miRNAs. Scanning was performed using the 3D-Gene Scanner 3000 (Toray Industries, Inc., Tokyo, Japan). The 3D-Gene extraction version 1.2 software (Toray Industries, Inc.) was used to calculate the raw signal intensity of the images. The raw data were analyzed using the GeneSpring GX 10.0 software (Agilent Technologies, Inc.) to assess miRNA expression. Quantile normalization was performed on raw data that were greater than the background level.

Table I. Clinical characteristics of participants in the microarray analysis.

Case	Sex	Age (years)	Genotype	HCV-RNA (log10 IU/ml)	AST (IU/l)	ALT (IU/l)	Platelet (x10^9/l)	AFP (ng/ml)	AFP-L3 (%)	PIVKA-II (AU/ml)	TNM stage	Treatment
1	M	56	2a	5.2	49	38	6.6	Negative	Negative	Negative	T1N0M0	RFA
2	M	61	1b	4.9	60	60	6.2	78	17.8	Negative	T2N0M0	Hepatectomy
3	M	82	2b	4.8	29	14	14.8	Negative	Negative	113	T2N0M0	Hepatectomy
4	F	77	1b	6.9	45	29	15.6	Negative	Negative	253	T2N0M0	RFA
5	M	68	1b	5.2	44	20	7.6	Negative	Negative	371	T1N0M0	RFA
6	M	57	1b	5.0	21	13	18.6	Negative	Negative	223	T2N0M0	Hepatectomy
7	M	76	2a	4.6	23	14	20.3	Negative	Negative	Negative	T1N0M0	RFA
8	M	83	1b	6.2	17	15	20.0	Negative	Negative	358	T2N0M0	RFA
9	F	76	1b	5.0	80	54	6.5	28	Negative	Negative	T2N0M0	RFA
10	F	79	1b	5.2	74	71	21.2	Negative	Negative	Negative	T1N0M0	RFA
11	M	69	2a	5.9	91	74	10.1	20	Negative	41	T1N0M0	RFA
12	F	70	1b	6.0	89	80	6.0	82	21	Negative	T2N0M0	RFA

The tumor marker is negative when within the following reference values: AFP <13 ng/ml; AFP-L3 <10%; and PIVKA-I I<40 AU/ml. TNM stage is based on the criteria of the Liver Cancer Study Group of Japan (24). M, male; F, female; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TNM, Tumor-Node-Metastasis; RFA, radiofrequency ablation.
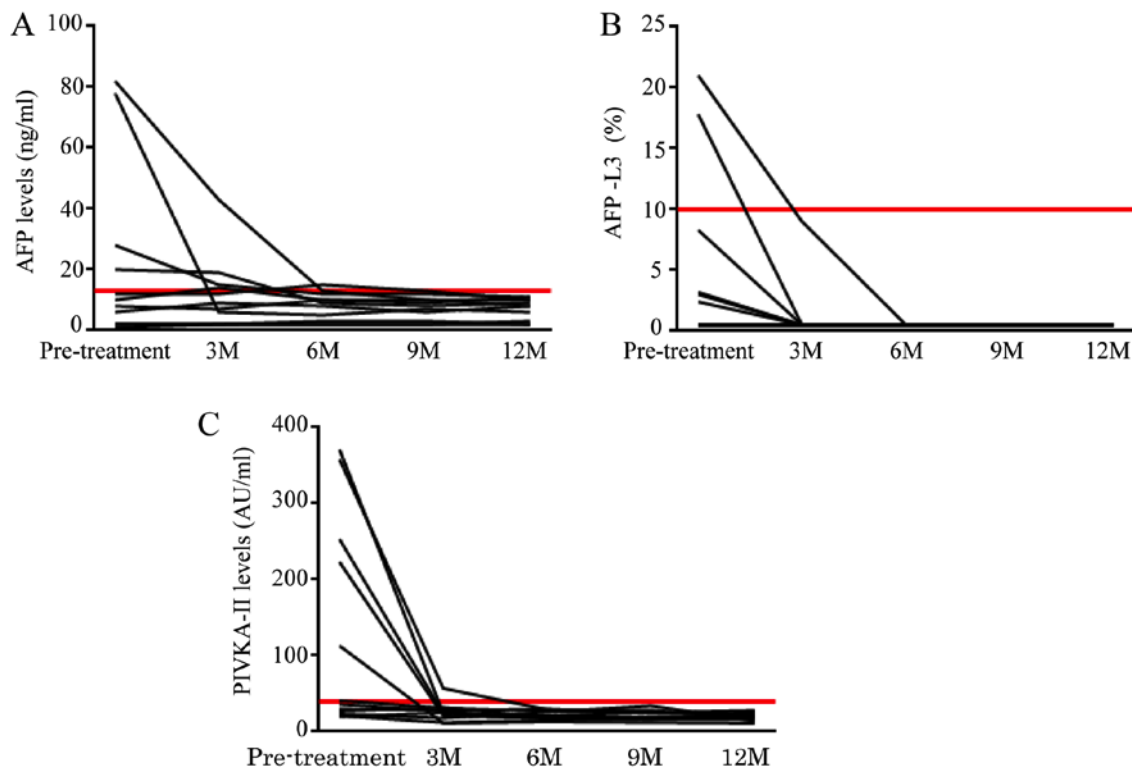
Figure 1. Changes in the levels of tumor markers in the serum of patients with hepatocellular carcinoma. (A) AFP levels. (B) AFP-L3 levels. (C) PIVKA-II levels. The black line graphs represent the change at 3, 6, 9 and 12M following treatment in 12 cases. The red horizontal lines represent the upper limits of normal at Kagawa University. Prior to treatment, 9 patients were positive for at least one tumor marker; however, they were negative for these markers following at least 6 months of treatment. M, months; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II.
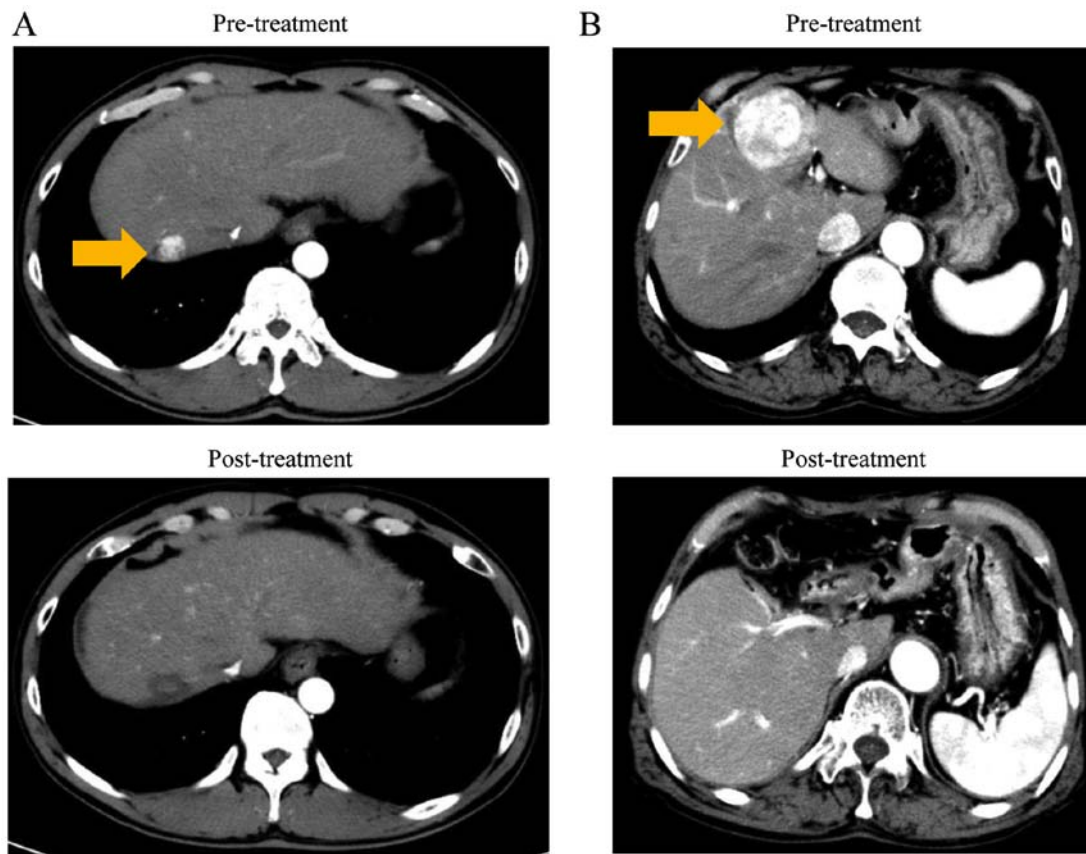

Figure 2. Dynamic computed topography images prior to and 12 months following treatment. (A) The images pre- and post-treatment in case 1. (B) The images pre- and post-treatment in case 2. Yellow arrows indicate the occupied lesions diagnosed as hepatocellular carcinoma. No local recurrence or no new lesion was exhibited following treatment in all patients.

Table II. Clinical characteristics of participants in the reverse transcription-quantitative polymerase chain reaction analysis.

Characteristics	CH-C	LC-C	HCC-C stage I or II	HCC-C stage IV
Individuals (n)	10	10	10	10
Male/female (n)	7/3	7/3	7/3	7/3
Mean age (years)	67.4±4.4	67.2±5.1	68.0±4.8	67.1±5.1
Laboratory data (median)				
AST (IU/l)	40 (20-75)	45 (13-77)	50 (29-106)	52 (40-485)
ALT (IU/l)	32 (13-99)	43 (16-75)	45 (17-104)	34 (20-180)
Alb (g/dl)	4.1 (2.9-4.7)	4.0 (3.4-4.7)	3.9 (3.4-5.2)	3.6 (2.6-3.8)
T.Bil (mg/dl)	0.7 (0.3-1.2)	1.0 (0.3-2.1)	1.1 (0.6-2.0)	0.8 (0.4-2.8)
PT (%)	87 (59-130)	78 (51-98)	99 (72-111)	79 (46-110)
Plt (x10^4/mm^3)	18.0 (13.0-21.0)	8.7 (5.9-9.8)	11.3 (7.2-14.0)	15.9 (8.4-22.9)
Tumor marker (n)				
AFP positive	1	2	2	7
AFP-L3 positive	0	0	1	8
PIVKA-II positive	0	0	4	10
Negative	9	8	5	0
Child-Pugh score (n)				
A	10	9	10	3
B	0	1	0	6
C	0	0	0	1
FIB-4 index (median)	2.87 (1.17-3.78)	5.12 (3.36-10.68)	5.09 (2.69-9.21)	4.03 (2.61-7.97)

The ages are given as the means ± standard deviation. AST, ALT, Alb, T-Bil, PT, Plt, and Fib-4 index are depicted as the medians and range. Fib-4 index is calculated using the formula (age x AST)/(platelet count x √ALT). Clinical stage of HCC is based on the criteria of the Liver Cancer Study Group of Japan (24). AST, aspartate aminotransferase; ALT, alanine aminotransferase; Alb, albumin; T.Bil, total bilirubin; PT, prothrombin time; Plt, platelet; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; Fib-4, fibrosis-4; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for miRNA validation. Due to the possibility of false positive results obtained from the miRNA array analysis, the present study performed qPCR using the same samples prior to and following treatment. Furthermore, RT-qPCR was performed for the analysis of the expression levels of specific miRNAs using 40 serum samples from patients with HCV-associated liver diseases, including CH, LC and HCC.

Initially, *Caenorhabditis elegans* miRNA, cel-miR-39 (miRNeasy Serum/Plasma Spike-in control; Qiagen GmbH) was added as an exogenous control during the process of total RNA extraction. TaqMan microRNA assays (Applied Biosystems; Thermo Fisher Scientific, Inc.) were adopted to determine the expression levels of four miRNAs (assay ID: 002198 and target sequence: 5'-UCCCUGAGACCCUUU AACCUGUGA-3' for hsa-miR-125a-5p; assay ID: 002340 and target sequence: 5'-UGAGGGGCAGAGAGCGAGACUUU-3' for hsa-miR-423-5p; assay ID: 46440 and target sequence: 5'-AGCCGCGGGGAUCGCCGAGGG-3' for hsa-miR-3648; and assay ID: 000200 and target sequence: 5'-UCACCG GGUGUAAAUCAGCUUG-3' for cel-miR-39). To examine another two miRNAs, TaqMan Advanced miRNA Assays were used (assay ID: 479553_mir and target sequence: 5'-CCC CGGGAACGUCGAGACUGGAGC-3' for hsa-miR-1247-3p; assay ID: 479574_mir and target sequence: 5'-UCUCACUGU AGCCUCGAACCCC-3' for hsa-miR-1304-3p; and assay

ID: 478293_mir and target sequence: 5'-UCACCGGGUGUA AAUCAGCUUG-3' for cel-miR-39). miRNAs were reverse transcribed using a TaqMan microRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a TaqMan Advanced miRNA cDNA Synthesis kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCRs were performed using a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems; Thermo Fisher Scientific, Inc.), and each well contained cDNA, 20X qPCR assay, nuclease-free water and TaqMan Fast Advanced Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to manufacturer's protocol. Using the ViiA7 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), samples were denatured by incubation at 95°C for 20 sec. This was followed by 40 cycles of 1 sec at 95°C and 20 sec at 60°C.

The raw expression level was determined by the cycle number at which the reaction crossed a predetermined quantification cycle (Cq) identified for the miRNA probe. For relative expression of each miRNA in each sample is determined using $2^{-\Delta\Delta Cq}$ method (25). For the validation of miRNA changes between the pre- and post-treatment serum, the values were calculated according to the following formula: $\Delta Cq = Cq_{\text{target miRNA}} - Cq_{\text{cel-miR-39}}$, and $\Delta\Delta Cq = \Delta Cq_{\text{post-treatment sample}} - \Delta Cq_{\text{pre-treatment sample}}$. For the analysis of the expression levels of specific miRNAs from individual patients with various liver diseases, the values were calculated according

Table III. Serum miRNA levels were significantly different between pre- and post-treatment.

miRNAs	Fold change post-/pre-treatment	SD	P-value	Chromosome location
miR-125a-5p	0.74	0.23	0.00989	19
miR-423-5p	0.61	0.20	0.00151	17
miR-1247-3p	0.73	0.19	0.0066	14
miR-1304-3p	0.69	0.26	0.00952	11
miR-3648	0.63	0.34	0.00384	21

Fold change represents the ratio of the post-treatment miRNA levels to the pre-treatment levels. SD, standard deviation; miRNA, microRNA.
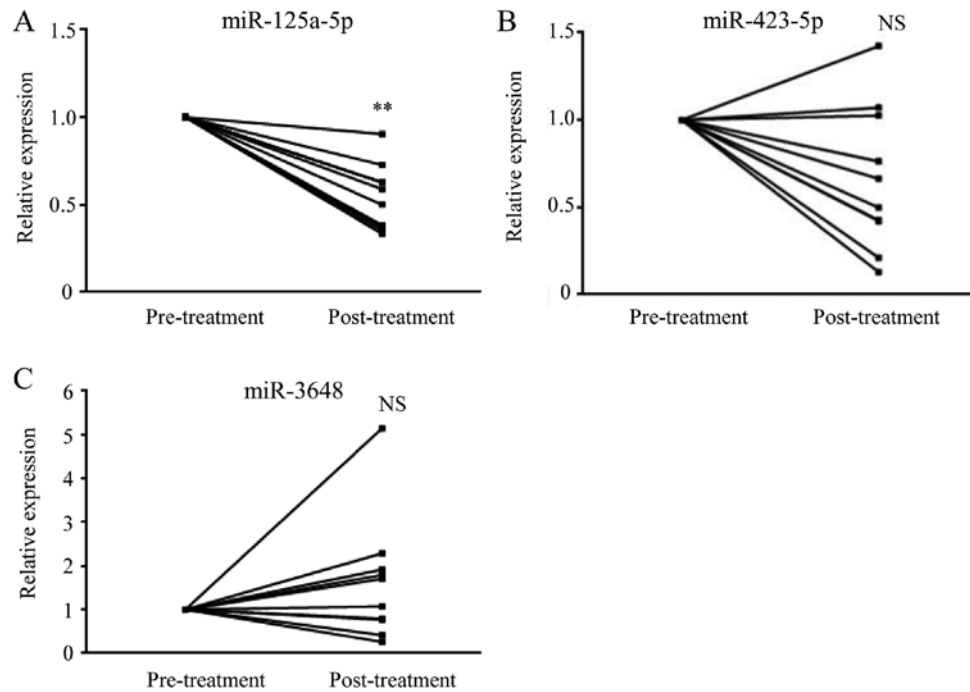

Figure 3. Relative expression levels in the serum of patients with hepatocellular carcinoma prior to and following treatment quantified by reverse transcription-quantitative polymerase chain reaction. (A) miR-125a-5p. (B) miR-423-5p. (C) miR-3648. miR-125a-5p was downregulated post-treatment in all 12 cases as compared to that in pre-treatment and the relative quantity value was 0.57 ± 0.27 (**$P<0.01$). For other miRNAs, no significant change was observed. The relative expression was determined by calculating $2^{-\Delta\Delta Cq}$, where $\Delta Cq=Cq_{target\ miRNA}-Cq_{cel-miR-39}$ and $\Delta\Delta Cq=\Delta Cq_{post-treatment\ sample}-\Delta Cq_{pre-treatment\ sample}$. miR, microRNA; NS, not significant.

to the following formula; $\Delta Cq=Cq_{target\ miRNA}-Cq_{cel-miR-39}$, and $\Delta\Delta Cq=\Delta Cq-mean\Delta Cq$ of control group patients.

Furthermore, the expression profile of each differentially-expressed miRNA was used to create receiver operator characteristic (ROC) curves. This method displays the discriminatory accuracy of the marker for distinguishing between the non-HCC (patients with CH and LC) and HCC (patients with early and advanced stage HCC) groups. Additionally, by using the ROC curve, the area under the curve (AUC) value and the optimal cutoff value were calculated.

Statistical analysis. All statistical analyses were performed using Prism software version 6.0 (Graph Pad Software, Inc., La Jolla, CA, USA). Normally distributed data were expressed as mean ± standard deviation. Skewed data were described by the median and range. The difference between normally distributed numeric variables was analyzed by the Student's t-test, while non-normally distributed variables were analyzed

by Mann-Whitney U test. When comparing multiple groups, one-way analysis of variance was conducted, followed by Dunnett post-hoc test. All P-values were two-sided, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

miRNA analysis pre- and post-curative treatment. To determine miRNA changes between pre- and post-curative treatment serums from the patients with early stage HCC, the present study exhaustively analyzed 2,555 miRNA molecules using a microarray. A total of 5 miRNAs were identified to be the most significantly changed molecules ($P<0.05$), including miR-125a-5p, miR-423-5p, miR-1247-3p, miR-1304-3p and miR-3648, all of which were downregulated (Table III).

The 5 miRNAs selected by exhaustive analysis were quantified by RT-qPCR. miR-125a-5p was downregulated post-treatment in all 12 cases (Fig. 3A) and the relative
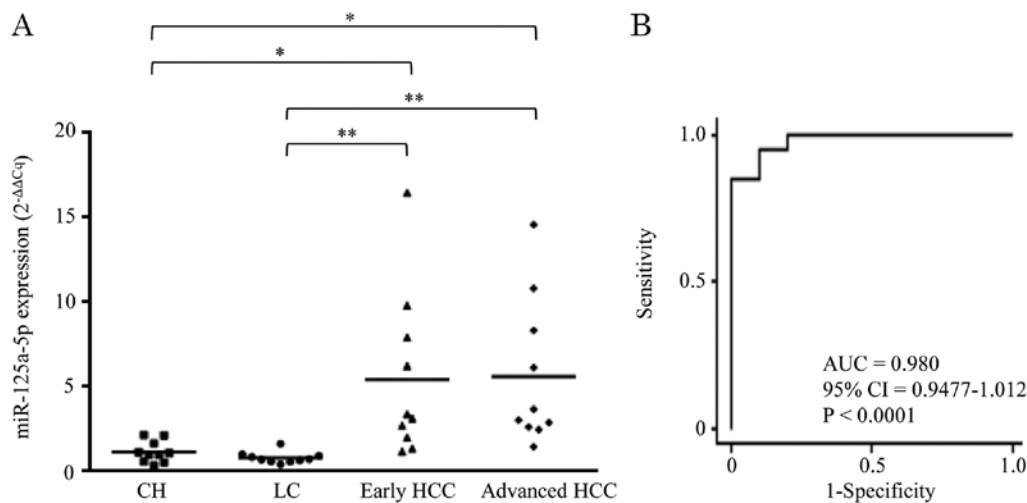
Figure 4. Expression of serum miR-125a-5p in patients with hepatitis C virus-associated liver diseases. (A) Relative expression levels. The expression levels of miR-125a-5p were significantly upregulated in patients with early and advanced stage HCC, compared with patients with CH or LC. There was no significant difference between patients with CH and LC. The relative expression levels of miR-125a-5p was determined by calculating $2^{-\Delta\Delta Cq}$, where $\Delta Cq=Cq_{miR-125a-5p}-Cq_{cel-miR-39}$, $\Delta\Delta Cq=\Delta Cq$-mean ΔCq of patients with CH. The horizontal lines represent the mean relative expression levels. $^{*}P<0.05$ and $^{**}P<0.01$. (B) Receiver operating characteristic curve analysis. The AUC was 0.980 for discriminating the HCC group from the non-HCC group. CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; AUC, area under the curve; CI, confidence interval; miR, microRNA.

quantity (RQ) value was 0.57±0.27 (P<0.01). miR-423-5p exhibited the opposite trend in expression in a few cases; however, no significant change was observed by RT-qPCR analysis (Fig. 3B), and the RQ value was 0.71±0.56 (not significant). For miR-3648, the measured values varied from case to case, and a certain trend could not be identified (Fig. 3C). miR-1247-3p and hsa-miR-1304-3p did not yield stable results in the method used and could not be detected in more than half of the cases.

Upregulation of miR-125a-5p in the serum of patients with HCC. In order to determine whether miR-125a-5p was differentially expressed in HCV-associated liver diseases, the serum levels of miR-125a-5p in patients with HCV-associated CH, LC and HCC were measured (Fig. 4A). The results demonstrated that the miR-125a-5p expression was significantly upregulated in patients with advanced stage HCC, compared with patients with CH, with a RQ value of 5.60±4.34 (P<0.05). Additionally, miR-125a-5p was also significantly upregulated in patients with early stage HCC when, compared with patients with CH, with a RQ value of 5.43±4.84 (P<0.05). There was no difference in miR-125a-5p expression between patients with LC and patients with CH, with a RQ value of 0.82±0.34 (not significant). Similar results were obtained in comparison with patients with LC, as the levels of miR-125a-5p expression were significantly upregulated in patients with advanced stage HCC, compared with patients with LC, with a RQ value of 7.36±5.69 (P<0.01). Additionally, miR-125a-5p was significantly upregulated in patients with early stage HCC, compared with patients with LC, with a RQ value of 7.11±6.35 (P<0.01).

Diagnostic value of miR-125a-5p in serum. In order to evaluate the diagnostic value of serum miRNA-125a-5p in discriminating the HCC group (patients with early and advanced stage HCC) from the non-HCC group (patients with CH and LC), the optimal cutoff value for miR-125a-5p was the ROC curve based on the RT-qPCR data. The AUC was 0.980 and the optimal cutoff value was 2.476, which demonstrated a sensitivity of 0.8 and a specificity of 1.0 (Fig. 4B).

Discussion

In the present study, serum samples obtained from pre- and post-treatment patients with HCC, miRNAs underwent a comprehensive examination and a number of miRNAs were selected as biomarker candidates for HCC. The present study demonstrated that serum miR-125a-5p levels are significantly reduced in post-treatment samples, and that the levels in patients in the early and advanced stages of HCC were significantly increased, compared with patients with HCV-associated chronic liver disease. These results indicated that miR-125a-5p has potential as a biomarker for early detection of HCV-associated HCC and evaluation following treatment. Additionally, among patients with early stage HCC, the elevation of miR-125a-5p level was observed in 4/5 cases, which were negative for tumor markers. These results indicated that serum miR-125a-5p is a valuable biomarker that could be in conjunction with conventional HCC tumor markers, including AFP and PIVKA-II. These events may reflect a longer increase in serum miR-125a-5p at cancer initiation rather than during progression.

Among the characteristic miRNAs contained in the serum of patients with cancer, circulating miR-21 plays an important role and has been reported to be associated with various carcinoma types, including colorectal (26), pancreatic (27), ovarian (28) and pharyngeal cancer (29). It was also reported that miR-21 is upregulated in the serum of patients with HCC (20,30). Furthermore, miR-718 has also been reported as a characteristic miRNA in the serum of patients with HCC (31); however, in the present study, comprehensive analysis revealed no significant change in miR-21 and miR-718. The discrepancy between the present data and previous reports may be

explained by the difference in the methods used, as in the present study, 2,555 miRNAs were comprehensively analyzed, which was a notably larger number of molecules, compared with the previous reports.

miR-125a is located at 19q13, and has been reported that miR-125a targeted genes that suppress and control cancer, including tumor protein P53 (32), cyclin dependent kinase inhibitor 1A (33), Erb-B2 receptor tyrosine kinase 2 (ERBB2) and ERBB3 (34). In HCC cell lines, studies also reported that miR-125a inhibits the migration and invasion via suppression of phosphoinositide 3-kinase/AKT/mechanistic target of rapamycin kinase signaling pathway (35), and miR-125a-5p inhibits cell proliferation by downregulation of ERBB3 (36). The results demonstrated that miR-125a-5p may serve a tumor-suppressive role in HCC carcinogenesis.

Previous studies also demonstrated that the expression of miR-125a-5p is downregulated in a number of human cancer types, including breast (37), ovarian (38), lung (39) and gastric cancer (40) tissues. miR-125a-5p is also downregulated in HCC tissues and may function as a tumor suppressor (41,42). However, the clinical significance of miR-125a-5p in serum of patients with HCC has yet to be completely elucidated. Additionally, it was previously unclear whether miRNA from cancer tissue are up- or downregulated in the serum of patients with HCC; however, the present study demonstrated that miR-125a-5p is upregulated in the serum of patients with HCC. Our hypothesis is that investigating the biological role of miR-125a-5p may be beneficial in understanding the pathology of HCC.

However, it should be considered that the present study examined the serum miR-125a-5p level in patients with chronic liver disease and only HCC caused by HCV infection. However, it has previously been reported that miR-125a-5p can also target a viral sequence and interfere with the expression of HBV surface antigen (43). Another independent study also reported that miR-125a-5p levels are correlated with HBV DNA concentrations in the liver and plasma, and that miR-125a-5p is upregulated in the patients with high viral load (44). As the level of miR-125a-5p may change due to the viral load and the degree of HBV-induced CH, the present study excluded the cases with persistent HBV infection. Therefore, the present study selected only the samples with HCV-associated HCC. Another limitation of the present study is that miR-125a-5p levels in healthy controls without liver diseases were not examined. Therefore, future investigations should examine whether the miR-125a-5p measurement is different in HBV-associated liver diseases, compared with healthy controls. However, in countries such as Japan, where the majority of HCC is caused by HCV infection, miR-125a-5p may be beneficial for diagnosis and follow-up following treatment.

In conclusion, miRNA expression profile in the serum of patients with HCV-associated HCC, and in particular, the serum miR-125a-5p levels changed pre- and post-treatment in patients with HCV-associated HCC. Irrespective of the clinical stage, the miR-125a-5p level was identified to be elevated in the serum of patients with HCC. Therefore, serum miR-125a-5p may serve as a noninvasive biomarker for the diagnosis of early carcinogenesis in HCV-associated chronic liver diseases.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from corresponding author on reasonable request.

Authors' contributions

KOu designed the study and wrote the manuscript. KOu, KF and AM carried out the major experiments. HI, MN, TT and TS analyzed and interpreted the data. TN, HY, SM, JT, HK, KOk, YS and TM designed the study and conducted the experiments. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Kagawa University Hospital (Kagawa, Japan) (Ethics approval: Heisei 22-063). Written informed consent was obtained from all participants.

Patient consent for publication

The patients provided written informed consent for the publication of any data.

Competing interests

The authors declare that they have no competing interests.

References

1. McGlynn KA and London WT: The global epidemiology of hepatocellular carcinoma: Present and future. Clin Liver Dis 15: 223-243, 2011.
2. Forner A, Llovet JM and Bruix J: Hepatocellular carcinoma. Lancet 379: 1245-1255, 2012.
3. El-Seraq HB: Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology 142: 1264-1273.e1, 2012.
4. Yoeman AD, Al-Chalabi T, Karani JB, Quaglia A, Devlin J, Mieli-Vergani G, Bomford A, O'Grady JG, Harrison PM and Heneghan MA: Evaluation of risk factors in the development of hepatocellular carcinoma in autoimmune hepatitis: Implications for follow-up and screening. Hepatology 48: 863-870, 2008.
5. Zhang W, He H, Zang M, Wu Q, Zhao H, Lu LL, Ma P, Zheng H, Wang N, Zhang Y, *et al*: Genetic features of aflatoxin-associated hepatocellular carcinoma. Gastroenterology 153: 249-262.e2, 2017.
6. Altekruse SF, Henley SJ, Cucinelli JE and McGlynn KA: Changing hepatocellular carcinoma incidence and liver cancer mortality rates in the United States. Am J Gastroenterol 109: 542-553, 2014.
7. Kim SR, Kudo M, Hino O, Han KH, Chung YH and Lee HS; Organizing Committee of Japan-Korea Liver Symposium: Epidemiology of hepatocellular carcinoma in Japan and Korea. A review. Oncology 75 (Suppl 1): S13-S16, 2008.

8. Murakami T, Kim T, Oi H, Nakamura H, Igurashi H, Matsushita M, Okamura J and Kozuka T: Detectability of hypervascular hepatocellular carcinoma by arterial phase images of MR and spiral CT. Acta Radiol 36: 372-376, 1995.

9. Park SJ, Jang JY, Jeong SW, Cho YK, Lee SH, Kim SG, Cha SW, Kim YS, Cho YD, Kim HS, *et al*: Usefulness of AFP, AFP-L3, and PIVKA-II, and their combinations in diagnosing hepatocellular carcinoma. Medicine (Baltimore) 96: e5811, 2017.

10. Tomimaru Y, Eguchi H, Nagano H, Wada H, Kobayashi S, Marubashi S, Tanemura M, Tomokuni A, Takemasa I, Umeshita K, *et al*: Circulating microRNA-21 as a novel biomarker for hepatocellular carcinoma. J Hepatol 56: 167-175, 2012.

11. Esquela-Kerscher A and Slack FJ: Oncomirs-microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269, 2006.

12. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.

13. Carleton M, Cleary MA and Linsley PS: MicroRNAs and cell cycle regulation. Cell Cycle 6: 2127-2132, 2007.

14. Harfe BD: MicroRNAs in vertebrate development. Curr Opin Genet Dev 15: 410-415, 2005.

15. Garzon R, Calin GA and Croce CM: MicroRNA in cancer. Annu Rev Med 60: 167-179, 2009.

16. Melo SA and Esteller M: Dysregulation of microRNAs in cancer: Playing with fire. FEBS Lett 585: 2087-2099, 2011.

17. Osaki M, Takeshita F and Ochiya T: MicroRNAs as biomarkers and therapeutic drugs in human cancer. Biomarkers 13: 658-670, 2008.

18. Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, Zanetti KA, Ye QH, Qin LX, Croce CM, *et al*: Identification of metastasis-related microRNAs in hepatocellular carcinoma. Hepatology 47: 897-907, 2008.

19. Morishita A, Iwama H, Fujihara S, Sakamoto T, Fujita K, Tani J, Miyoshi H, Yoneyama H, Himoto T and Masaki T: MicroRNA profiles in various hepatocellular carcinoma cell lines. Oncol Lett 12: 1687-1692, 2016.

20. Morishita A and Masaki T: MicroRNAs as possible biomarkers for hepatocellular carcinoma. Hepatol Res 48: 499-501, 2018.

21. Gibbings DJ, Ciaudo C, Erhardt M and Voinnet O: Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. Nat Cell Biol 11: 1143-1149, 2009.

22. Yin C, Zhou X, Yan J and Zhang G: Potential role of circulating miR-21 in the diagnosis and prognosis of digestive system cancer: A systematic review and meta-analysis. Medicine (Baltimore) 94: e2123, 2015.

23. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, *et al*: Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA 105: 10513-10518, 2008.

24. Liver Cancer Study Group of Japan. In: General rules for the clinical and pathological study of primary liver cancer, 3rd English edition. Tokyo, Kanehara, 2010.

25. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Method 25: 402-408, 2001.

26. Ogata-Kagata H, Izumiya M, Kurioka D, Honma Y, Yamada Y, Furuta K, Gunji T, Ohta H, Okamoto H, Soneda H, *et al*: Circulating exosomal microRNAs as biomarkers of colon cancer. PLoS One 9: e92921, 2014.

27. Que R, Ding G, Chen J and Cao L: Analysis of serum exosomal microRNAs and clinicopathologic features of patients with pancreatic adenocarcinoma. World J Surg Oncol 11: 219, 2013.

28. Taylor DD and Gercel-Talor C: MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 110: 13-21, 2008.

29. Wang J, Zhou Y, Lu J, Sun Y, Xiao H, Liu M and Taian L: Combined detection of serum exosomal miR-21 and HOTAIR as diagnostic and prognostic biomarkers for laryngeal squamous cell carcinoma. Med Oncol 31: 148, 2014.

30. Wang H, Hou L, Li A, Duan Y, Gao H and Song X: Expression of serum exosomal microRNA-21 in human hepatocellular carcinoma. Biomed Res Int 2014: 864894, 2014.

31. Sugimachi K, Matsumura T, Hirata H, Uchi R, Ueda M, Ueo H, Shinden Y, Iguchi H, Eguchi H, Shirabe K, *et al*: Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation. Br J Cancer 112: 532-538, 2015.

32. Zhang Y, Gao JS, Tang X, Tucker LD, Quesenberry P, Rigoutsos I and Ramratnam B: MicroRNA 125a and its regulation of the p53 tumor suppressor gene. FEBS Lett 583: 3725-3730, 2009.

33. Wu S, Huang S, Ding J, Zhao Y, Liang L, Liu T, Zhan R and He X: Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3' untranslated region. Oncogene 29: 2302-2308, 2010.

34. Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS and Benz CC: Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. J Biol Chem 282: 1479-1486, 2007.

35. Tang H, Li RP, Liang P, Zhou YL and Wang GW: miR-125a inhibits the migration and invasion of liver cancer cells via suppression of the PI3K/AKT/mTOR signaling pathway. Oncol Lett 10: 681-686, 2015.

36. Li G, Zhang W, Gong L and Huang X: MicroRNA-125a-5p inhibits cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma by downregulation of ErbB3. Oncol Res 27: 449-458, 2019.

37. O'Day E and Lal A: MicroRNAs and their target gene in breast cancer. Breast Cancer Res 12: 201, 2010.

38. Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, Kim JW and Kim S: MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res 14: 2690-2695, 2008.

39. Wang G, Mao W, Zheng S and Ye J: Epidermal growth factor receptor-regulated miR-125a-5p-a metastatic inhibitor of lung cancer. FEBS J 276: 5571-5578, 2009.

40. Nishida N, Mimori K, Fabbri M, Yokobori T, Sudo T, Tanaka F, Shibata K, Ishii H, Doki Y and Mori M: MicroRNA-125a-5p is an independent prognostic factor in gastric cancer and inhibits the proliferation of human gastric cancer cells in combination with Trastuzumab. Clin Cancer Res 17: 2725-2733, 2011.

41. Li W, Xie L, He X, Li J, Tu K, Wei L, Wu J, Guo Y, Ma X, Zhang P, *et al*: Diagnostic and prognostic implications of microRNAs in human hepatocellular carcinoma. Int J Cancer 123: 1616-1622, 2008.

42. Bi Q, Tang S, Xia L, Du R, Fan R, Gao L, Jin J, Liang S, Chen Z, Xu G, *et al*: Ectopic expression of miR-125a inhibits the proliferation and metastasis of hepatocellular carcinoma by targeting MMP11 and VEGF. PLoS One 7: e40169, 2012.

43. Potenza N, Papa U, Mosca N, Zerbini F, Nobile V and Rosso A: Human microRNA hsa-miR-125a-5p interferes with expression of hepatitis B virus surface antigen. Nucleic Acid Res 39: 5157-5163, 2011.

44. Coppola N, Potenza N, Pisaturo M, Mosca N, Tonziello G, Signoriello G, Messina V, Sagnelli C, Russo A and Sagnelli E: Liver microRNA hsa-miR-125a-5p in HBV chronic infection: Correlation with HBV replication and disease progression. PLoS One 8: e65336, 2013.