Relevance of MicroRNA-18a and MicroRNA-199a-5p to Hepatocellular Carcinoma Recurrence After Living Donor Liver Transplantation

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There are few reports about recurrence-related microRNAs (miRNAs) after liver transplantation (LT) for hepatocellular carcinoma (HCC). The purpose of this study was to identify novel recurrence-related miRNAs after living donor liver transplantation (LDLT) for HCC. First, we performed microarray analyses of samples from a liver with primary HCC, a liver that was noncancerous, and a liver that had recurrence-metastasis from 3 patients with posttransplant recurrence. Then we selected miRNAs with consistently altered expression in both primary HCC and recurrence as potential candidates of recurrence-related miRNAs. Expression of the miRNAs in HCC and noncancerous livers was assessed in 70 HCC patients who underwent LDLT. The target genes regulated by the recurrence-related miRNAs were identified. MicroRNA-18a (miR-18a) expression was increased, and microRNA-199a-5p (miR-199a-5p) expression was decreased in both primary HCC and recurrence. Increased miR-18a expression correlated with high levels of tumor markers, large tumor size, and a high recurrence rate. Decreased miR-199a-5p expression correlated with high levels of tumor markers, portal venous invasion, and a high recurrence rate. In HCC cells, miR-18a regulated the expression of tumor necrosis factor alpha–induced protein 3 (TNFAIP3), and miR-199a-5p regulated the expression of hypoxia-inducible factor 1 alpha (HIF1A), vascular endothelial growth factor A (VEGFA), insulin-like growth factor 1 receptor, and insulin-like growth factor 2. In conclusion, increased miR-18a levels and decreased miR-199a-5p levels are relevant to HCC recurrence after LDLT. MiR-18a and miR-199a-5p could be novel therapeutic targets of recurrent HCC after LDLT.

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Abbreviations: AFP, alpha-fetoprotein; DCP, des-gamma-carboxy-prothrombin; DDLT, deceased donor liver transplantation; HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIF1A, hypoxia-inducible factor 1 alpha; IGF1R, insulin-like growth factor 1 receptor; IGF2, insulin-like growth factor 2; LDLT, living donor liver transplantation; LT, liver transplantation; M, recurrence-metastasis; miR-18a, microRNA-18a; miR-199a-5p, microRNA-199a-5p; miRNA, microRNA; mRNA, messenger RNA; miR-NC, microRNA inhibitor negative control; N, noncancerous liver; NF-κB, nuclear factor kappa B; NL, normal liver; nt, nucleotide; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; T, primary hepatocellular carcinoma; TACE, transarterial chemoembolization; TNFAIP3, tumor necrosis factor alpha–induced protein 3; UTR, untranslated region; VEGFA, vascular endothelial growth factor A; vp, histopathological portal venous invasion; vv, histopathological venous invasion.

Hepatocellular carcinoma (HCC) is the most frequent cancer of the liver and the third most common cause of cancer deaths worldwide.[1] Curative treatment is limited to hepatic resection and liver transplantation (LT). However, the overall cumulative recurrence rate after hepatic resection is approximately 80% at 5 years.[2] Among various recurrent patterns of HCC, the prognosis of extrahepatic recurrence-metastasis (M) is much poorer than that of intrahepatic metastasis and multicentric recurrence.[3-5] LT can overcome the problems of intrahepatic metastasis and multicentric recurrence theoretically. However, the recurrence of HCC after LT is a critical problem to be solved because the outcomes of patients with HCC recurrence after LT are extremely poor.[6]

MicroRNAs (miRNAs) are small noncoding RNAs, approximately 21-22 nucleotides (nts) in length, that regulate gene expression by forming partial or completely complementary heteroduplexes with the 3′ untranslated region (UTR) of target messenger RNAs.[7-9] MiRNAs are implicated in many biological processes and diverse diseases, such as viral infections and cancers.[10-13] Recently, metastasis-related miRNAs in HCC were analyzed by comparing primary hepatocellular carcinoma (T) and noncancerous liver (N)[14] or T, adjacent venous tumor thrombus, and N.[15] In HCC, cancer metastasis is very complex with multiple steps, including local invasion, intravasation, survival in the circulation, extravasation, and colonization, which is accompanied by the accumulation of genetic and epigenetic alterations.[16] Furthermore, metastatic clones are derived from only a few clones in the genetically heterogeneous primary cancer.[17,18] Therefore, an analysis of miRNA expression in metastatic foci would be thought-provoking. We considered the miRNAs with consistently altered expression in both T and M as promising and reliable candidates of recurrence-related miRNAs after living donor liver transplantation (LDLT).

There are few reports about recurrence-related miRNAs after LT for HCC.[19,20] In the present study, first, we focused on miRNA expression in recurrent foci of 3 patients with recurrent HCC after LDLT. Then, we selected miRNAs with consistently altered expression in both T and M as promising and reliable candidates of recurrence-related miRNAs after LDLT. Finally, we identified novel recurrence-related miRNAs after LDLT for HCC and their novel target genes.

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Patients and Methods

PATIENT CHARACTERISTICS AND HUMAN TISSUE SAMPLES

Liver specimens were obtained from 70 patients who had undergone LDLT for HCC at the Department of Surgery and Science, Kyushu University Hospital between December 1999 and May 2007. This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The study protocol was approved by the institutional review board, and informed consent was obtained from each patient. Cancerous and noncancerous liver specimens from the explanted liver were snap-frozen in liquid nitrogen and stored at −80 °C. Samples from living liver donors were obtained during intraoperative biopsy. In the patients with multiple HCCs, the tissue sample from the largest HCC in each patient was assayed. All patients were under immunosuppression after LT according to our usual protocols.[21]

RNA ISOLATION

Total RNA containing miRNA was isolated from the frozen liver tissues as previously described.[10]

MicroRNA MICROARRAY ANALYSIS

In our institute, surgical resection for recurrent HCC after LDLT is indicated if possible.[21] The RNA samples from 3 patients who underwent resection of recurrences after LDLT were made available for miRNA microarray analysis. The 3 patients included a man (patient 1) with peritoneal recurrence and infected by hepatitis B virus (HBV), and a woman (patient 2) and a man (patient 3) with lung recurrences and hepatitis C virus (HCV) infection. Microarray analysis was performed for each RNA sample from T, N in the explanted liver, and M. A sample containing equal amounts of RNAs from histologically normal livers (NLs) of 3 living donors was analyzed as a control. Microarray analysis was performed by EXIQON (Copenhagen, Denmark) via B-bridge (Tokyo, Japan) using an miRCURY LNA™ microRNA Array, version 10.0, which represented all human miRNAs known when this study was conducted (miRBase 11.0). Each sample was labeled with Hy3™ and hybridized with a reference sample labeled with Hy5™ using a

miRCURY LNA Array Power labeling kit (EXIQON). A reference sample contained equal amounts of RNAs from all 10 samples. MiRNAs with consistently altered expression were selected as potential candidates of recurrence-related miRNAs after LDLT according to the following changes in miRNA signals: T/N > 2 and M/N > 2 in all 3 patients; T/NL > 2 and M/NL > 2 in all 3 patients; T/N < 0.5 and M/N < 0.5 in all 3 patients; T/NL < 0.5 and M/NL < 0.5 in all 3 patients. Unsupervised hierarchical cluster analyses were performed. The microarray data have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus repository under the accession number GSE41874.

QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (QRT-PCR) FOR miRNA EXPRESSION

The expression of miRNAs was quantified by qRT-PCR as previously described.[10] The expression levels of microRNA-18a (miR-18a) and microRNA-199a-5p (miR-199a-5p) were quantified by the relative standard curve method using a control sample (histopathologically NL sample from a patient with a metastatic liver tumor) and normalized according to the level of expression of RNU6B.

PREDICTION OF TARGET GENES OF miRNAS

The prediction of target genes was performed using the algorithms of TargetScan,[22] miRanda,[23] and miRBase.[24]

CELL CULTURE

Human HCC cell lines, PLC/PRF/5, Hep3B, and Huh7 (Riken Cell Bank, Tsukuba, Japan) were cultured as previously described.[25,26]

CELL TRANSFECTION WITH miRNA INHIBITORS OR PRECURSORS

Anti-miR-18a inhibitor, pre-miR-199a-5p precursor, anti–microRNA inhibitor negative control (miR-NC), and pre-miR-NC (Ambion, Austin, TX) were used to transfect PLC/PRF/5 cells using siPORT NeoFX transfection agent (Ambion) in accordance with the manufacturer's protocol.

LUCIFERASE REPORTER ASSAYS

The target sites within the 3′ UTRs of tumor necrosis factor alpha–induced protein 3 (TNFAIP3), hypoxia-inducible factor 1 alpha (HIF1A), vascular endothelial growth factor A (VEGFA), insulin-like growth factor 1 receptor (IGF1R), and insulin-like growth factor 2 (IGF2) messenger RNAs (mRNAs) were each cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI). Each cloned pmirGLO vector and anti-miR-18a or pre-miR-199a-5p were cotransfected into PLC/PRF/5 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Luciferase signals were measured and expressed as a Firefly signal/Renilla signal using the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer's protocol.

WESTERN BLOT ANALYSES

Western blot analyses were performed 24 hours after transfection of the HCC cells with anti-miR-18a or pre-miR-199a-5p as described.[25,26] TNFAIP3 expression was assayed in PLC/PRF/5 cells and that of HIF1A and VEGFA in Huh7 cells 6 hours after culture under hypoxic conditions (1% oxygen) 24 hours after transfection. IGF1R and IGF2 expression was assayed in Hep3B cells. The primary antibodies for TNFAIP3 (#ab45366), IGF1R (#ab39675), IGF2 (#ab9574; Abcam, Cambridge, MA), HIF1A (#NB100-105; Novus Biologicals, Littleton, CO), and VEGFA (#sc-152; Santa Cruz Biotechnology Inc., Santa Cruz, CA) were used according to the manufacturer's protocol.

CELL PROLIFERATION ASSAYS

PLC/PRF/5 cells were plated at a density of 1.0×10^5 cells/well in three 6-cm plates 24 hours after transfection of anti-miR-18a or pre-miR-199a-5p and were harvested and counted on days 1, 4, and 7. The medium was changed every 72 hours. This experiment was repeated 3 times.

CELL INVASION ASSAYS

PLC/PRF/5 cells were plated at a density of 1.0×10^4 cells/well in the upper chamber of 24-well Transwell culture chambers coated with Matrigel (Becton Dickinson, Franklin Lakes, NJ) 24 hours after transfection of anti-miR-18a or pre-miR-199a-5p. Invasion assays were performed as previously described.[25,26]

STATISTICAL ANALYSES

The Fisher's exact test was used for analysis of categorical data; the independent *t* test was used for continuous parametric data; and the Mann-Whitney U test was used for continuous nonparametric data. Survival data were computed using the Kaplan-Meier method and compared between groups using the log-rank test. Multivariate analysis was performed using Cox's proportional hazard regression model to evaluate the independent factors predictive of recurrence. Multivariate analysis included the clinicopathological factors as follows: age, male sex, serum alpha-fetoprotein (AFP) > 300 ng/mL, serum des-gamma-carboxyprothrombin (DCP) > 300 mAU/mL, poorly differentiated HCC, positive for histopathological portal venous invasion (vp), positive for histopathological venous invasion (vv), beyond Milan criteria,[27] and high miR-18a and low miR-199a-5p expression groups. Kendall's rank correlation was used to determine the correlation between miR-18a expression and inflammatory activity. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed using StatView 5.0 software (Abacus Concepts, Berkeley, CA) and JMP 11.0 software (SAS Institute Inc., Cary, NC).

Results

RECURRENCE SITES AND MEDIAN TIME TO RECURRENCE

As shown in Table 1, 14 patients developed HCC recurrence. The most common sites of recurrence were lung, bone, lymph nodes, and the transplanted liver. The median time to recurrence was 0.715 years (range, 0.15-4.95 years). None of the recurrent patients had recurrent liver cirrhosis. In our institute, we experienced only 1 case of de novo HCC.[28] The de novo case was not included in the present study.

MICROARRAY ANALYSIS OF 3 RECURRENT PATIENTS AFTER LDLT FOR HCC

At the first step, microarray analysis was performed for each RNA sample from T, N, and M of 3 patients (patients 1-3) and control NL. Unsupervised hierarchical clustering analysis successfully segregated T, N, NL, and M. Interestingly, the profile of recurrence was closely related to that of noncancerous liver and control NL rather than that of HCC (Fig. 1A). MiRNAs with consistently altered expression were selected according to the criteria described in the Patients and Methods. The pattern of expression of miR-18a was T/NL > 2 and M/NL > 2 and that of miR-199a-5p was T/N < 0.5 and M/N < 0.5 (Fig. 1B). Additionally, the pattern of expression of miR-768-5p was T/NL < 0.5 and M/NL < 0.5 and that of miR-199a-3p/3b was T/N < 0.5 and M/N < 0.5. There were no miRNAs with the pattern of T/N > 2 and M/N > 2. There were few reports on the relevance between miR-768-5p and cancer. Few potential target gene candidates of miR-199a-3p/3b were found using the algorithms of TargetScan,[22] miRanda,[23] and miRBase.[24] Therefore, miR-18a and miR-199a-5p were selected for further analyses.

ANALYSIS OF miR-18A AND miR-199A-5P EXPRESSION IN HCC AND NONCANCEROUS LIVERS IN 70 LDLT PATIENTS

The miR-18a expression in samples of noncancerous liver and HCC was 1.12 ± 0.74 and 1.63 ± 1.28, respectively ($P < 0.001$). The mean ratio of the miR-18a expression in HCC to the miR-18a expression in the paired noncancerous liver (T/N) was 1.80. The miR-199a-5p expression in samples of noncancerous liver and HCC was 3.42 ± 2.49 and 1.00 ± 1.28, respectively ($P < 0.001$). The mean ratio of the miR-199a-5p expression in HCC to the miR-199a-5p expression in the paired noncancerous liver (T/N) was 0.31 (Fig. 1C).

ASSOCIATION BETWEEN THE miR-18A AND miR-199A-5P EXPRESSION AND CLINICOPATHOLOGICAL FACTORS

The 70 patients were grouped into miR-18a high/low and miR-199a-5p high/low expression groups according to each miRNA expression ratio (T/N). The cutoff value was each mean expression ratio (miR-18a, 1.80; miR-199a-5p, 0.31), as described above. The clinicopathological factors with respect to the miR-18a high/low or miR-199a-5p high/low groups are shown in Tables 2 and 3. Serum AFP and DCP levels in the miR-18a high expression group were significantly higher than those in the miR-18a low expression group ($P < 0.001$). The maximum tumor size in the miR-18a high expression group was significantly larger than that of the miR-18a low expression group ($P = 0.04$). The serum DCP level in the miR-199a-5p low expression group was significantly higher than that of the miR-

TABLE 1. Characteristics of the Patients Who Had HCC Recurrence After LDLT

Patients	Age at LDLT, years	Sex	Time to Recurrence, years	Recurrence Sites
1	49	Male	0.27	Lung
2	60	Male	0.41	Lung, bone, kidney
3	57	Male	0.52	Liver, peritoneum
4	48	Female	1.35	Lung
5	56	Male	4.95	Lymph nodes
6	62	Male	0.20	Lung, bone
7	61	Male	0.92	Lymph nodes
8	58	Female	1.13	Lymph nodes
9	55	Female	2.09	Lung, bone, liver
10	51	Male	0.60	Lung, bone
11	65	Male	0.83	Peritoneum
12	47	Female	0.15	Lung, bone, brain
13	60	Male	0.30	Lung, liver
14	59	Female	1.50	Lung

199a-5p high expression group ($P = 0.006$). Portal venous invasion in the miR-199a-5p low expression group was significantly more frequent than in the miR-199a-5p high expression group ($P = 0.04$).

RELEVANCE OF miR-18A AND miR-199A-5P EXPRESSION LEVELS TO HCC RECURRENCE AFTER LDLT

Five-year recurrence-free survival rates were 65.0% versus 82.7% (miR-18a high versus low; $P = 0.02$; Fig. 2A) and 72.0% versus 85.1% (miR-199a-5p low versus high; $P = 0.049$; Fig. 2B). Furthermore, the 70 patients were grouped into 4 groups by combining the expression levels of miR-18a and miR-199a-5p as follows:

1. MiR-18a high/miR-199a-5p high
2. MiR-18a low/miR-199a-5p high
3. MiR-18a high/miR-199a-5p low
4. MiR-18a low/miR-199a-5p low

The recurrence-free survival rate of the miR-18a high/miR-199a-5p low expression group was the lowest in the 4 groups ($P = 0.007$; Fig. 2C). Multivariate analysis revealed that lesions beyond Milan criteria (odds ratio, 15.60; $P = 0.02$) and miR-18a high/miR-199a-5p low expression group (odds ratio, 4.23; $P = 0.04$) were independent factors for HCC recurrence (Table 4). In the present study, only 1 patient had HCC recurrence within Milan criteria. Therefore, recurrence-free survival analysis was added for the patients beyond Milan criteria. The number of patients with miR-18a high/miR-199-5p high was small (n = 1), the other 3 groups were clearly stratified ($P = 0.004$; Fig. 2D).

IDENTIFICATION OF TARGET GENES REGULATED BY miR-18A AND miR-199A-5P

To investigate the signaling pathways regulated by miR-18a and miR-199a-5p in HCC, target genes were predicted using algorithms such as TargetScan,[22] miRanda[23] and miRBase.[24] As the target gene of miR-18a, cancer-suppressive genes were explored. On the other hand, as the target gene of miR-199a-5p, cancer-progressive genes were explored. TNFAIP3 was predicted as the target of miR-18a, and HIF1A, VEGFA, IGF1R, and IGF2 were predicted as the targets of miR-199a-5p. The target sites (nt numbers) in the 3′ UTR sequences of each were as follows: TNFAIP3, 674-680; HIF1A, 31-37; VEGFA, 467-473; IGF1R, 2701-2707; and IGF2, 1987-1993 (Fig. 3A, bold sequences).

Anti-miR-18a inhibitor and pre-miR-199a-5p precursor were transfected with luciferase reporter expression constructs containing the 3′ UTR target sequences of each of the genes described above. Anti-miR-18a inhibitor significantly augmented the luciferase signal of the vector containing the target site of TNFAIP3 ($P = 0.02$; Fig. 3B). In contrast, pre-miR-199a-5p precursor significantly suppressed the luciferase signals of the vectors including the target sites in HIF1A, VEGFA, IGF1R, and IGF2 mRNAs ($P = 0.04$, $P < 0.001$, $P < 0.001$, and $P = 0.005$; Fig. 3C). Similarly, Western blot analyses revealed that anti-miR-18a augmented TNFAIP3 expression in PLC/PRF/5 cells. Pre-miR-199a-5p suppressed the expression of HIF1A and VEGFA in Huh7 cells, the expression of IGF1R and IGF2 in Hep3B cells, respectively (Fig. 4A).

EFFECTS OF miR-18A AND miR-199A-5P ON THE PROLIFERATION AND INVASIVENESS OF THE PLC/PRF/5 CELL LINE

Cell proliferation assay revealed that the anti-miR-18a inhibitor significantly suppressed the proliferation of PLC/PRF/5 cells to a greater extent than anti-miR-NC ($P = 0.049$). The pre-miR-199a-5p precursor also

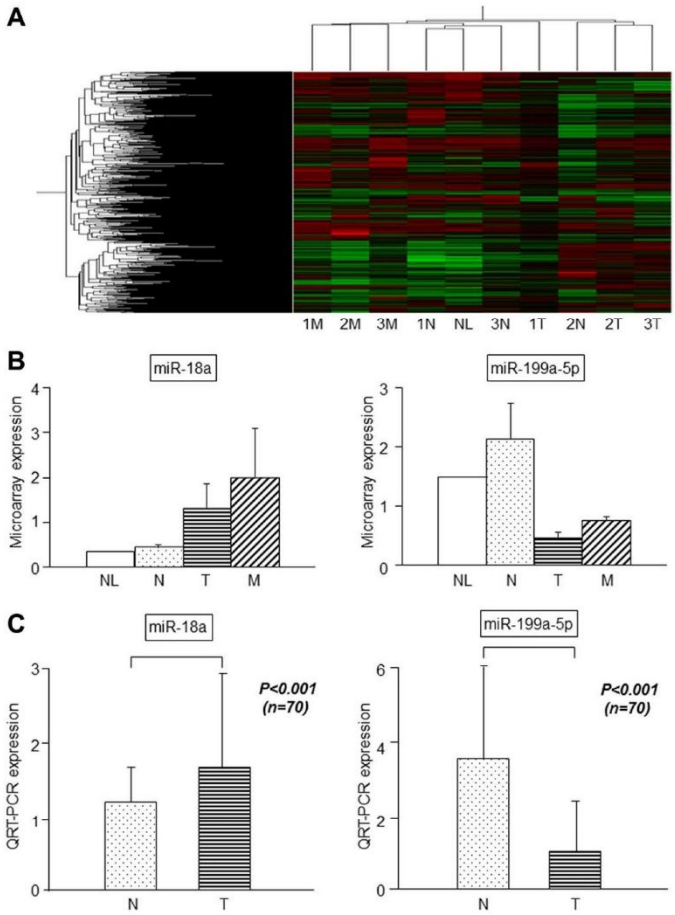

FIG. 1. (A) Microarray analysis of T, N, and M of the 3 patients (patients 1-3) and control NL. (B) MiR-18a and miR-199a-5p expression in microarray analysis. (C) Analysis of miR-18a and miR-199a-5p expression in HCC and noncancerous liver using qRT-PCR for 70 patients.

significantly suppressed the proliferation of the PLC/PRF/5 cells more than pre-miR-NC ($P = 0.049$; Fig. 4B). Furthermore, the results of the invasion assay revealed that anti-miR-18a suppressed invasion of PLC/PRF/5 cells to a greater extent than anti-miR-NC ($P = 0.049$). Expression of pre-miR-199a-5p suppressed invasion of PLC/PRF/5 cells to a greater extent than pre-miR-NC ($P = 0.049$; Fig. 4C).

ASSOCIATION BETWEEN miR-18A EXPRESSION AND HISTOPATHOLOGICAL INFLAMMATION ACTIVITY GRADE

TNFAIP3 was identified as the target of miR-18a. Then we investigated the association between miR-18a expression and histopathological inflammation

TABLE 2. Clinical and Histopathological Characteristics of the Patients According to MiR-18a Expression Levels

Factor	MiR-18a Low Group (n = 46)	MiR-18a High Group (n = 24)	*P* Value
Age, years	58.0 ± 5.7	57.0 ± 6.5	0.53
Sex, male/female	29/17	15/9	0.96
Viral infection status			
HBsAg, −/+	39/7	20/4	0.87
HCV antibody, −/+	11/35	7/17	0.63
Milan criteria, within/beyond	23/23	11/13	0.74
Tumor marker			
AFP, log ng/mL	1.32 ± 0.80	2.20 ± 1.24	<0.001
DCP, log mAU/mL	1.78 ± 0.70	2.49 ± 0.83	<0.001
Histopathological HCC			
Tumor number	19.8 ± 68.5	25.5 ± 42.5	0.72
Maximum tumor size, cm	2.60 ± 1.19	3.33 ± 1.69	0.04
Differentiation			0.12
Well	3	0	
Moderate	30	12	
Poor	13	12	
Vp, −/+	28/18	9/15	0.06
Vv, −/+	37/9	20/4	0.77

TABLE 3. Clinical and Histopathological Characteristics of the Patients According to MiR-199a-5p Expression Levels

Factor	MiR-199a-5p High Group (n = 28)	MiR-199a-5p Low Group (n = 42)	*P* Value
Age, years	57.8 ± 5.9	57.5 ± 6.1	0.89
Sex, male/female	16/12	28/14	0.42
Viral infection status			
HBsAg, −/+	22/6	37/5	0.28
HCV antibody, −/+	10/18	8/34	0.12
Milan criteria, within/beyond	15/13	19/23	0.49
Tumor marker			
AFP, log ng/mL	1.33 ± 0.76	1.82 ± 1.18	0.05
DCP, log mAU/mL	1.70 ± 0.63	2.24 ± 0.87	0.006
Histopathological HCC			
Tumor number	8.9 ± 15.0	30.5 ± 77.1	0.15
Maximum tumor size, cm	2.56 ± 1.04	3.04 ± 1.60	0.17
Differentiation			0.97
Well	1	2	
Moderate	17	25	
Poor	10	15	
Vp, −/+	19/9	18/24	0.04
Vv, −/+	24/4	33/9	0.45

activity grade in the explanted liver. There was no significant correlation between miR-18a expression in N and the inflammation activity grade. However, miR-18a expression ratio (T/N) was significantly positively correlated with the inflammation activity grade ($P = 0.043$; $\tau = 0.16$; Fig. 5).

Discussion

In the present study, we identified novel recurrence-related miRNAs after LDLT for HCC. We revealed that increased miR-18a levels and decreased miR-199a-5p levels are relevant to HCC recurrence after LDLT. Moreover, we identified novel target genes regulated by these miRNAs.

Published studies on miRNAs and metastatic HCC are mainly based on the comparison between T and N and lack analysis of the metastatic foci. At the first step, we focused on miRNA expression in the recurrent foci (M) in 3 recurrent patients after LDLT for HCC. Then we selected miR-18a and miR-199a-5p with consistently altered expression for both T and M as promising and reliable candidates of recurrence-related miRNAs.

At the second step, miR-18a and miR-199a-5p expression was assessed in HCC and N in 70 patients. Increased expression of miR-18a and decreased expression of miR-199a-5p, respectively, were associated with high levels of tumor markers, large tumor size, portal venous invasion, and HCC recurrence after LDLT. MiR-18a is present in the plasma of patients with pancreatic cancer and could be a novel tumor marker.[29] Thus, increased plasma miR-18a might also serve as a novel marker for HCC recurrence after LDLT. In the present study, all patients were under immunosuppression after LT. The immunosuppression might have some effects on prognosis[21] and miRNA expression.

Our present study revealed that miR-18a regulates the levels of TNFAIP3 in HCC, which is known to be decreased in HCC and associated with early intrahepatic recurrence after hepatic resection.[30] TNFAIP3 negatively regulates nuclear factor kappa B (NF-κB) activation, and decreased TNFAIP3 expression is involved in the progression of B cell lymphomas and glioblastomas.[31,32] MiR-18a is also involved in interleukin 6/signal transducer and activator of transcription 3 signaling pathway that mediates inflammation in human hepatocytes.[33] In contrast, it is well known that HCC is closely associated with inflammation, such as that induced by viral hepatitis, alcoholic, or nonalcoholic steatohepatitis.[34] In the present study, there was no significant correlation between miR-18a expression in the noncancerous liver and the inflammation activity grade. However, there was a positive correlation between miR-18a expression ratio (T/N) and inflammation activity grade. MiR-18a is induced by tumor necrosis factor alpha and is part of a positive feedback loop in the NF-κB signaling pathway in rheumatoid arthritis synovial fibroblasts.[35] Increased levels of expression of miR-18a in HCC may reflect

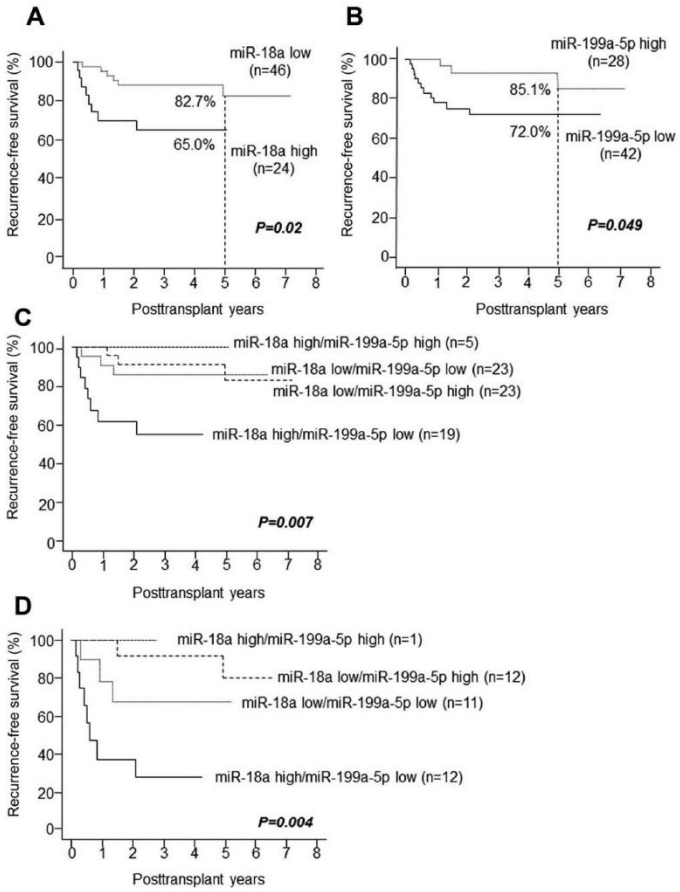

FIG. 2. Comparison of recurrence-free survival (A) in the miR-18a high/low expression groups, (B) in the miR-199a-5p high/low expression groups, (C) in the miR-18a high/low and miR-199a-5p high/low expression groups, and (D) in the miR-18a high/low and miR-199a-5p high/low expression groups beyond Milan criteria.

the association between HCC and hepatic inflammation. Furthermore, the inflammation-induced hepatocarcinogenesis may be the trigger of a positive feedback loop in the miR-18a and NF-κB signaling pathway.

Furthermore, we revealed that miR-199a-5p regulates HIF1A, VEGFA, IGF1R, and IGF2. HIF1A and VEGFA are generally overexpressed in HCC and required for tumor survival under hypoxic conditions.[36] VEGF expression levels correlate with clinicopathological factors, such as proliferation, vascular invasion, and tumor multiplicity.[37] Increased expression of HIF1A and VEGFA is associated with poor prognosis.[38,39] IGF1R and IGF2 are known to be involved in cancer progression.[40–42] Therefore, our present findings indicated that miR-199a-5p can negatively regulate these very important molecular targets in HCC. In the present study, 39 of 70 (55.7%) patients underwent transarterial chemoembolization (TACE) prior to LDLT. TACE could increase the expression of HIF1A and VEGF.[43] Therefore, the

TABLE 4. Multivariate Analysis of Recurrence

Factors	Odds Ratio (95% Confidential Interval)	*P* Value
Age	0.94 (0.85-1.04)	0.24
Sex, male	0.58 (0.14-2.37)	0.45
AFP > 300 ng/mL	0.69 (0.15-3.26)	0.64
DCP > 300 ng/mL	5.31 (0.52-54.30)	0.16
Poorly differentiated	3.21 (0.76-13.62)	0.11
Positive for vp	0.63 (0.06-6.19)	0.69
Positive for vv	2.19 (0.49-9.69)	0.30
Beyond Milan criteria	15.60 (1.56-155.91)	0.02
MiR-18a high and miR-199a-5p low	4.23 (1.04-17.28)	0.04

previous TACE might affect the expression of these genes in the present study.

There are several reports comparing LDLT with deceased donor liver transplantation (DDLT) for HCC.[44-46] These previous reports revealed that there was no significant difference in the recurrence-free survival between LDLT and DDLT. We previously reported that the longterm outcomes after LDLT for HCC were similar between left lobe graft recipients and right lobe graft recipients.[47] These findings might suggest that liver regeneration does not affect

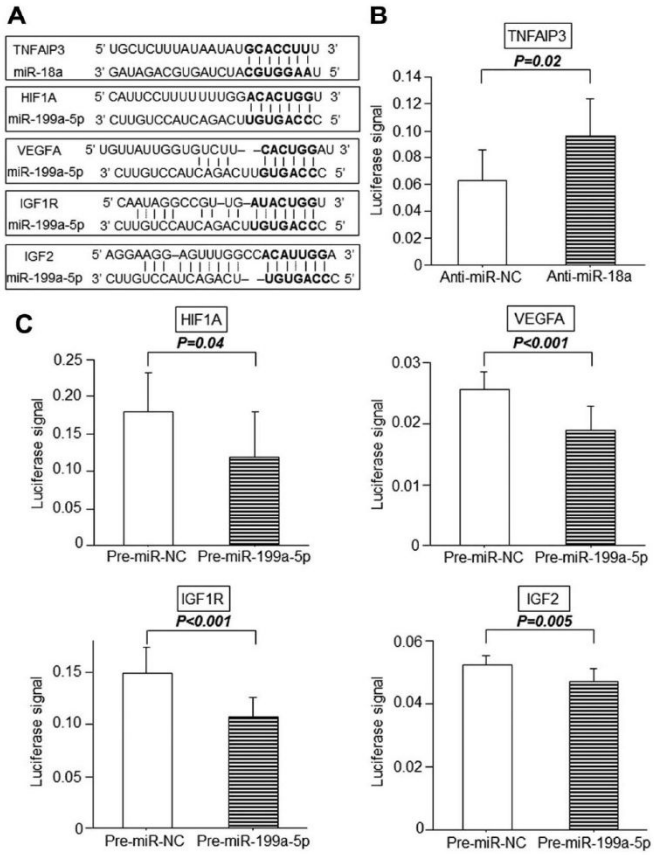

FIG. 3. (A) Target genes were predicted with the target site (bold sequence) in the 3′ UTR using prediction algorithms. (B) The effect of anti-miR-18a inhibitor on the luciferase signal of the reporter vector, which contained the target site of TNFAIP3. (C) The effect of pre-miR-199a-5p precursor on the luciferase signals of the vectors, including the target site of HIF1A, VEGFA, IGF1R, and IGF2.

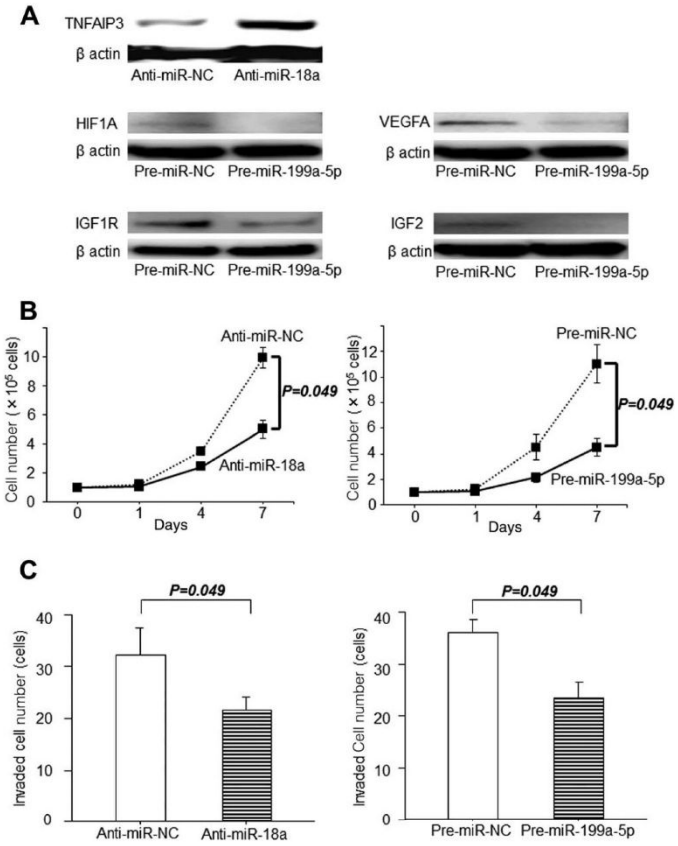

FIG. 4. (A) Western blot analyses. The effect of anti-miR-18a on TNFAIP3 expression. The effect of pre-miR-199a-5p on HIF1A, VEGFA, IGF1R, and IGF2 expression. (B) Cell proliferation assays. The effect of anti-miR-18a inhibitor and pre-miR-199a-5p precursor on the proliferation of the PLC/PRF/5 cells. (C) Invasion assays. The effect of anti-miR-18a inhibitor and pre-miR-199a-5p precursor on the invasion of PLC/PRF/5 cells.

the HCC recurrence after LT. Therefore, our present findings would be applicable in DDLT.

There are few reports about recurrence-related miRNAs after LT for HCC. MiR-18a and miR-199a-5p were not identified as recurrence-related miRNAs after LT for HCC in the few previous reports. Our miRNA candidates such as miR-18a, miR-199a-5p, miR-768-5p, and miR-199a-3p/3b were not included in the recurrence-related miRNAs in the previous reports. On the other hand, the recurrence-related miRNAs in the previous reports did not meet our microarray criteria.[19,20] Although it was reported that miR-18a and miR-199a-5p are associated with HCC, the clinical significance and prognostic impact of these miRNAs were not evaluated in the previous reports.[48,49] Here we revealed that miR-18a and miR-199a-5p are relevant to HCC recurrence after LDLT. Moreover, we identified novel target genes regulated by these miRNAs. The sample number of microarray was small, and prospective validation was not performed. Therefore, further studies are needed.

In conclusion, increased expression levels of miR-18a and decreased expression levels of miR-199a-5p are relevant to the HCC recurrence after LDLT.

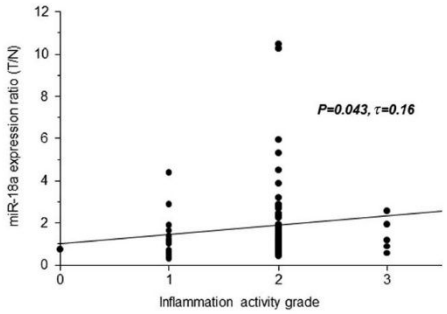

FIG. 5. Correlation between miR-18a expression ratio (T/N) and histopathological inflammation activity grade in the explanted liver.

MiR-18a and miR-199a-5p could be novel therapeutic targets of recurrent HCC after LDLT.

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