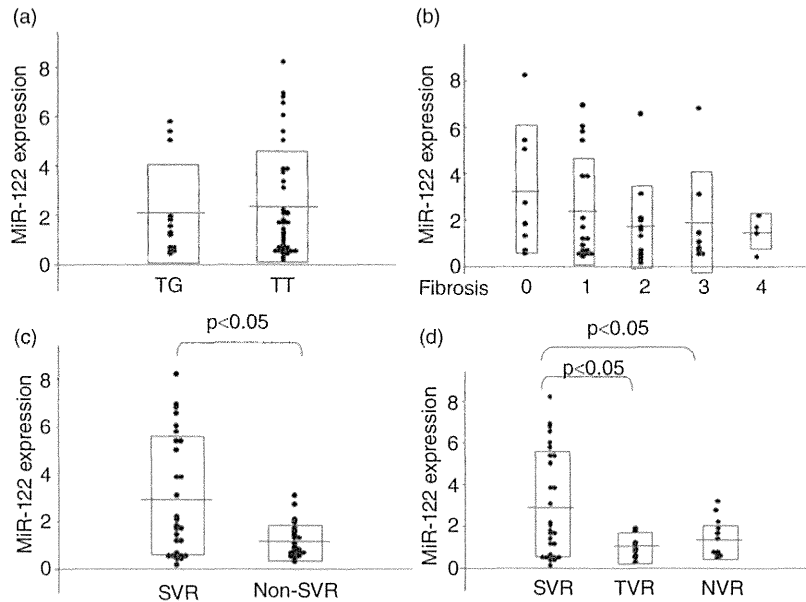

Figure 1 Association between hepatic miRNA-122(miR-122) expression of serotype 1 hepatitis C virus (HCV)-infected liver and IL28B single nucleotide polymorphisms (SNP). There is no correlation with miR-122 expression between IL28B SNP TT and TG/GG (a). Also, we compared with the fibrotic stage. Although there is a tendency for miR-122 expression to decrease if fibrosis progressed, there is no significant difference (b). According to viral response to interferon (IFN) therapy, there is a significant difference between sustained virological response (SVR) and non-SVR ($P < 0.05$, c). Furthermore, we investigate the correlation miR-122 expression between SVR and TVR and undetectable HCV DNA (NR). There are significant difference between SVR and TVR ($P < 0.05$), and SVR and NR ($P < 0.05$) (Fig. 1d).

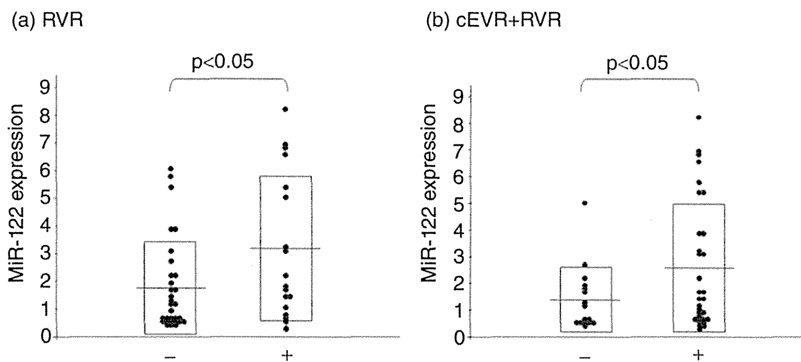

Figure 2 Correlation between miR-122 expression and rapid (RVR)/early virological response (EVR). RVR was defined as patients who respond to interferon (IFN) therapy with a decrease in viral load at week 4. EVR was defined as patients who respond to IFN therapy with a decrease in viral load at week 12. According to the virological response, patients who achieved RVR had significantly higher miR-122 expression level than those that did not achieve RVR (a, $P < 0.05$). The same tendency can be said between patients who achieved and did not achieve EVR (Fig. 2b, $P < 0.05$). We examined the relationship between miR-122 expression and several clinical parameters (white blood cell count, red blood cell count, platelets, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, albumin, pre-albumin, ferritin, type IV collagen and hyaluronic acid), there are no significant differences.

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Table 2 Relation with miR-122 expression level in liver and clinical items

Items	Relation	*P*
Age (year)	0.01	NS
BMI (kg/m2)	0.105	NS
BTR (ratio)	0.175	NS
WBC (cells/μL)	0.102	NS
RBC (cells/μL)	0.219	NS
PLT ($\times 10^4$ platelets/μL)	0.090	NS
AST (IU/L)	0.045	NS
ALT (IU/L)	0.020	NS
γ-GT (IU/L)	0.004	NS
Albumin (g/dL)	0.059	NS
PreAlb (mg/dL)	0.138	NS
Ferritin (ng/mL)	0.191	NS
Type IV collagen (ng/mL)	0.214	NS
Hyaluronic acid (ng/mL)	0.178	NS
FPG (mg/dL)	0.284	<0.05
FFA (mEq/L)	0.190	NS
HOMA-R	0.08	NS
HbA1c (%)	0.167	NS
TC (mg/dL)	0.124	NS
HDL (mg/dL)	0.044	NS
LDL (mg/dL)	0.128	NS
TG (mg/dL)	0.146	NS
REE	0.352	<0.05
RQ	0.550	<0.05
IFN adherence, >80%	0.222	NS
RBV adherence, >60%	0.038	NS
HCV RNA (logIU/mL)	0.088	NS

Analyzed using the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.
Normal values in laboratory tests: body mass index (BMI) calculated as bodyweight (kg)/height (m)^2; white blood cell count (WBC, cells/μL), 3500–9000; platelets (PLT, $\times 10^4$ platelets/μL), 12–33; aspartate aminotransferase (AST, IU/L), 10–40; alanine aminotransferase (ALT, IU/L), 5–40; γ-glutamyltransferase (γ-GT, IU/L), <70 in males, <30 in females; albumin (Alb, g/dL), 4.0–5.0; total cholesterol (TC, mg/dL), 128–220; triglyceride (TG, mg/dL), 38–150; low-density lipoprotein cholesterol (LDL-C, mg/dL), 70–139; high-density lipoprotein cholesterol (HDL-C, mg/dL), 40–80; free fatty acid (FFA, mEq/L), 100–800; pre-albumin (preAlb), 22–40; hemoglobin A1c (HbA1c), <5.8%.
BTR, branched-chain amino acids to tyrosine ratio; FPG, fasting plasma glucose; HCV, hepatitis C virus; HOMA-IR, Homeostasis Model of Assessment – Insulin Resistance; IFN, interferon; NR, undetectable HCV RNA; NS, not significant; RBV, ribavirin; REE, resting energy expenditure; RQ, respiratory quotient; SD, standard deviation; SVR, sustained virological response.

disease activity score (NAS). There was a significant difference in NAS among patients in the 0–5% and more than 5% groups (Fig. 3b, $P < 0.05$). Furthermore, we determined whether a viral or host factor was responsible for miR-122 expression by examining the correlation of miR-122 expression with several clinical parameters, namely, the presence of hypertension and diabetes mellitus, obesity (body mass index, >25), total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, fasting plasma glucose, free fatty acids, Homeostasis Model of Assessment – Insulin Resistance, hemoglobin A1c, resting energy expenditure (REE) and respiratory quotient (RQ). We found that only hypertension, fasting plasma glucose, RQ and REE correlated with miR-122 expression (Table 2).

Factors contributing to a CHC serotype 1 SVR to IFN therapy

We further investigated factors contributing to a CHC serotype 1 SVR to IFN therapy. In univariate analysis, more male than female patients achieved SVR. The prevalence of type 2 diabetes was also significantly higher among patients who did not achieve SVR. miR-122 expression was higher among patients with SVR than among those without such a response. Multivariate analysis indicated that miR-122 expression was an independent predictor for SVR (Table 3a).

Patients who did not achieve SVR could be divided into two further groups: those who responded to IFN therapy and momentarily had undetectable HCV RNA but who then relapsed (transient responders, TVR) and those who did not respond to IFN therapy and never had any undetectable HCV RNA (null responders, NVR). Therefore, we divided the 51 patients into two groups: those who responded to IFN therapy and had undetectable HCV RNA at least once (SVR + TVR), and those who did not respond to IFN therapy and never had undetectable HCV RNA (NR). Univariate analysis indicated that patients with minor *IL28B* SNP were less likely to achieve SVR or TVR than those with major *IL28B* SNP. Females were also less likely to achieve SVR or TVR than males. Multivariate analysis indicated that *IL28B* SNP were independent predictors of a null response (Table 3b).

DISCUSSION

IN OUR STUDY, we found that hepatic miR-122 expression correlated with virological response to IFN therapy. However, there was no significant difference in miR-122 expression between minor and major *IL28B* SNP. We also determined whether other factors predictive of response to IFN therapy, including *IL28B* SNP, correlated with miR-122 expression, but no such corre-

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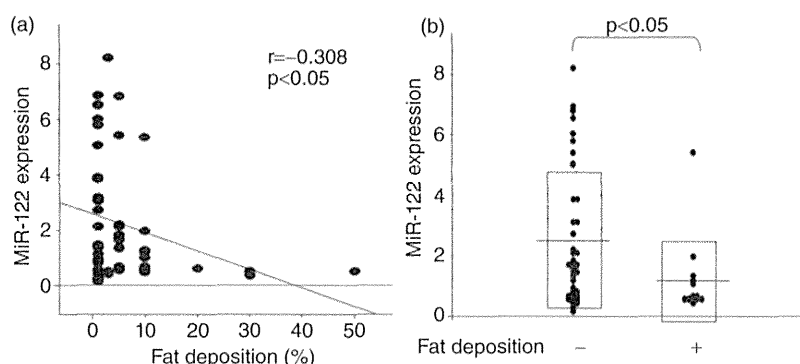

Figure 3 miRNA122 (miR-122) expression was correlated with fat deposition in liver. By using liver biopsy specimen, we scored the degree of fat deposition in liver and examined the relationship miR-122 expression. miR-122 expression significantly decreased as the extent of fat deposition in the liver increased (a, $P < 0.05$). We divided patients into those whose fat deposition was 0–5% and >5% in proportion to non-alcoholic fatty liver disease activity score (NAS). There was a significant difference between 0–5% and >5% (b, $P < 0.05$).

lation was found. These findings suggest that miR-122 is an independent factor predictive of response to IFN therapy and affects the second phase of IFN therapy.

In CHC patients, miR-122 reportedly facilitates the replication of HCV by binding to the 5′-UTR of HCV RNA *in vitro*.[19,22] However, in our study, no correlation was observed between HCV load and miR-122 expression, supporting previous findings of the lack of any such correlation. Why miR-122 expression is not correlated with the HCV load is not currently understood. Many factors have been reported to promote HCV replication and production, including cyclophilin B[27], FBL2, FK506 binding-protein 8, heat shock protein 90,[28] heat shock cognate protein 70,[29] fatty acid synthesis, geranylgeranylation,[30,31] fatty acids[32] and lipid droplets.[33,34] Given that miR-122 is abundant in the human liver, HCV replication would likely be dependent on miR-122. However, miR-122 expression is decreased as liver injury progresses and, hence, HCV replication must be dependent on other factors.

miR-122 is reportedly associated with lipid and iron metabolism in healthy individuals.[35–37] In our study, miR-122 was inversely correlated with the extent of hepatic fat deposition. We also determined whether host- or virus-related factors were responsible for fat deposition in CHC patients. We found no correlation between hepatic fat deposition and host factors such as the presence or absence of hypertension, obesity and type 2 diabetes. Thus, it was clear that fat deposition was induced by a virus-related factor. Also, patients with low miR-122 expression had low RQ and REE. Therefore, we hypothesized that as miR-122 expression was reduced, fat was deposited in the liver, which might have been associated with increased oxidation of fatty acids. This would lead to the use of fat as an energy source and decrease RQ.

Hepatitis C virus infection is associated with non-alcoholic fatty liver disease.[38] Once a host is infected with HCV, the virus begins to replicate in the host's liver using miR-122. This hijack of miR-122 may decrease lipid metabolism, which is its primary function. Indeed, it has been reported that a 4-week therapy session with an antisense nucleotide of miR-122 (miravirsen; Santaris Pharma, San Diego, CA, USA) in treatment-naïve patients with HCV genotype 1 infection resulted in lowered total cholesterol as well as suppression of viremia in chimpanzees.[24] We believe that as hepatic fat deposition progresses, lipid droplets are formed and these act as sites for replication of HCV RNA. If this hypothesis is correct, then inhibition of viral propagation by targeting miR-122 using an antisense approach may have a positive effect on circulating cholesterol and HCV-associated lipid abnormalities and, hence, decrease the number of lipid droplets available for HCV replication.

In conclusion, miR-122 expression is correlated with response to IFN therapy in CHC patients with HCV serotype 1 infection and is independent of other predictors of response, including *IL28B* SNP. miR-122 expression is also correlated with hepatic fat deposition and a patient's RQ, which may be associated with fat deposition in the liver. Hereafter, it is necessary to evaluate miR-122 expression in blood samples to determine how

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Table 3 Study of predictive factors for IFN treatment in serotype 1 chronic hepatitis C patients

A. Contributing factor to SVR in all patients

Variable	Univariate analysis		Multivariate analysis	
	P	odds ratio (95% CI)	*P*	Odds ratio (95% CI)
Age (years)	0.234	1.03 (0.97–1.10)		
Sex (F)	0.054	0.31 (0.09–1.02)		
BMI	0.472	0.93 (0.78–1.12)		
Fibrosis	0.700	1.09 (0.67–1.78)		
Activity	0.599	0.72 (0.22–2.38)		
Fat deposition	0.455	1.02 (0.96–1.09)		
HCV RNA (logIU/mL)	0.892	1.04 (0.55–1.94)		
Albumin (g/dL)	0.897	0.88 (0.12–6.03)		
AST (IU/L)	0.203	0.98 (0.96–1.00)		
ALT (IU/L)	0.084	0.98 (0.97–1.00)		
γ-GT (IU/L)	0.121	0.98 (0.96–1.00)		
PLT (×10⁴/μL)	0.898	1.00 (0.91–1.10)		
Ferritin (mEq/L)	0.569	0.99 (0.99–1.00)		
PreAlb (g/dL)	0.272	0.93 (0.82–1.05)		
HbA1c (%)	0.022	0.08 (0.01–0.71)	NS	
IFN adherence, >80%	0.716	0.80 (0.25–2.53)		
RBV adherence, >60%	0.773	1.35 (0.17–10.41)		
miRNA-122	0.012	0.55 (0.34–0.88)	0.029	0.401 (0.17–0.91)
IL28B rs8099917	0.127	0.36 (0.09–1.33)		

B. Contributing factor to NVR in all patients

Variable	Univariate analysis		Multivariate analysis	
	P	Odds ratio (95% CI)	*P*	Odds ratio (95% CI)
Age (years)	0.178	0.93 (0.85–1.02)		
Sex (male)	0.035	5.02 (1.11–22.6)	0.088	4.38 (0.80–23.9)
BMI (kg/m²)	0.944	1.00 (0.81–1.24)		
Fibrosis	0.414	1.29 (0.69–2.41)		
Activity	0.570	1.55 (0.33–7.15)		
Fat deposition (%)	0.104	0.95 (0.90–1.00)		
HCV RNA (logIU/mL)	0.659	1.18 (0.56–2.48)		
Albumin (g/dL)	0.358	3.39 (0.25–46.0)		
AST (IU/L)	0.656	0.99 (0.97–1.01)		
ALT (IU/L)	0.779	1.00 (0.98–1.01)		
γ-GT (IU/L)	0.525	1.00 (0.98–1.02)		
PLT (×10⁴/μL)	0.233	0.92 (0.82–1.04)		
Ferritin (ng/mL)	0.479	0.99 (0.99–1.00)		
PreAlb (g/dL)	0.412	1.06 (0.91–1.25)		
HbA1c (%)	0.406	1.83 (0.43–7.66)		
IFN adherence, >80%	0.508	0.60 (0.13–2.68)		
RBV adherence, >60%	0.778	1.40 (0.13–15.1)		
miRNA-122	0.141	1.51 (0.87–2.64)	0.239	1.42 (0.78–2.58)
IL28B rs8099917	0.009	7.28 (1.61–32.7)	0.016	7.77 (1.45–41.7)

Normal values in laboratory tests: body mass index (BMI) calculated as bodyweight (kg)/height (m)^2; white blood cell count (WBC, cells/μL), 3500–9000; platelets (PLT, ×10^4 platelets/μL), 12–33; aspartate aminotransferase (AST, IU/L), 10–40; alanine aminotransferase (ALT, IU/L), 5–40; γ-glutamyltransferase (γ-GT, IU/L), <70 in males, <30 in females; albumin (Alb, g/dL), 4.0–5.0; total cholesterol (TC, mg/dL), 128–220; triglyceride (TG, mg/dL), 38–150; low-density lipoprotein cholesterol (LDL-C, mg/dL), 70–139; high-density lipoprotein cholesterol (HDL-C, mg/dL), 40–80; free fatty acid (FFA, mEq/L), 100–800; pre-albumin (preAlb), 22–40; hemoglobin A1c (HbA1c), <5.8%.
CI, confidence interval; HCV, hepatitis C virus; IFN, interferon; NR, undetectable HCV RNA; NVR, non-virological response; RBV, ribavirin; SVR, sustained virological response.

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fatty liver and lipid metabolism are involved in the pathogenesis of chronic hepatitis.

Thus, miR-122 may be a therapeutic target as well as a predictive marker of response to IFN therapy. Targeting miR-122 may have a positive effect not only by directly inhibiting viral propagation but also by ameliorating cholesterol and lipid abnormalities and reducing the number of sites available for HCV replication.

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REVIEW

Antitumor function of microRNA-122 against hepatocellular carcinoma

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Abstract MicroRNA-122 (miR-122), a highly abundant and liver-specific miRNA, acts as a tumor suppressor against hepatocellular carcinoma (HCC). Decreased expression of miR-122 in HCC is frequently observed and is associated with poor differentiation, larger tumor size, metastasis and invasion, and poor prognosis. Mutant mice with knockout (KO) of the miR-122 locus developed steatohepatitis due to increased triglyceride (TG) synthesis and decreased TG secretion from hepatocytes, and eventually developed HCC. Exogenic miR-122 introduction into miR-122 KO mice inhibited the development of HCC. Target genes of miR-122, including cyclin G1, a disintegrin and metalloprotease (ADAM)10, serum response factor, insulin-like growth factor-1 receptor, ADAM17, transcription factor CUTL1, the embryonic isoform of pyruvate kinase (Pkm2), Wnt1, pituitary tumor-transforming gene 1 binding factor, Cut-like homeobox 1, and c-myc, are involved in hepatocarcinogenesis, epithelial mesenchymal transition, and angiogenesis. MiR-122 expression is regulated by liver-enriched transcription factors such as hepatocyte nuclear factor (HNF)1α, HNF3β, HNF4α, HNF6, and CCAAT/enhancer-binding protein (C/EBP)α. A positive feedback loop exists between C/EBPα and miR-122 and between HNF6 and miR-122, whereas a negative feedback loop exists between c-myc and miR-122. Since cotreatment of 5-Aza-Cd and histone deacetylase inhibitor restored miR-122 expression in HCC cells, epigenetic modulation of miR-122 expression is involved in the suppression of miR-122 in HCC. Several experiments suggest that increasing miR-122 levels in HCC with or without antitumor agents may be a promising strategy for HCC treatment.

Keywords MicroRNA-122 (miR-122) · Hepatocellular carcinoma (HCC) · Steatohepatitis

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Introduction

MicroRNA122 (miR-122) accounts for 70 % of the total liver miRNA population, but it is undetectable in other tissues [1–3]. MiR-122 plays important roles in regulating hepatocyte development, differentiation, lipid metabolism, and stress response [2–5]. With regard to liver diseases, miR-122 stimulates hepatitis C virus (HCV) replication by direct binding to HCV 5′UTR of HCV RNA [4, 6, 7], whereas miR-122 inhibits replication of hepatitis B virus (HBV) by p53-mediated inhibition of HBV transcription [7, 8]. MiR-122 acts as a tumor suppressor and represses hepatocellular carcinoma (HCC) development by binding to target genes involved in cell proliferation, migration, differentiation, apoptosis and angiogenesis in HCC [3, 5]. In this review, we focus on the antitumor activity of miR-122 against HCC and describe miR-122 expression in HCC, hepatocarcinogenesis in miR-122 knockout mice, mechanisms of antitumor function of miR-122, regulation of miR-122 gene expression, and therapeutic application of miR-122 against HCC.

Down-regulation of miR-122 in HCC

Down-regulation of miR-122 in HCC tissue as compared to adjacent normal tissue has been reported in several studies

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[9–15]. Loss of miR-122 expression in HCC is associated with metastasis and poor prognosis [10, 12–15]. Coulouarn et al. reported that the overall survival of patients with low and high miR-122 expression in HCC was 30.3 ± 8.0 and 83.7 ± 10.3 months, respectively ($p < 0.001$), and that miR-122 repression was associated with poor differentiation status and large tumor size. They also found that the loss of miR-122 expression in HCC tissue was correlated with high proliferation and low apoptotic index [10]. Down-regulation of miR-122 is also observed in numerous human HCC cell lines, although the levels of miR-122 are variable with more than 1000-fold expression differences among cell lines [10]. Lower expression levels of miR-122 are related to the migration and invasion activity of HCC cells [11].

Serum levels of miR-122 have been determined in patients with chronic liver disease including HCC [16–18]. The miR-122 levels did not significantly differ between patients with and without HCC, but were positively correlated with liver transaminases and negatively correlated with the Model for End-Stage Liver Disease (MELD) score [18], suggesting that serum miR-122 is a novel biomarker for liver injury but not specifically for HCC.

Hepatocarcinogenesis in miR-122 knockout mice

To elucidate the relevance of miR-122 depletion and HCC development, mutant mice with germ line knockout (KO) or liver-specific knockout (LKO) of the miR-122 locus were generated in two studies [19, 20], and the pathophysiological changes in these mutant mice were investigated. Both KO and LKO mice develop normally and are viable and indistinguishable from wild-type (WT) mice. Both mice exhibit reduced serum cholesterol and triglyceride (TG) levels and develop hepatic steatosis due to TG accumulation and reduced glycogen storage, as well as inflammation and fibrosis. These histological features are similar to steatohepatitis. Eventually, HCC developed in 10-month-old KO mice and 12-month-old LKO mice. The incidence of HCC exhibited significant sex differences; HCC developed in 17 of 19 (89 %) male KO mice and 6 of 26 (23 %) female KO mice [19], and in 13 of 26 (50 %) male LKO mice and 2 of 20 (10 %) female LKO mice [20].

In the miR-122 KO liver, the expression of Agpatl, which catalyzes TG biosynthesis [21], was up-regulated, and the expression of Cidec (Fsp27), a lipid droplet-binding protein that promotes TG accumulation in hepatocytes [22], was also elevated. 3′UTRs of both Agpatl and Cidec mRNA contain miR-122 binding sites [20]. MiR-122 significantly repressed luciferase expression from reporter plasmids containing 3′UTRs of these genes [20]. In addition, the expression of microsomal TG transfer protein (MTTP), which plays a crucial role in hepatic very low-density lipoprotein (VLDL) assembly and secretion [23], was reduced in both mRNA and protein levels. Hydrodynamic injection of the MTTP gene in miR-122 KO mice resulted in an increase in serum VLDL as well as in normalization of serum levels of cholesterol and TG. Surprisingly, the MTTP-restored liver showed significant reduction of hepatic steatosis, inflammation, and fibrosis, as well as recovery of glycogen storage [19]. These results suggest that TG accumulation in the miR-122 KO liver is caused by an increase of TG synthesis in hepatocytes and a decrease of TG secretion from hepatocytes.

In the setting of chronic liver injury, Ccl2, a monocyte-chemotactic protein induced in the injured liver, recruits monocytes and dendritic cells to the sites of inflammation [24]. In the miR-122 KO liver, Ccl2 expression increased, and inflammatory cells including monocytes producing IL-6 infiltrated. It was confirmed that miR-122 negatively regulates Ccl2 expression by binding to the 3′UTR of Ccl2 mRNA. Park et al. [25] demonstrated that enhanced production of the tumor-promoting cytokines IL-6 and TNF-α causes hepatic inflammation and activation of the oncogenic transcription factor STAT3. Taken together, these results suggest that over-production of Ccl2 and consequential IL-6 over-secretion from inflammatory cells contribute to hepatocarcinogenesis in miR-122 KO mice.

The initial sign of epithelial mesenchymal transition (EMT), including loss of the portal distribution of E-cadherin and gain of vimentin expression was observed in the livers of young miR-122 KO mice, followed by the loss of E-cadherin expression in the tumors of aged mice. Similarly, the expression of oncofetal genes such as α-fetoprotein (AFP), insulin-like growth factor-1 (IGF-1), and Src, as well as cancer stem cell marker genes such as Prom1, Thy1, and Epcam, were detected in the livers of young miR-122 KO mice, followed by further increases in the tumors of aged mice. The activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) signaling pathways was also detected in the livers of young miR-122 KO mice, followed by strong activation in the tumors of aged mice [19]. These data suggest that tumor-related genes are already activated early in the tumor-free livers of young miR-122 KO mice and attributed to tumor initiation and progression.

Mechanisms of antitumor function of miR-122

Recently, various target genes of miR-122 have been identified to be involved in hepatocarcinogenesis and EMT. Of these, miR-122 directly down-regulates cyclin G1 expression, and an inverse correlation between miR-122 and cyclin G1 expression exists in HCC tissues [26]. Since

cyclin G1 negatively regulates p53 protein stability by acting on the B' subunit of phosphatase 2A, miR-122 increases the expression of p53 and its transcriptional activities [27].

A disintegrin and metalloprotease 10 (ADAM10), serum response factor (SRF), and insulin-like growth factor-1 receptor (IGF-1R), which promote tumorigenesis, are validated as targets of miR-122 and are repressed by miR-122. ADAM10, SRF, and IGF-1R are up-regulated in HCC tissue compared with the adjacent normal tissue [12]. Zeng et al. suggest the following regulatory circuitry: miR-122 suppresses IGF-1R expression and attenuates IGF-1R/Akt signaling, which sustains glycogen synthase kinase-3 beta (GSK-3β) activity and in turn represses cyclin D1 expression and cell proliferation. The activated GSK-3β maintains high levels of miR-122 through activating CCAAT/enhancer-binding protein (C/EBP)α, a transcription factor of the miR-122 gene, which enforces IGF-1R suppression. Disruption of this regulatory circuitry may result in uncontrolled cell proliferation and hepatocarcinogenesis [13]. ADAM17 is another target of miR-122 and is involved in HCC metastasis. Silencing of ADAM17 resulted in a dramatic reduction of in vitro migration, invasion, in vivo tumorigenesis, and angiogenesis, which is similar to that which occurs with the restoration of miR-122 [11].

CUTL1, a transcriptional repressor of genes specifying differentiation during development, is a target of miR-122. The amount of CUTL1 protein gradually disappeared during the progression of liver development, which was inversely correlated with the expression of miR-122 [28]. Zinc finger and BTB domain-containing 20 (ZBTB20) is a repressor of AFP gene transcription in normal liver. ZBTB20 directly binds to a region of the AFP promoter and represses its activity [29]. Recently, it was shown that miR-122 indirectly modulates the expression of ZBTB20 and regulates AFP expression [30]. The miR122-silenced HCC cells exhibit a more invasive phenotype and produce more abundant AFP. In these cells, the expression of Cut-like homeobox 1 (CUX1), which is a target of miR-122 and regulates multiple processes including cell-cycle progression, is up-regulated. CUX1 is a positive regulator of miR-214. Since ZBTB20 is a target of miR-214, the elevated expression of miR214 represses ZBTB20 translation, followed by increased expression of AFP [30]. The embryonic isoform of pyruvate kinase (Pkm2) is a target of miR-122 and is highly expressed in human embryonic stem cells (hESCs) and HCC cells. During the differentiation process of hESCs into mature hepatocytes, a reciprocal expression pattern is observed between miR-122 and Pkm2. Depleting hESCs and HCC cells of Pkm2, or overexpressing miR-122, leads to a common deficiency in self-renewal and proliferation [31].

Various signaling pathways are deregulated in HCC. The Wnt/β-catenin pathway is activated in approximately 30 % of HCCs [32]. Wnt1 is a direct target of miR-122. miR-122 suppresses the expression of Wnt1 protein and subsequently leads to down-regulation of β-catenin and TCF-4, resulting in the attenuation of the Wnt/β-catenin signaling pathway [33]. Pituitary tumor-transforming gene 1 (PTTG1) binding factor (PBF) is a target of miR-122 [34] Overexpression of PBF is observed in 68 % of HCC (13 of 19). PBF increases HCC cell proliferation and invasive ability and promotes tumor growth in nude mice. PBF interacts with PTTG1 and promotes its transcriptional activities by facilitating PTTG1 nuclear translocation, which in turn stimulates the transcription of tumor-promoting genes such as VEGF, FGF-2, c-myc, and MMP-2 [34].

Regulation of miR-122 gene expression

The expression of miR-122 is correlated with liver-enriched transcription factors (LETFs), such as hepatocyte nuclear factor (HNF)1α, HNF3β, HNF4α, HNF6, and C/EBPα [10, 13, 28, 35, 36]. These LETFs are coordinately involved in the transcriptional regulation of miR-122 by binding to the miR-122 promoter as transcriptional activators. Hepatocyte differentiation is directed by a positive feedback loop that includes C/EBPα, HNF6, and miR-122 [13, 36]. As described above, miR-122 indirectly activates C/EBPα through IGF-1R suppression and resultant GSK-3β activation [13]. MiR-122 stimulates HNF6 expression, although the mechanism is unclear [36].

The expression of miR-122 is suppressed in HCC cells, which is, at least in part, explained by epigenetic modulation of miR-122. The promoter region of miR-122 is

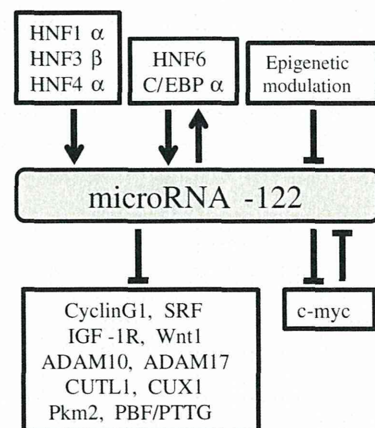

Fig. 1 Regulators of miR-122 expression (*upper box*), and miR-122 target genes involved in HCC development (*lower box*)

hypermethylated in HCC cells, but not in human primary hepatocytes [31]. The treatment of HCC cells with a demethylating agent, 5-Aza-2′ deoxycytidine (5-Aza-Cd), significantly increases the gene expression of miR-122 [37]. Moreover, in HCC cells, a H3K9 histone methyl transferase down-regulates the gene expression of miR-122 by inhibiting PPARγ/RXRα-mediated promoter activity, which is cancelled by cotreatment with 5-Aza-Cd and histone deacetylase inhibitor [37].

c-myc can repress miR-122 gene expression by associating with miR-122 promoter and by down-regulating HNF3β expression, whereas miR-122 indirectly inhibits c-myc transcription by targeting the transcriptional activator E2f1 and coactivator Tfdp2 [38].

Figure 1 shows the regulators of miR-122 expression and the downstream target molecules relevant to antitumor activity of miR-122.

Therapeutic application of miR-122 against HCC

Since miR-122 is a liver-specific tumor suppressor microRNA, increasing miR-122 levels in HCC with or without antitumor agents may be a promising strategy for HCC treatment. In fact, gene transfer of miR-122 into cultured HCC cells induces apoptosis and cell-cycle arrest [39–41]. Hydrodynamic injection of miR-122 into 3-month-old miR-122 KO mice effectively impaired hepatocarcinogenesis and tumor progression, as reflected by a reduction in tumor occurrence and size [19]. Intratumor injection of miR-122 encapsulated in cationic lipid nanoparticles suppressed the growth of HCC xenograft by 50 %, which was correlated with repression of target genes and impairment of angiogenesis [42]. In addition, miR-122 sensitizes HCC cells to antitumor agents including doxorubicin [27, 43, 44], vincristine [43], cisplatin [44], and sorafenib [12] by modulating the expression of multidrug resistance genes [43] and the unfolded protein response [44].

Conflict of interest The authors declare that they have no conflict of interest.

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METABOLIC AND STEATOHEPATITIS

Significance of serum and hepatic microRNA-122 levels in patients with non-alcoholic fatty liver disease

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Abstract

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is believed to be a type of metabolic syndrome. MicroRNA-122 (miR-122) is the most abundant microRNA in the liver and is an important factor for the metabolism of glucose and lipids. In the present study, we examined the correlation between the hepatic and serum miR-122 expression levels and the clinicopathological factors of patients with NAFLD. *Methods:* We extracted the total RNA, along with preserved miRNAs, from liver biopsy samples of 67 patients with NAFLD. In 52 of these 67 patients, the total RNA was extracted from serum. The miR-122 that was obtained by quantitative reverse transcription-polymerase chain reaction was quantified using TaqMan MicroRNA assays. *Results:* A significant correlation was detected between serum and hepatic miR-122 expression (correlation coefficient, 0.461; $P = 0.005$). Patients with mild steatosis (<33%) showed significantly lower levels of hepatic miR-122 compared with patients with severe steatosis (>33%) (hepatic miR-122: mild/severe = 2.158 ± 1.786/4.836 ± 7.506, $P = 0.0473$; serum miR-122: mild/severe = 0.002 ± 0.005/0.007 ± 0.001, $P = 0.0491$). Moreover, hepatic and serum miR-122 levels were significantly higher in patients with mild fibrosis than in those with severe fibrosis (hepatic miR-122: mild/severe = 5.201 ± 7.275/2.394 ± 1.547, $P = 0.0087$; serum miR-122: mild/severe = 0.008 ± 0.011/0.002 ± 0.004, $P = 0.0191$). *Conclusions:* We found that the hepatic and serum miR-122 levels were associated with hepatic steatosis and fibrosis. The serum miR-122 level can be a useful predictive marker of liver fibrosis in patients with NAFLD.

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease worldwide (1–8). NAFLD is considered to represent the hepatic manifestation of metabolic syndrome. In Japan, an increase in the incidence of metabolic syndrome has led to an increase in the prevalence of NAFLD (5). NAFLD was traditionally considered as a relatively benign liver disease. However, some patients with NAFLD progress to liver fibrosis, cirrhosis and hepatocellular carcinoma (8–13). Therefore, the precise diagnosis and staging of NAFLD patients is clinically important. Liver biopsy is the gold standard for the evaluation of NAFLD patients in terms of staging. However, liver biopsy is an invasive technique, and the identification of non-invasive biomarkers is required.

Micro-RNAs (miRNAs) are endogenous, small, non-coding RNAs of approximately 21–22 nucleotides that have important gene regulatory functions in animals and plants. miRNAs bind to the messenger RNAs of protein coding genes to direct their post-transcriptional repression (14–16). miRNAs have been reported to play important roles in cell proliferation (17) and apoptosis (18), lymphocyte development (19), and adipocyte differentiation (20). Several recent studies have indicated that miRNAs play important roles in metabolism and metabolic diseases (21–23). MicroRNA-122 (miR-122) is the most abundant miRNA in the liver, and it regulates metabolic pathways, including cholesterol biosynthesis, fatty acid synthesis and oxidation (22, 23).

Recently, extracellular miRNAs were detected in serum, plasma and other body fluids. These circulating miRNAs have been reported to be predictive biomarkers for various cancers and in liver diseases (24, 25). However, the significance of miR-122 expression in the serum and liver of NAFLD patients has not been studied in detail.

In the present study, we analysed the relationship between the clinicopathological features and the expression of miR-122 in the serum and liver of NAFLD patients.

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Patients and methods

Patient groups

In this study, we examined consecutive NAFLD patients who visited the Department of Gastroenterology and Hepatology at Nagasaki University Hospital. The patients who exhibited positive results for hepatitis B virus surface antigen or hepatitis C virus antibody, or those showing evidence of inherited, autoimmune, cholestatic or drug-induced liver disease were excluded using clinical, laboratory, imaging and histological criteria. In addition, patients with a history of current or past excessive alcohol intake, as defined by an average daily consumption of more than 20 g of alcohol, were excluded from the study.

Non-alcoholic fatty liver disease was diagnosed by percutaneous liver biopsy and ultrasonography. Liver biopsy specimens were fixed in 10% formalin, cut to a thickness of 4 μm and subjected to haematoxylin–eosin and Azan-Mallory staining. Steatosis was classified as mild (>30%) or severe (30%). Inflammation was scored on a scale of 0–9 according to the standards proposed by the Non-alcoholic Steatohepatitis Clinical Research Network (26). Fibrosis staging was performed using a five-grade scale as follows: F0, no fibrosis; F1, pericellular fibrosis in zone 3; F2, pericellular fibrosis in zone 3 with periportal fibrosis; F3, bridging fibrosis; and F4, cirrhosis defined as mild fibrosis (F0 or F1) and severe fibrosis (>F1).

miRNA extraction and quantification

RNA was extracted from a total of 67 liver biopsy specimens. Total RNA, including the miRNA, was isolated from formalin-fixed paraffin-embedded (FFPE) liver biopsy specimens using the Recover All Total Nucleic Acid Isolation Kit for FFPE (Ambion, Carlsbad, CA, USA) according to the manufacturer's protocol. In 52 of 67 patients, total RNA, along with preserved miRNAs, was extracted from 400 μL of serum using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Synthetic miR-39 was added to serum samples prior to RNA extraction as an internal control.

The miR-122 obtained by quantitative reverse transcription-polymerase chain reaction was quantified using TaqMan MicroRNA assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. miR-122 expression was calculated by the relative standard curve method and normalized to RNU6 expression in the liver and cell-miR39 expression in the serum.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Data were analysed by the Student's *t*-test for comparison of paired data. Correlations were analysed using the Spearman rank correlation coefficient. A *P* value of <0.05 was considered statistically significant.

Results

The characteristic of this study population are show in Table 1.

Correlation between hepatic and serum miR-122 expression and clinical factors

No significant correlations were observed between clinical factors and the expression of hepatic (Table 2) or serum (Table 3) miR-122. However, a significant correlation was observed between the serum and hepatic miR-122 expression levels (Fig. 1).

Correlation between hepatic miR-122 level and the pathological findings of NAFLD patients

Patients with mild steatosis (<33%) showed significantly lower levels of hepatic miR-122 than patients with severe steatosis (>33%) (mild/severe = 2.158 ± 1.786/4.836 ± 7.506; *P* = 0.0473). No significant correlation between serum miR-122 level and the NAFLD activity score (NAS) was observed. In contrast, hepatic miR-122 level showed a significant negative correlation with the fibrosis stage [correlation coefficient: −0.292 (−0.497 to −0.056); *P* = 0.0161] (Table 2). Moreover, hepatic miR-122 expression was significantly higher in patients with no or mild fibrosis than in those with severe fibrosis (mild/severe = 5.201 ± 7.275/2.394 ± 1.547; *P* = 0.0087) (Fig. 2).

Correlation between serum miR-122 level and the pathological findings of NAFLD patients

Patients with mild steatosis (<33%) showed significantly lower levels of serum miR-122 than patients with severe steatosis (>33%) (mild/severe = 0.002 ± 0.005/0.007 ± 0.001; *P* = 0.0491). No significant correlation was

Table 1. Clinical characteristics of liver samples (67 cases)

Patient age (years)	51.8 ± 17.4
Male:female	27:40
BMI	28.5 ± 4.2
Type 2 diabetes	46 cases
AST (IU/L)	71.7 ± 42.4
ALT (IU/L)	102.7 ± 64.1
ALP (IU/L)	286.3 ± 117.3
γ-GTP (IU/L)	103.6 ± 121.6
T-cho (mg/dl)	195.1 ± 45.4
TG (mg/dl)	144.7 ± 60.1
Plt (10^4/mm^3)	21.7 ± 7.4
FBS (mg/dl)	115.7 ± 41.4
HbA1c (%)	6.7 ± 2.0

γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, free blood sugar; HbA1c, glyco haemoglobin A1c; Plt, platelet; T-cho, total cholesterol; TG, triglyceride.

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Table 2. Relation between hepatic microRNA-122 level and clinical factors

	Correlation coefficient	*P*-value
Age	0.025 (−0.216 to 0.264)	0.8385
BMI	−0.107 (−0.342 to 0.141)	0.3984
AST	−0.142 (−0.369 to 0.102)	0.2541
ALT	−0.042 (−0.279 to 0.201)	0.7390
ALP	−0.072 (−0.307 to 0.142)	0.5657
γ-GTP	−0.082 (−0.318 to 0.163)	0.5125
T-cho	0.054 (−0.199 to 0.300)	0.6785
TG	0.125 (−0.119 to 0.354)	0.3152
Plt	0.123 (−0.121 to 0.352)	0.3422
FBS	0.224 (−0.034 to 0.454)	0.0878
HbA1c	0.250 (−0.017 to 0.483)	0.0660
NAS	0.053 (−0.190 to 0.289)	0.6732
Fibrosis	−0.292 (−0.497 to −0.056)	0.0161

γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, free blood sugar; HbA1c, glyco haemoglobin A1c; NAS, NAFLD activity score; Plt, platelet; T-cho, total cholesterol; TG, triglyceride.

Table 3. Relation between serum microRNA-122 level and clinical factors

	Correlation coefficient	*P*-value
Age	−0.183 (−0.434 to 0.095)	0.1959
BMI	−0.042 (−0.314 to 0.236)	0.7708
AST (IU/L)	−0.049 (−0.317 to 0.386)	0.7340
ALT (IU/L)	0.126 (−0.152 to 0.136)	0.3750
ALP (IU/L)	−0.143 (−0.400 to 0.136)	0.3146
γ-GTP (IU/L)	−0.125 (−0.387 to 0.156)	0.3849
T-cho	0.089 (−0.194 to 0.358)	0.5420
TG	−0.061 (−0.329 to 0.215)	0.6667
Plt	−0.035 (−0.305 to 0.240)	0.8044
FBS	0.212 (−0.087 to 0.476)	0.1626
HbA1c	0.114 (−0.193 to 0.401)	0.4695
NAS	0.138 (−0.140 to 0.396)	0.3312
Fibrosis	−0.316 (−0.543 to 0.048)	0.0218

γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; FBS, free blood sugar; HbA1c, glyco haemoglobin A1c; NAS, NAFLD activity score; Plt, platelet; T-cho, total cholesterol; TG, triglyceride.

detected between serum miR-122 levels and the NAS. Serum miR-122 expression in the liver showed a significant inverse correlation with fibrosis stage [correlation coefficient: −0.316 (−0.543 to 0.048); $P = 0.0218$] (Table 3). Moreover, serum miR-122 levels were significantly higher in patients with mild fibrosis than in those with severe fibrosis (mild/severe = $0.008 \pm 0.011/0.002 \pm 0.004$; $P = 0.0191$) (Fig. 3).

To compare the ability of the blood tests to predict the fibrotic stage, we constructed receiver operating characteristics (ROC) curves for serum miR-122, hyaluronic acid and type IV collagen; the area under the ROC curves for miR-122, hyaluronic acid and type IV collagen were 0.82, 0.74 and 0.72, respectively (Fig. 4).

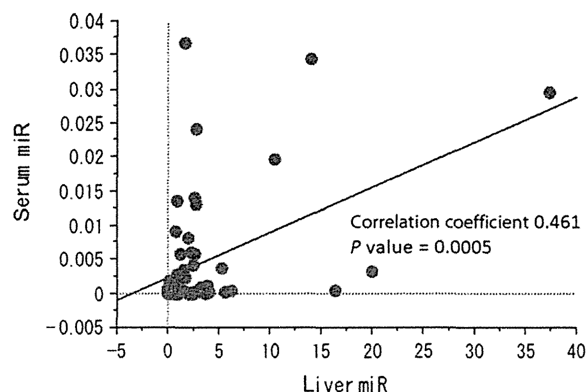

Fig. 1. Correlation between liver and serum miR-122 levels. The serum miR-122 levels were significantly correlated with hepatic miR-122 levels (Spearman correlation coefficient: 0.461; $P = 0.0005$).

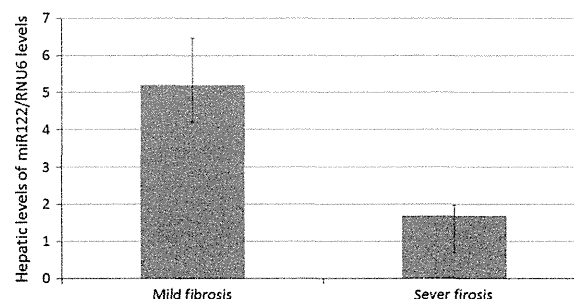

Fig. 2. Correlation between hepatic miR-122 level and the fibrosis stage. Comparisons between groups were performed using the Student's *t*-test ($P = 0.0087$).

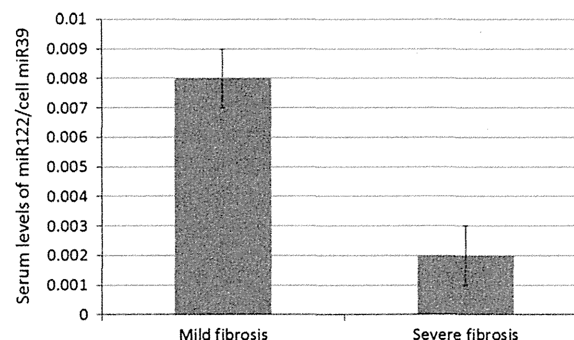

Fig. 3. Correlation between serum miR-122 level and the fibrosis stage. Comparisons between groups were performed using the Student's *t*-test ($P = 0.0191$).

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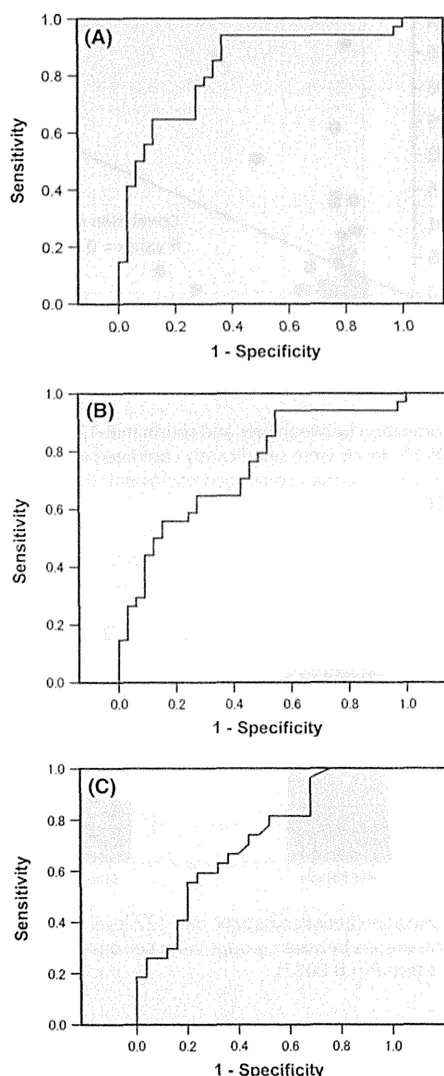

Fig. 4. Receiver operating characteristic (ROC) curve for serum miR-122, hyaluronic acid and Type IV collagen. The area under the ROC curve for serum miR-122 (A), hyaluronic acid (B) and type IV collagen (C) are 0.82, 0.74 and 0.72, respectively.

Discussion

Recent studies have indicated the value of the miR-122 level as a predictive factor of liver disease (27–30). The progression of NAFLD is associated with visceral fat deposition and insulin resistance. miR-122 is a key factor of lipid metabolism (23, 24). In the present study, patients with severe fat deposition showed high miR-122 expression levels in the liver. The role of miR-122 in lipid metabolism has been demonstrated in vitro and in vivo. In in vitro studies using HEP G2 cells, silencing of miR-122 led to the upregulation of the expression of lipid metabolism genes such as fatty acid synthase (FAS), 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and sterol binding element binding protein (SREBP), whereas overexpression of miR-122 led to a significant decrease in the levels of these genes (31). In *in vivo* studies, inhibition of miR-122 expression in mice led to the promotion of hepatic fatty acid (FA) oxidation, decreased FA levels, and decreased liver steatosis (23). Thus, these results support our finding that the expression of miR-122 is correlated with liver steatosis.

However, the liver and serum miR-122 levels did not correlate with the NAS and alanine aminotransferase levels. Several recent studies showed that the miR-122 level is associated with liver inflammation (27–29), which was not observed in the present study. However, the previous studies included patients with other liver diseases such as viral hepatitis. In the present study, most of patients had mild inflammation, which may contribute to the lack of a significant difference in miR-122 expression. Moreover, the NAS—established as a scoring system for NAFLD—evaluates not only inflammation but also steatosis. Thus, this discrepancy could be attributed to the different categories of liver disease included in each study.

In the present study, liver miR-122 levels significantly correlated with the liver fibrosis stage. This result is in agreement with those of previous studies, which reported a decrease in liver miR-122 levels at the later stage of fibrosis in patients with liver disease (27–29). Persistent liver injury results in liver cell death, loss of hepatic cells and the accumulation of extracellular matrix. Moreover, the liver miR-122 levels did not correlate with the NAS, which was reflected the inflammation grade of the NAFLD patients. However, hepatocytes are the main source of miR-122. Thus, the progression of liver fibrosis results in the replacement of hepatocytes by extracellular matrix, and thus leads to a decrease in the levels of hepatic miR-122.

Recently, Li *et al.* reported that miR-122 suppressed collagen maturation in hepatic stellate cells and inhibited the proliferation of activated hepatic stellate cells (32). Therefore, decreased miR-122 expression appears to lead to increased collagen maturation and extracellular matrix production, which is consistent with the present results.

In the present study, decreased serum miR-122 levels were detected in association with mild steatosis and advanced fibrosis stage. These results are similar to those noted for hepatic miR-122 expression. Moreover, serum miR-122 expression was well-correlated with hepatic miR-122 expression, which suggests that the miR-122 released from hepatic cells enters into the bloodstream.

The evaluation of liver fibrosis is important to predict the prognosis of patients with NAFLD. Follow-up liver biopsies or repeat liver stiffness assessment is currently necessary to assess liver fibrosis. However, these methods have some limitations. Liver biopsy is an invasive technique and is associated with certain complications

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(33, 34). In addition, the utility of liver stiffness measurement is low in obese patients and in those with ascites and hepatic inflammation (35, 36). In the present study, serum miR-122 levels inversely correlated with liver fibrosis, and decreased miR-122 expression was associated with advanced fibrosis stage. Moreover, the ROC curves showed that the ability of the serum miR-122 to predict fibrosis was superior to that of hyaluronic acid and type IV collagen. Therefore, serum miR-122 may be a valuable tool to predict liver fibrosis.

In conclusion, hepatic and serum miR-122 levels are associated with hepatic steatosis and fibrosis, and the serum miR-122 level can serve as a useful predictive marker of liver fibrosis in patients with NAFLD.

Acknowledgements

Conflicts of interest: The authors do not have any disclosures to report.

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下まで低下，24週間の治療を完遂しSVRが得られた（Fig. 右）．

考案と結論：IFN-βはうつ病を合併したIFN-α製剤不耐容のC型肝炎難治例に対する有効性と安全性が確認されており[1]，うつ病の既往・合併，うつ病の疑いのある症例に対してはIFN-β＋RBV併用療法が考慮されるべきとされている[2]．しかしセロタイプ2型でうつ症状の副作用が懸念される症例に関しては充分な検討はなされていない．一方，統合失調症に対するIFN治療に関しては米国の後ろ向き研究によりは禁忌にはならないと報告されているが[3]，本邦では報告例は少ない．斉藤らの統合失調症を合併したセロタイプ2型の3例にPegIFN-α(±RBV)を用いた治療を行い全例にSVRを認めた報告[4]があるが，IFN-βの使用も含めさらなる症例報告の蓄積が必要であると考える．2014年度中にプロテアーゼ阻害剤とNS5A阻害剤による経口2剤治療の承認が予定されておりゲノタイプIbに対して高いSVR率が期待されている．また，近い将来本邦でもセロタイプ2型に対する経口剤治療が可能になると目されているが，いずれの新薬においても耐性ウイルスの出現，治療抵抗例が懸念される．副作用が少ない経口剤による治療を希望する患者にはこれまでIFN不耐容群とされてきた症例も少なくないと考えられるため，将来のC型慢性肝炎治療の選択肢としてIFN-βの治療経験を蓄積しておくことが必要であると考える．

索引用語：C型慢性肝炎，インターフェロンβ，精神疾患

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本論文内容に関連する著者の利益相反：なし

英文要旨

Interferon-β therapy for five chronic hepatitis C patients with mental disorder

Hidetaka Shibata[1)*], Moto Kitayama[1)], Yasuhiro Kamo[1)], Takuya Honda[1)], Satoshi Miuma[1)], Hisamitsu Miyaaki[1)], Naota Taura[1)], Tatsuki Ichikawa[1)2)], Kazuhiko Nakao[1)]

We report of interferon-β (IFN-β) therapy with or without ribavirin in five patients with chronic hepatitis and mental disorder. Two of three patients diagnosed with depression and one of two patients diagnosed with chronic schizophrenic psychosis had developed a viral response after IFN-β therapy. Symptoms of depression or psychosis are well-known side effects of IFN-α therapy. Therefore, patients with mental disorders are often hesitant toward receiving interferon therapy. Direct acting antivirals (DAAs) will probably become mainstream therapy for IFN-α intolerant patients in the near future. However, the risk of developing mutations related to drug resistance should not be neglected. Therefore, experience with IFN-β therapy used as an alternative to DAAs is required.

Key words: chronic hepatitis type C, interferon-β, mental disorder

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World Journal of Surgery

Laparoscopy-Assisted Hybrid Left-Side Donor Hepatectomy: Rationale for Performing LADH

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We are pleased to have the opportunity to respond to the letter to the editor and would like to thank Dr. Ishizawa and his colleagues for their interest in our study and very important comments. We agree with their concerns about donor safety. As we have stated in our article [1], the most important issue in donor hepatectomy is 'donor safety'. If donor safety is threatened for any reason in a new procedure as compared with the traditional procedure, it would not be acceptable.

On the other hand, most liver donors experience postoperative difficulties arising from the mental and physical changes caused by a big skin incision [2]. As surgeons, we cannot help but consider alleviation of such stress in donors by making efforts to minimize the skin incision, while ensuring donor safety.

Fortunately, owing to the development of surgical devices and the accumulating experience of surgeons, laparoscopic hepatectomy has become established and is now widely practiced routinely all over the world. Among the techniques for laparoscopic hepatectomy, the hybrid technique is a combination of laparoscopic mobilization of the liver and open hepatectomy under direct vision through the skin incision, and that carries the benefits of both safety and minimal invasiveness. One can easily realize after sufficient experience in using the hybrid technique that a large skin incision is not necessary for performing hepatectomy after the liver is adequately mobilized from the retroperitoneum, which is the point of this procedure.

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Although we experienced two incidental injury episodes, it was not difficult to manage them promptly under direct vision through a skin incision. Besides, we were always prepared to extend the incision to perform traditional open donor hepatectomy in the event of any unexpected trouble with the laparoscopy-assisted hybrid donor hepatectomy (LADH) procedure.

In view of the live liver donor mortalities and potentially life-threatening events reported in the literature [3], we cannot be too careful when securing donor safety during surgery.

We again emphasize our approach to establishing the new technique. First, we already had adequate experience of both open donor hepatectomy and laparoscopic hepatectomy when we started this study [4, 5]. We analyzed the incidence of morbidities in our open donor surgery [4], and found that the operative time was shortened and the blood loss decreased according to the surgeons' experience; furthermore, we have not encountered any case of bile leak in donor hepatectomy since 2007. We have performed a sufficient number of laparoscopic hepatectomies, including hybrid hepatectomy [5], so that our center was approved as one of the leading centers for highly advanced medical treatment (laparoscopy-assisted hepatectomy) by the Ministry of Health, Labour and Welfare, Japan, in April 2008. We think that mastery of both donor hepatectomy and laparoscopic hepatectomy is a minimal requirement for safe performance of LADH. Second, we have taken a step-by-step approach to introducing LADH from left lateral sectionectomy to left lobectomy. Left lateral sectionectomy in LADH was simpler than left lobectomy in terms of mobilization of the liver and parenchymal dissection. In fact, our experience in left lateral sectionectomy was quite useful in performing left lobectomy. This step-by-step approach is quite important

when introducing any new technique. Third, this study was conducted with the approval of the institutional review board after discussing the ethics of performing LADH. Furthermore, the risks of LADH as well as of donor surgery were explained to the donor and his/her family in detail, and informed consent was obtained from each donor.

Thus, we carefully planned the application of LADH using these three approaches in our study, as described in the article.

We do not propose that all donor surgeries should be changed to LADH based on the results of our study. Careful approaches and the best practice of each surgical team, needless to say, are necessary in live-donor hepatectomy to minimize morbidity. Another important message from our study is our belief that only experienced surgical teams can be allowed to perform LADH safely and effectively.

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