blinded to patient clinical and biochemical data. Diagnosis of each case was independently and histologically confirmed by liver pathologists according to the Japanese chronic hepatitis classification (New Inuyama classification). Furthermore, we scored the degree of fat deposition in the liver. Histological characteristics of the patients are also shown in Table 1.

Methods

RNA isolation

Total RNA containing miRNA was isolated from formalin-fixed paraffin-embedded (FFPE) liver biopsy specimens using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol.

Quantitative reverse transcription polymerase chain reaction (PCR)

miR-122 expression was quantified using TaqMan MicroRNA assays (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocols. Reverse transcription was performed using 10 μg of RNA isolated from the liver FFPE specimens. Quantitative PCR was performed using the Light Cycler 480 system (Roche Diagnostics, Basel, Switzerland). miR-122 expression was calculated by the relative standard curve method and normalized to RNU6B expression. The *IL28B* SNP rs8099917 was also examined. SNP were detected by pyrosequencing analysis. The sense, antisense and pyrosequencing primers used were B-TCCTCCTTTTGTTTTCCTTTCTG, AAAAAGCCAGC TACCAAACTGT, and TGGTTCCAATTTGGG, respectively, where "**B**" indicates a biotin-labeled sequence.

Statistical analysis

Data were processed on a personal computer and analyzed using StatFlex (Artech, Tokyo, Japan). Differences in each laboratory parameter were analyzed using the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Correlation of miR-122 expression with virological response to IFN therapy

WE COMPARED MIR-122 expression between *IL28B* rs8099917 TT and TG/GG. No significant difference was observed in hepatic miR-122 expression between *IL28B* SNP TT and TG/GG (Fig. 1a). We also compared miR-122 expression and stage of fibrosis. Although there was a tendency toward a decrease in miR-122 expression as fibrosis progressed, no statistically significant difference was detected (Fig. 1b). In terms of virological response to IFN therapy, a significant difference was observed in the extent of miR-122 expression and the number of patients who were classified as achieving an SVR or not ($P < 0.05$, Fig. 1c). We also investigated the correlation of miR-122 expression with SVR, transient response (TVR) and no response (NVR). Significant differences were observed between SVR and TVR ($P < 0.05$) and between SVR and NVR ($P < 0.05$) (Fig. 1d). As stated above, decrease of miR-122 was associated with progression of fibrosis. Therefore, we thought to compare the correlation of miR-122 expression to viral response to INF in patients matched for stage of fibrosis. Although we found that patients achieving SVR were likely to have higher expression of miR-122 than those who failed to achieve SVR, statistical difference was not observed due to limited patient numbers.

Correlation of miR-122 expression with rapid/early virological response

miR-122 expression was significantly higher in patients with a strong response to IFN and undetectable HCV RNA at week 4 (i.e. those with a rapid virological response [RVR]) than in patients who did not achieve RVR (Fig. 2a, $P < 0.05$). Patients who responded to IFN therapy and who had undetectable HCV RNA at week 12 were regarded as achieving an early virological response (EVR). miR-122 expression showed the same tendency in these patients, namely, it was higher among those with EVR than among those without such a response (Fig. 2b, $P < 0.05$). No significant correlation was observed between miR-122 expression and several other clinical parameters including 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 (Table 2).

Fat deposition in the liver was associated with miR-122 expression

In addition, we scored the degree of fat deposition in liver biopsy specimens and examined its correlation with miR-122 expression. miR-122 expression significantly decreased as the extent of fat deposition in the liver increased (Fig. 3a, $P < 0.05$). We then divided the patient's biopsy specimens into groups based on the extent of fat deposition (0–5% and >5%) and determined their association with non-alcoholic fatty liver

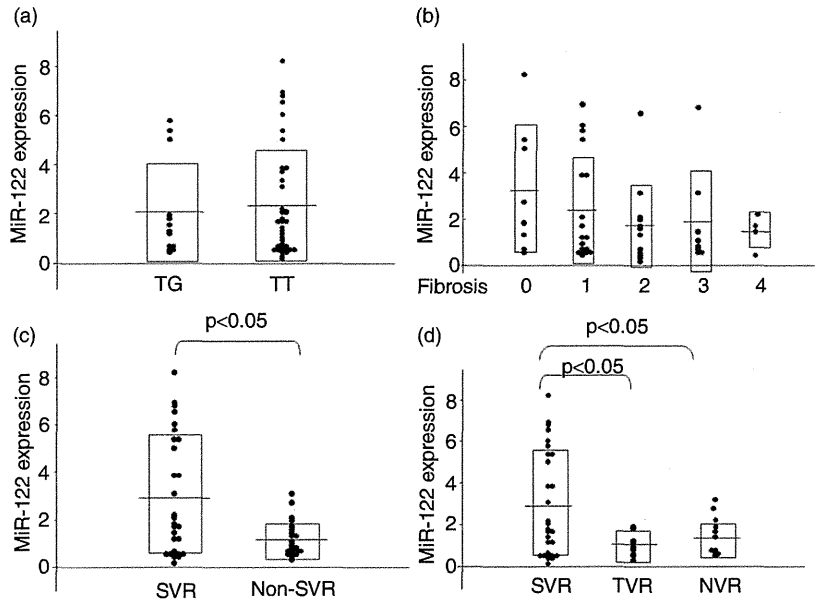

Figure 1 Association between hepatic miRNA-122(miR-122) expression of serotype 1 hepatitis C virus (HCV)-infected liver and IL28B single nucleotide polymorphisms (SNP). There is no correlation with miR-122 expression between IL28B SNP TT and TG/GG (a). Also, we compared with the fibrotic stage. Although 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).

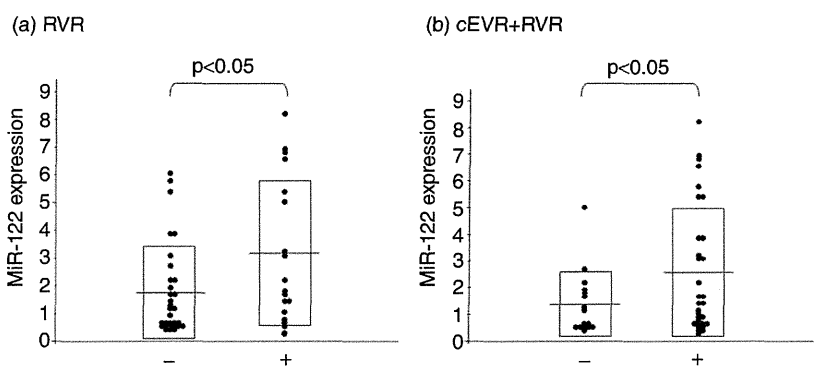

Figure 2 Correlation between miR-122 expression and rapid (RVR)/early virological response (EVR). RVR was defined as patients who respond to interferon (IFN) therapy with a decrease in viral load at week 4. EVR was defined as patients who respond to IFN therapy with a decrease in viral load at week 12. 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.

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-

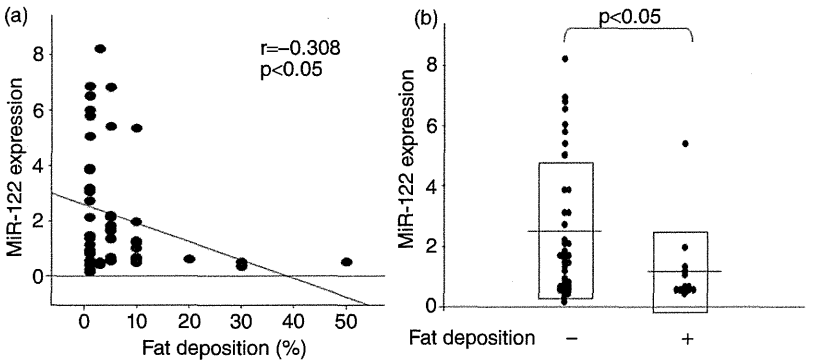

Figure 3 miRNA122 (miR-122) expression was correlated with fat deposition in liver. By using liver biopsy specimen, we scored the degree of fat deposition in liver and examined the relationship miR-122 expression. miR-122 expression significantly decreased as the extent of fat deposition in the liver 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

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 ($\times 10^4/\mu L$)	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/m^2)	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 ($\times 10^4/\mu L$)	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, $\times 10^4$ 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%.

CI, confidence interval; HCV, hepatitis C virus; IFN, interferon; NR, undetectable HCV RNA; NVR, non-virological response; RBV, ribavirin; SVR, sustained virological response.

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.

REFERENCES

1 Ascione A, Tartaglione T, Di Costanzo GG. Natural history of chronic hepatitis C virus infection. *Dig Liver Dis* 2007; **39** (Suppl 1): S4–7.
2 Bruno S, Facciotto C. The natural course of HCV infection and the need for treatment. *Ann Hepatol* 2008; **7**: 114–9.
3 Forestier N, Zeuzem S. Telaprevir for the treatment of hepatitis C. *Expert Opin Pharmacother* 2012; **13**: 593–606.
4 Vachon ML, Dieterich DT. The era of direct-acting antivirals has begun: the beginning of the end for HCV? *Semin Liver Dis* 2011; **31**: 399–409.
5 Quer J, Buti M, Cubero M, Guardia J, Esteban R, Esteban JI. New strategies for the treatment of hepatitis C virus infection and implications of resistance to new direct-acting antiviral agents. *Infect Drug Resist* 2010; **3**: 133–45.
6 Sarrazin C, Hézode C, Zeuzem S, Pawlotsky JM. Antiviral strategies in hepatitis C virus infection. *J Hepatol* 2012; **56** (Suppl 1): S88–100.
7 Asselah T, Marcellin P. New direct-acting antivirals' combination for the treatment of chronic hepatitis C. *Liver Int* 2011; **31** (Suppl 1): 68–77.
8 Mira JA, Valera-Bestard B, Arizcorreta-Yarza A *et al.* Rapid virological response at week 4 predicts response to pegylated interferon plus ribavirin among HIV/HCV-coinfected patients. *Antivir Ther* 2007; **12**: 523–9.
9 Shirakawa H, Matsumoto A, Joshita S *et al.* Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. *Hepatology* 2008; **48**: 1753–60.
10 Asselah T, Estrabaud E, Bieche I *et al.* Hepatitis C: viral and host factors associated with non-response to pegylated interferon plus ribavirin. *Liver Int* 2010; **30**: 1259–69.
11 Harrison SA, Rossaro L, Hu KQ *et al.* Serum cholesterol and statin use predict virological response to peginterferon and ribavirin therapy. *Hepatology* 2010; **52**: 864–74.
12 Kitamura S, Tsuge M, Hatakeyama T *et al.* Amino acid substitutions in core and NS5A regions of the HCV genome can predict virological decrease with pegylated interferon plus ribavirin therapy. *Antivir Ther* 2010; **15**: 1087–97.
13 Kurosaki M, Tanaka Y, Nishida N *et al.* Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in IL28B and viral factors. *J Hepatol* 2011; **54**: 439–48.
14 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet 2009*; **41**: 1105–9.
15 He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004; **5**: 522–31.
16 Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281–97.
17 Ambros V. The functions of animal microRNAs. *Nature* 2004; **431**: 350–5.
18 Shen J, Cho CH. miRNA in the pathogenesis and Therapy for Gastrointestinal and Hepatic Cancer. *Curr Pharm Des* 2013; **19**: 1179.
19 Jopling CL, Norman KL, Sarnow P. Positive and negative modulation of viral and cellular mRNAs by liver-specific microRNA miR-122. *Cold Spring Harb Symp Quant Biol* 2006; **71**: 369–76.
20 Roberts AP, Lewis AP, Jopling CL. miR-122 activates hepatitis C virus translation by a specialized mechanism requiring particular RNA components. *Nucleic Acids Res* 2011; **39**: 7716–29.
21 Jangra RK, Yi M, Lemon SM. Regulation of hepatitis C virus translation and infectious virus production by the microRNA miR-122. *J Virol 2010*; **84**: 6615–25.
22 Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 2005; **309**: 1577–81.
23 Henke JI, Goergen D, Zheng J *et al.* microRNA-122 stimulates translation of hepatitis C virus RNA. *EMBO J 2008*; **27**: 3300–10.
24 Lanford RE, Hildebrandt-Eriksen ES, Petri A *et al.* Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 2010; **327**: 198–201.
25 Stenvang J, Petri A, Lindow M, Obad S, Kauppinen S. Inhibition of microRNA function by antimiR oligonucleotides. *Silence* 2012; **3**: 1.
26 Sarasin-Filipowicz M, Krol J, Markiewicz I, Heim MH, Filipowicz W. Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. *Nat Med* 2009; **15**: 31–3.
27 Watashi K, Ishii N, Hijikata M *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 2005; **19**: 111–22.
28 Okamoto T, Nishimura Y, Ichimura T *et al.* Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J* 2006; **25**: 5015–25.
29 Parent R, Qu X, Petit MA, Beretta L. The heat shock cognate protein 70 is associated with hepatitis C virus particles and modulates virus infectivity. *Hepatology* 2009; **49**: 1798–809.
30 Wang C, Gale M, Keller BC *et al.* Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol Cell* 2005; **18**: 425–34.

31 Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci U S A* 2005; **102**: 2561–6.
32 Yang W, Hood BL, Chadwick SL *et al.* Fatty acid synthase is up-regulated during hepatitis C virus infection and regulates hepatitis C virus entry and production. *Hepatology* 2008; **48**: 1396–403.
33 Herker E, Ott M. Unique ties between hepatitis C virus replication and intracellular lipids. *Trends Endocrinol Metab* 2011; **22**: 241–8.
34 Miyanari Y, Atsuzawa K, Usuda N *et al.* The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 2007; **9**: 1089–97.
35 Castoldi M, Vujic Spasic M, Altamura S *et al.* The liver-specific microRNA miR-122 controls systemic iron homeostasis in mice. *J Clin Invest* 2011; **121**: 1386–96.
36 Moore KJ, Rayner KJ, Suárez Y, Fernández-Hernando C. The role of microRNAs in cholesterol efflux and hepatic lipid metabolism. *Annu Rev Nutr* 2011; **31**: 49–63.
37 Rottiers V, Näär AM. MicroRNAs in metabolism and metabolic disorders. *Nat Rev Mol Cell Biol* 2012; **13**: 239–50.
38 Cheung O, Puri P, Eicken C *et al.* Nonalcoholic steatohepatitis is associated with altered hepatic MicroRNA expression. *Hepatology* 2008; **48**: 1810–20.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The morbidity and associated risk factors of cancer in chronic liver disease patients with diabetes mellitus: a multicenter field survey

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Abstract

Background and aims Diabetes mellitus is associated with various cancers; however, little is known of the relationship between cancer and diabetes in chronic liver disease (CLD) patients. The aim of this study is to investigate the morbidity and associated factors of cancer, including the use of anti-diabetics, in CLD patients with diabetes.

Patients and methods We performed a multicenter survey in 2012 and 478 CLD patients with diabetes were enrolled (age 64.3 ± 12.1 years, female/male 187/291). A frequency analysis of cancer and antidiabetic use was performed. Independent factors for cancer were analyzed using logistic regression and decision-tree analysis.

Results The morbidity of cancer was 33.3 %. Hepatocellular carcinoma (HCC) and extra-hepatic cancer were diagnosed in 24.7 and 11.3 % of enrolled patients, respectively. The frequency of antidiabetic use was 66.5 %. Of prescribed antidiabetics, 39 % were dipeptidyl-peptidase 4 inhibitors; however, their use was not significantly associated with cancer. In contrast, the use of exogenous insulin (OR 2.21; 95 % CI 1.16–4.21, $P = 0.0165$) and sulfonylurea (OR 2.08; 95 % CI 1.05–3.97, $P = 0.0353$) were independently associated with HCC and extra-hepatic cancer, respectively. In decision-tree analysis, exogenous insulin and sulfonylurea were also identified as a divergence factor for HCC and extra-hepatic cancer, respectively.

Conclusions We found a high morbidity of not only HCC, but also extra-hepatic cancer in CLD patients with diabetes. We also showed a possible association between the use of antidiabetics and the morbidity of cancer. Thus, a large-scale cohort study is needed to establish a therapeutic strategy for diabetes to suppress carcinogenesis in CLD patients.

Keywords Diabetes mellitus · Chronic liver disease · Morbidity · Risk factor

Abbreviations

HCC	Hepatocellular carcinoma
CLD	Chronic liver disease
DPP-4	Dipeptidyl peptidase-4
AST	Aspartate aminotransferase
APRI	AST to platelet ratio index
ALT	Alanine aminotransferase
GGT	Gamma-glutamyl transpeptidase
HbA1c	Hemoglobin A1c

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HCV Hepatitis C virus
HBV Hepatitis B virus
MAPK Mitogen-activated protein kinase
IGF Insulin-like growth factor

Introduction

Diabetes mellitus is a known independent risk factor for a number of different cancers [1]. Recently, population-based studies and meta-analyses demonstrated that diabetes mellitus is a potent risk factor for hepatocellular carcinoma (HCC) [2, 3]. In addition, diabetes mellitus is a risk factor for extra-hepatic cancers including pancreatic cancer, bile duct cancer, and colon cancer [4–6], and is also known to increase the risk of other extra-hepatic cancers, including gynecologic cancers, respiratory tumors, and hematological malignancies [7–9].

Diabetes mellitus consists of a number of diverse diseases, including impaired insulin secretion and insulin resistance. Patients with chronic liver disease (CLD) often develop increased insulin resistance and pancreatic β cells consequently secrete excess insulin in order to maintain glucose homeostasis [10, 11]. Thus, hyperinsulinemia is a feature of CLD patients with diabetes. Insulin is a potent mitogen and promotes cell proliferation [12], and hyperinsulinemia is a risk factor for the development of cancer in patients with diabetes mellitus [1, 13]. These previous findings suggest a possible association between cancer and diabetes in CLD patients; however, no practical data are available for the morbidity of cancer in CLD patients with diabetes.

Established risk factors for carcinogenesis include age, sex, smoking, excessive alcohol intake, and chronic viral infection [14]. In addition, we, along with others, have reported a possible association between the use of anti-diabetic agents and carcinogenesis [15, 16]. The use of sulfonylurea, an insulin secretagogue, and exogenous insulin are associated with HCC and extra-hepatic cancers including pancreatic cancer, colon cancer, and breast cancer [15–18]. Recently, dipeptidyl peptidase-4 (DPP-4) inhibitor has become widely used to treat diabetes mellitus because of its ability to lower glucose levels with a low risk of hypoglycemia; however, a possible association between the use of DPP-4 inhibitors and cancer has never been investigated in CLD patients with diabetes.

The aims of this study were to investigate the morbidity of cancer and cancer-associated factors, including the use of anti-diabetics, in CLD patients with diabetes.

Subjects and methods

Ethics

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, as reflected in the prior approval given by each institutional review board. None of the subjects were institutionalized.

Study design

In 2012, we performed a multicenter cross-sectional study to investigate the morbidity of cancer and cancer-associated factors, including the use of anti-diabetics, in CLD patients with diabetes.

Subjects

Inclusion criteria were patients with (1) 20 years of age or more, (2) CLD complicated with diabetes mellitus, and (3) regular medical consultations with a hepatologist. Exclusion criteria were (1) type 1 diabetes mellitus, juvenile diabetes mellitus, or gestational diabetes mellitus, (2) severe pancreatitis, (3) adrenal gland disease, (4) pituitary disease, and (5) a gonadal disorder. We enrolled 478 CLD patients with diabetes in this study from five medical institutions in Japan.

Definition of CLD and its etiology

Regardless of the etiology of liver disease, chronic liver disease was diagnosed on the basis of hepatic inflammation that had lasted for more than 6 months, and findings of histopathology, ultrasonography, computed tomography, or magnetic resonance imaging.

The etiology of CLD was examined by biochemical tests, imaging examinations, and/or liver biopsy as previously described [19–23]. Briefly, chronic hepatitis C was diagnosed by positive results of anti-hepatitis C virus (HCV) and/or HCV RNA [20]. Chronic hepatitis B was diagnosed by positive results of hepatitis B surface antigen and/or hepatitis B virus (HBV) DNA [20]. Autoimmune hepatitis was diagnosed by the Diagnostic Criteria of the International Autoimmune Hepatitis Group [21]. Primary biliary cirrhosis was diagnosed based on the Clinical Guideline of Primary Biliary Cirrhosis by the Intractable Hepato-Biliary Disease Study Group [22]. Non-alcoholic fatty liver disease was diagnosed based on the Practice Guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology [19]. Alcoholic liver disease was diagnosed according to the Diagnostic

Criteria of Alcoholic Liver Disease by the Japanese Society for Biomedical Research on Alcohol [23].

Definition of liver cirrhosis

Liver cirrhosis was diagnosed by aspartate aminotransferase (AST) to platelet ratio index (APRI); serum AST level (U/L)/upper limit of normal AST (33 U/L) × 100/platelet count ($\times 10^6$/mL). APRI is a noninvasive index and can predict liver cirrhosis. Patients with APRI values above 2 were diagnosed as with liver cirrhosis as previously described [24].

Definition of diabetes mellitus

Diabetes mellitus was diagnosed on the basis of fasting blood glucose levels >126 mg/dL or HbA1c levels >6.5 % according to the Diagnostic Criteria for Diabetes Mellitus [25], or by the use of anti-diabetic agents.

Definition of cancer

Cancer was defined as any type of malignant neoplasm including epithelial and non-epithelial tumors. The diagnosis of cancer was based on finding(s) of histopathology and/or by a combination of serum tumor makers and imaging procedures such as ultrasonography, computed tomography, magnetic resonance imaging, endoscopy, and/or angiography.

Diagnosis of HCC

HCC was diagnosed by a combination of tests for serum tumor makers such as alpha-fetoprotein and des-gamma-carboxy prothrombin, and imaging procedures such as ultrasonography, computed tomography, magnetic resonance imaging, and/or angiography.

Definition of extra-hepatic cancer, digestive cancer, and non-digestive cancer

Extra-hepatic cancer was defined as cancer in any organ except for the liver, and was further classified as either digestive cancer or non-digestive cancer. Digestive cancer was defined as cancer in the oral cavity, esophagus, stomach, colon, gallbladder, or pancreas. Cancer other than digestive cancer was defined as non-digestive cancer. The diagnosis of each cancer was based on finding(s) of histopathology and/or by a combination of serum tumor makers and imaging procedures such as ultrasonography, computed tomography, magnetic resonance imaging, endoscopy, and/or angiography.

Definition of cardiovascular event

A cardiovascular event was defined as acute myocardial infarction or stroke, the diagnosis of which was based on clinical symptoms and findings of electrocardiogram recordings, biochemical tests, echocardiography, coronary angiography, computed tomography, or magnetic resonance imaging as previously reported [26].

Diagnosis of diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy

Diagnosis of diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy was based on findings of urine and biochemical tests, ophthalmoscopy, tendon reflex tests, and vibration sense tests as previously described [27–29].

Database

Using on medical records, a database of 478 CLD patients with diabetes was created on the basis of the following six categories:

Category 1: age, sex, body mass index, and blood pressure.
Category 2: any type of cancer, HCC, extra-hepatic cancer, digestive cancer, and non-digestive cancer.
Category 3: cardiovascular disease, diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy.
Category 4: chronic hepatitis C, chronic hepatitis B, non-alcoholic fatty liver disease, alcoholic liver disease, autoimmune hepatitis, and primary biliary cirrhosis.
Category 5: platelet count, serum AST level, serum alanine aminotransferase (ALT) level, serum gamma-glutamyl transpeptidase (GGT) level, serum albumin level, serum total bilirubin level, prothrombin activity, serum total cholesterol level, and serum triglyceride level.
Category 6: disease duration of diabetes mellitus, fasting blood glucose level, blood hemoglobin A1c (HbA1c; National Glycohemoglobin Standardization Program; NGSP), and use of a DPP-4 inhibitor, sulfonylurea, exogenous insulin, α-glucosidase inhibitor, biguanide, glinide, thiazolidine, and glucagon-like peptide 1 agonist.

Statistical analysis

Data are expressed as the number or mean ± standard deviation (SD). Nonparametric comparisons were made using the Wilcoxon signed-rank test, and categorical comparisons were made using Fisher's exact test. Independent factors for cancer were analyzed using logistic regression and decision-tree analysis as described