Table 2 Postoperative complications

	Hx ($n = 29$)	PD ($n = 32$)	HPD ($n = 14$)	p value*
Morbidity	10 (34)	14 (44)	8 (57)	.37
Liver failure	4 (14)		3 (21)	
Pancreatic fistula		8 (25)	3 (21)	
Bile leak	3 (10)		3 (21)	
Abscess	2 (7)	1 (3)	1 (7)	
Pleural effusion/ascites	2 (7)		2 (14)	
Sepsis	1 (3)	1 (3)		
DGE		2 (6)	1 (7)	
Cerebral infarction		2 (6)		
Mortality (in-hospital)	3 (10)	1 (3)	0	.28
Hospital stay, days (median)	32	35	44	.40

Numbers in parentheses indicate the percentages

DGE delayed gastric empting

* p values compare variables among the three surgical methods

Table 3 Histopathologic factors

	Hx ($n = 29$)	PD ($n = 32$)	HPD ($n = 14$)	p value*
Tumor grade				
wel[a]	9 (31)	13 (41)	5 (36)	.12
mod[b]	19 (66)	15 (47)	5 (36)	
por[c]	1 (3)	4 (13)	4 (29)	
Perineural invasion	23 (79)	16 (50)	11 (79)	.031
Arterial invasion	11 (38)	3 (9)	3 (21)	.029
Portal vein invasion	8 (28)	4 (12)	2 (14)	.29
Node involvement	16 (55)	12 (38)	6 (43)	.38
Positive ductal margin	8 (28)[d]	2 (6)	2 (14)	.075
Positive radial margin	3 (10)	4 (13)	2 (14)	.93
R-classification				
R0	17 (59)	27 (84)	9 (64)	.074
R1/2	12 (41)	5 (16)	5 (36)	
T-classification				
≤2	5 (17)	9 (28)	7 (50)	.081
3, 4	24 (83)	23 (72)	7 (50)	
Stage				
0, I	5	8	5	.58
II	12	16	5	
III	5	4	1	
IV	7	4	3	

Numbers in parentheses indicate the percentages

* p values compare variables among three surgical methods

[a] Well differentiated, including papillary adenocarcinoma

[b] Moderately differentiated

[c] Poorly differentiated

[d] Six hepatic side bile ducts, 2 duodenal side bile ducts

severe liver failure developed in 4 patients undergoing Hx, contributing to the relatively high mortality rate in this group. Liver failure and bile leakage rates were higher in the HPD group than the Hx group. The pancreatic fistula rate and DGE rate did not differ between the HPD group and the PD group. Reoperation was required in 1 patient after PD for refractory bleeding due to rupture of the gastroduodenal artery secondary to pancreatic fistula and in a second patient following HLPD who underwent re-anastomosis of the pancreaticojejunostomy due to a sustained pancreatic fistula requiring readmission (5 months after HLPD). The causes of mortality were liver failure in 2 patients (on POD 32 and POD 116) and sepsis due to cholangitis in 1 patient (on POD 44) in the Hx group, and cerebral infarction in 1 patient (POD 42) in the PD group who suffered from prolonged DGE. Hospital stay was approximately 10 days longer after HPD than after the other procedures, but the differences in hospital stay between groups were not significant.

Patency of the vascular reconstruction was questionable in 2 patients who underwent HAR; blood flow was not seen postoperatively at the anastomotic site. However, there were no associated complications because collateral flow was well established. There were no complications in patients with PVR in this study.

Histopathologic factors

Histopathologic features of the tumors were compared between groups. Tumor grade, portal vein invasion, nodal involvement, surgical margin status, R-classification, UICC T-classification, and UICC stage did not differ significantly between groups (Table 3). Perineural invasion and associated arterial invasion occurred more frequently in the Hx and HPD groups than in the PD group ($p = .031$ and $p = .029$, respectively). Cancer invasion into the portal vein wall was histologically proven in 14 of 22 patients (64%) who underwent PVR. Although curability did not differ statistically between groups, R0 (histologically curative) resection was achieved in 84% of patients who underwent PD, but in only 59% of patients who underwent Hx and 64% of those who underwent HPD. Histologically positive bile duct margins (R1 resection) were present in 8 patients who underwent Hx, but in only 2 patients each who underwent HPD and PD. Radial margins were comparable between groups. Histologic para-aortic lymph nodes metastases accounted for R2 resections (macroscopically residual cancer) in 3 patients who underwent Hx, 3 patients who underwent PD, and 2 patients who underwent HPD, with no statistical differences between groups. Other factors contributing to R2 resection were liver metastases (Hx group, $n = 3$; HPD group, $n = 1$) and localized peritoneal dissemination around the PTBD catheter (Hx group, $n = 2$).

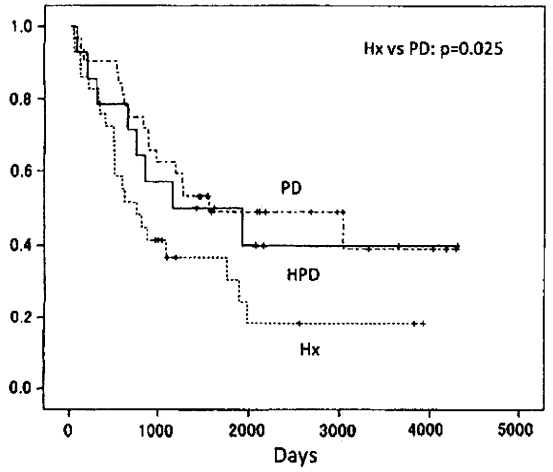

Fig. 3 Cumulative survival of patients according to operative procedure. *Hx* hepatectomy, *PD* pancreatoduodenectomy, *HPD* hepatopancreatoduodenectomy

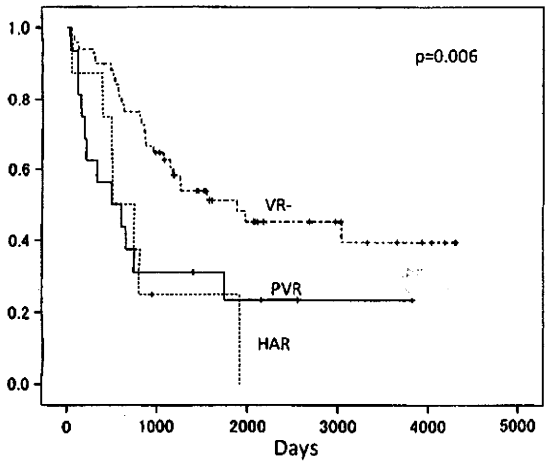

Fig. 4 Cumulative survival of patients according to vascular reconstruction. *VR–* no vascular reconstruction, *PVR* portal vein reconstruction, *HAR* hepatic artery reconstruction

Survival

Overall median survival time was 39 months, and the 5-year survival rate was 42%. Median survival time and 5-year survival per group were as follows: 24 months and 31% in the Hx group, 51 months and 49% in the PD group, and 63 months and 50% in the HPD group (Fig. 3). The difference in survival rate between the Hx group and the PD group was significant ($p = .025$), but that between the Hx group and the HPD group was not ($p = .21$).

Five-year survival rates were comparable between patients who underwent PVR (23%) and those who underwent HAR (25%), but the 5-year survival rate was substantially increased (51%) for patients who did not undergo vascular reconstruction (Fig. 4).

According to the types of invasion in HPD, survival time associated with each of 3 types of cancer spread did not differ significantly ($p = .78$), and 5-year survival rates were the same (50% each).

Sites of recurrence were identified in 17 patients after Hx, 15 patients after PD, and 7 patients after HPD. Liver metastases occurred in 3 patients (18%) after Hx, 5 patients (33%) after PD, and 2 patients (29%) after HPD. Local recurrence (with or without peritoneal dissemination) was the most prevalent pattern of recurrence, seen in 12 patients (71%) after Hx, 6 patients (40%) after PD, and 4 patients (57%) after HPD. Other types of recurrence were lymph

Table 4 Univariate analysis of risk factors for all patients

	No. (months)	MST (%)	5-year survival	*p* value
Tumor grade				
wel[a]	27	100	57	.079
mod[b]	39	28	33	
por[c]	9	21	33	
Perineural invasion				
No	25	–	75	<.0001
Yes	50	24	25	
Arterial invasion				
No	58	58	50	<.0001
Yes	17	19	18	
Portal vein invasion				
No	61	51	47	.003
Yes	14	24	14	
Node involvement				
No	41	100	55	.0012
Yes	34	24	23	
Positive ductal margin				
No	63	51	47	.011
Yes	12	20	13	
Positive radial margin				
No	66	51	48	<.0001
Yes	9	19	0	
R-classification				
R0	53	63	56	<.0001
R1/2	22	19	12	
T-classification				
≤2	21	–	71	.009
3, 4	54	28	30	

MST median survival time

[a] Well differentiated including papillary adenocarcinoma

[b] Moderately differentiated

[c] Poorly differentiated

node metastasis ($n = 11$), lung metastasis ($n = 2$), and bone metastasis ($n = 1$) (including the overlapping data).

Risk factors

Univariate analysis showed perineural invasion ($p < .0001$), arterial invasion ($p < .0001$), R classification (R1/2 versus R0, $p < .0001$), a positive radial margin ($p < .0001$), nodal involvement ($p = .0012$), portal vein invasion ($p = .003$), UICC T classification (T3/4 versus T0/1/2, $p = .009$), and a positive ductal margin ($p = .011$) to significantly influence survival (Table 4). Absence of perineural invasion was associated with a high 5-year survival rate (75%). Patients with lymph node metastases had relatively good survival, with a median survival time of 24 months and a 5-year survival rate of 23%. When positive nodes were present in the *hepatoduodenal ligament* ($n = 13$) and *peripancreatic space* ($n = 13$) mean survival times were 35 and 31 months, respectively, but when positive *para-aortic* ($n = 8$) nodes (leading to R2 resection) were present, mean survival was only 16 months, with no patient living more than 2 years.

When factors shown by univariate analysis to be statistically significant were entered into the Cox regression model, only perineural invasion (relative risk [RR], 3.39; 95% confidence interval [CI] 1.40–8.19; $p = .007$) and R classification (RR, 2.30; 95%CI, 1.16–4.53; $p = .017$) were shown to be independent predictors of outcome.

Discussion

Many institutions are now starting to treat perihilar and distal cholangiocarcinomas by hepatectomy with either bile duct resection or PD as an attempt at curative resection [1–6]. Bile duct resection alone should be restricted to early-stage papillary carcinoma present in a limited area [4]. With the idea that radical surgery could contribute to better survival, we have performed radical surgeries including hepatecomy with bile duct resection, PD, and HPD for biliary malignancies over the last several years [17, 18]. Regarding HPD, in the early 1990 s, morbidity and mortality associated with this surgery were approximately 90–100% and 25–29%, respectively [8, 9]. These numbers have decreased over time to 30–43% and 0–14%, respectively [19, 20]. The Japanese Society of Biliary Surgery recently reported that HPD was performed for 29 of 255 hilar cholangiocarcinomas (11.4%) in selected institutions in Japan during the period 1998–2002 [21]. These data indicate that HPD is going to be the standard surgery for biliary malignancies. Appropriate preoperative biliary drainage following portal vein embolization to optimize residual liver function as standard preoperative treatment before extended surgery, such as HPD, has improved outcomes [22–25]. Portal vein embolization introduced by Makuuchi [26], in particular, has become key to improving the safety of major hepatectomy, and approximate 10% increase in residual liver volume was reported [27, 28].

Only a few articles have addressed the clinical significance of HPD in comparison to hepatectomy or PD for cholangiocarcinoma [29]. According to our single institutional study, morbidity associated with HPD was higher than that associated with the other procedures, but the difference did not reach statistical significance. Additional complications with HPD were related to the number of required reconstructions in the alimentary tract. Morbidity associated with Hx was lower than anticipated (34%), but the associated mortality was much higher (10%). We speculate that liver function after Hx is typically most significantly affected by bilateral or segmental obstruction of the intrahepatic bile ducts, which might cause postoperative septicemia, and also some patients with Hx had far advanced stage of disease, which might decrease the patient's immunologic defenses against surgical stress. In contrast, HPD is typically performed for cases in which liver function is relatively good and the UICC T-classification is relatively low, indicating limited cancer cell invasion around the tumor, with a concomitant decrease in the need for vascular reconstruction. In addition, our criteria for HPD, in which the proximal extent of the residual hepatic duct by the tumor had to be limited within the first order branch, might decrease the operative risk, which was seen in the Hx group. Hence, no in-hospital mortality occurred among our study patients who underwent HPD. Incidences of pancreatic fistula and also DGE were not different between patients with PD and HPD; therefore, we believe a two-stage operation [30], in which reconstruction of the pancreatic duct is the second step, is not necessary for HPD.

Histopathologic analysis demonstrated perineural invasion, and the associated arterial invasion was significantly different between the groups, with the Hx and HPD group revealing greater prevalence of these factors than the PD group. This difference in incidence may be related to anatomic differences of the perihilar and distal portion of the bile duct conferring an inherent difference in distance from adjacent vessels. Although the incidences of portal vein invasion and nodal involvement did not differ significantly between groups, both were more prevalent in the Hx group. Positive ductal margin was also more frequently identified in the Hx group than in the others. Although R-classification and the stage of disease were not different statistically between the groups, these data strongly suggested that the Hx group showed more extensive disease and received less curable surgery than the other groups.

The 5-year survival rate of 31% in our Hx group, in comparison to the 49% in our PD group and 50% in our

HPD group, was the worst among our patients. The 31% was similar to other reported rates for Hx [25], despite the high incidence of very advanced tumors in our patient series. Distant metastases apparently prevented many R0 resections in the Hx group; however, aggressive resection was possible in many cases in this group because of the limited extent of metastatic disease. Unexpectedly high survival rates were achieved with PD and HPD and may be due to the comparatively early stage at which our patients' tumors were treated and the aggressive surgical tactics we used. Bile duct resection was not performed in isolation, and concomitant vascular resection was vigorously applied [31–33]. However, the rate at which R0 resection was achieved with HPD was not as high as expected. In general, it might be difficult to secure negative surgical margins in cases of biliary malignancy; the recently reported rate of positive surgical margins is about 60%, regardless of the type of surgery [34]. When survival was examined in relation to vascular reconstruction, it was clear that survival associated with PVR and HAR was low in comparison to that associated with absence of vascular reconstruction. Between PVR and HAR, survival was equivalent, contrary to some reports indicating no survival benefit with HAR [35]. We suppose our surgical tactics, i.e., en bloc resection of the tumor with the adherent vessels, might confer favorable results. In this study, HAR was almost done using the mobilized artery to keep a safe distance from the tumor. Historically, HPD has been performed for superficially spreading perihilar cholangiocarcinoma or retropancreatic lymph node metastases [36]. In our patient series, HPD was performed for any of 3 indications: widespread intramural invasion, superficial spread, and hepatoduodenal ligament invasion. Survival did not differ statistically between these three groups. The number of patients in our study was too low to determine the pertinent indications for HPD among the types of cholangiocarcinoma, but our results suggest preliminarily that any type of cholangiocarcinoma in which curative resection can be obtained is a possible candidate for surgery. Miwa et al. [37] reported results similar to ours: in their report 14 patients with diffuse bile duct cancer underwent HPD with no postoperative mortality and 51.9% 5-year survival. Concomitant vascular resection including HLPD was applied to decrease the incidence of positive radial margins in our patient series. It is possible that HLPD for highly advanced intrahepatic cholangiocarcinoma may prove beneficial, providing survival beyond 5 years and a positive palliative effect [38].

Risk factors were analyzed to identify the biologic behaviors of extrahepatic cholangiocarcinomas following the three aggressive surgical approaches. We determined that R classification, a positive surgical stump, perineural invasion, UICC T-classification, arterial invasion, lymph node metastasis, and portal vein invasion significantly influenced survival. As Sakamoto et al. [34] pointed out, the radial margin rather than the duct margin significantly influenced survival, and there was no 5-year survival with the positive radial margin in the present study. Unlike other researchers [24, 25, 39, 40], we did not find tumor grade and lymph node metastasis to be statistically significant by multivariate analyses, and only perineural invasion and R classification (R1/2 versus R0) were shown to be independent risk factors. Most studies performed outside Japan did not include perineural invasion as a prognostic factor; thus, a consensus definition for positive perineural invasion has not yet been established worldwide. In the present study, positive perineural invasion was defined as an intermediate or greater degree of invasion as set forth in the *Japanese General Rules for Surgical and Pathological Studies on Cancer of the Biliary Tract* [16]. As previously reported [41, 42], we found perineural invasion to be one of the major obstacles to obtaining histologically curative resection for biliary malignancies. In addition, our results indicate that regional (periductal and peripancreatic) lymph node metastasis does not have to be a contraindication for surgery; when positive nodes were present in the regional nodes mean survival times was over 31 months. However, patients with para-aortic lymph node metastases did not survive beyond 2 years. Randomized controlled trials examining the survival benefits of adjuvant chemotherapies in patients with biliary cancer are underway. Perhaps a standard adjuvant chemotherapy regimen for biliary cancer will be established in the near future.

In conclusion, our aggressive surgical strategy provides a long-term survival benefit to patients with perihilar or distal cholangiocarcinoma. The zero in-hospital mortality and favorable long-term survival among our 14 consecutive patients who underwent HPD suggest that HPD is worthy of consideration as a standard operation for cholangiocarcinoma with limited tumor extent, regardless of the type of tumor invasion. Because of the small number of patients in this study, further investigation in larger study populations is needed to confirm our policy.

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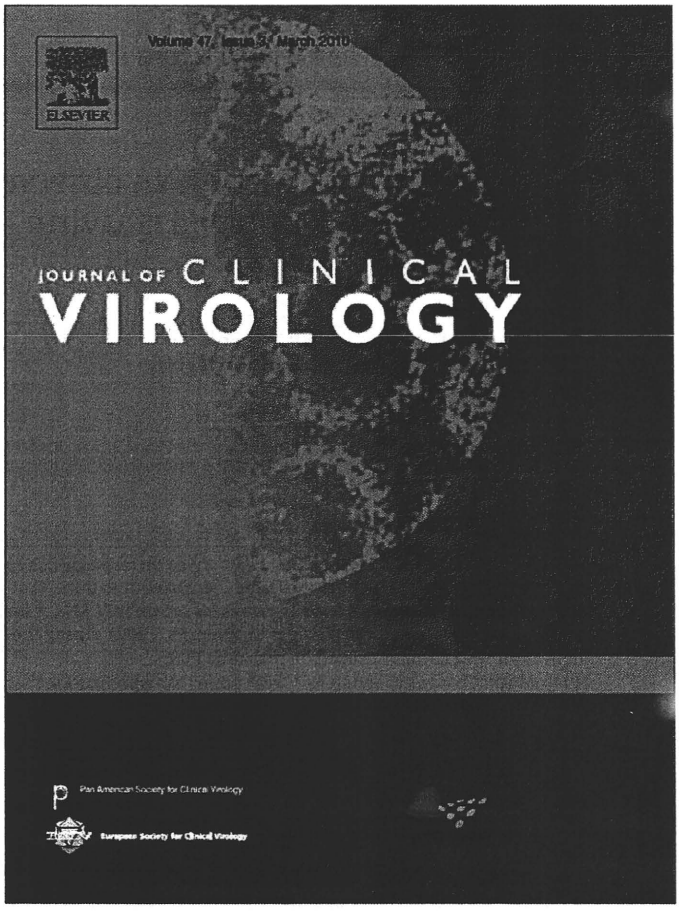

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Transient reappearance of serum hepatitis C virus RNA observed by real-time PCR during antiviral therapy with peginterferon and ribavirin in patients with HCV genotype 1b

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ABSTRACT

Background: The "response-guided therapy" based on response of hepatitis C virus (HCV) during antiviral combination therapy with peginterferon and ribavirin is important for patients with HCV genotype 1. However, the sensitivity of previous assays for serum HCV RNA is limited.
Objectives: We evaluated the changes in serum HCV RNA during the combination therapy using a novel method for measurement based on real-time PCR.
Study design: Changes in serum HCV RNA during the combination therapy were reanalyzed using TaqMan PCR assay in 144 patients with chronic HCV genotype 1b infection who underwent the therapy under HCV RNA monitoring with the Amplicor Monitor assay. Treatment duration was elongated from 48 weeks to 72 weeks in 17 patients based on the time when serum HCV RNA became negative.
Results: In 9 of 144 (6.3%) patients, serum HCV RNA transiently appeared again on the TaqMan PCR assay after having previously become negative. At the point of reappearance, the Amplicor Monitor assay gave a negative result in all patients, and no flare of alanine aminotransferase activity was observed. Each of the 9 patients achieved an end-of-treatment response but relapsed after the end of treatment, including 3 patients in whom the treatment duration was elongated to 72 weeks.
Conclusions: Attention should be paid to this phenomenon in the antiviral treatment for patients with HCV infection. The transient reappearance of HCV RNA in the serum indicates a high likelihood of relapse, and is likely to be missed without frequent measurements by a sensitive detection method.

1. Background

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin.[1] Although the rate of sustained virologic response (SVR), which indicates the eradication of hepatitis C virus (HCV), has increased with the use of the current regimen, it is still only around 50% in patients infected with HCV genotype 1.[2–8] The response of HCV during combination therapy, i.e., the changes in serum HCV RNA after the start of therapy have been reported to be predictors of the therapeutic outcome[9–12]; therefore "response-guided therapy" based on this response is now favored,[12,13] especially for patients with HCV genotype 1.

To improve outcome prediction and the selection of treatment duration for response-guided therapy, more precise and sensitive evaluation of serum HCV RNA is necessary. Serum HCV RNA concentration has previously been measured by the branched-DNA probe assay and, more recently, by the Amplicor Monitor assay.[14,15] However, the sensitivity of these assays is limited. Very recently, a novel method for measurement of serum HCV RNA, based on real-time PCR, has been established, and is reported to have high sensitivity for the detection of serum HCV RNA.[16–18]

2. Objectives

In the present study, we used the real-time PCR-based TaqMan assay to reanalyze the changes in serum HCV RNA from stored serum samples of patients with chronic HCV genotype 1 infection. These patients had undergone antiviral combination therapy with PEG-IFN and ribavirin under monitoring of serum HCV RNA using the Amplicor Monitor assay. In some patients, we observed a reappearance of serum HCV RNA during the treatment after hav-

Abbreviations: HCV, hepatitis C virus; PEG-IFN, peginterferon; SVR, sustained virologic response; RVR, rapid virologic response; cEVR, complete early virologic response; ETR, end-of-treatment response.

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Table 1
Baseline characteristics of study patients ($n = 144$).

Age (years)	58.3 ± 8.9
Sex (female/male)	71 (49.3)/73 (50.7)
History of interferon therapy (naive/retreatment)	109 (75.7)/35 (24.3)
Body weight (kg)	58.7 ± 10.1
Alanine aminotransferase (IU/L)	62.1 ± 58.2
Aspartate aminotransferase (IU/L)	52.7 ± 40.4
Gamma-glutamyl transpeptidase (IU)	52.6 ± 58.3
Alkaline phosphatase (IU/L)	260.6 ± 83.9
Albumin (g/dL)	4.15 ± 0.35
Total bilirubin (mg/dL)	0.68 ± 0.30
White blood cell count (/μL)	5130 ± 1327
Hemoglobin (g/dL)	14.2 ± 1.4
Platelet count ($\times 10^3$/μL)	167 ± 51
Liver histology-activity (A0/A1/A2/A3)[a]	3 (2.3)/73 (54.9)/45 (33.8)/12 (9.0)
Liver histology-fibrosis (F0/F1/F2/F3)[a]	5 (3.8)/79 (59.4)/33 (24.8)/16 (12.0)
Pretreatment HCV RNA concentration ($\log_{10}$ IU/mL)	6.28 ± 6.16
Reduction of the peginterferon dose	40 (27.8)
Reduction of the ribavirin dose	71 (49.3)

HCV, hepatitis C virus. Percentages are shown in parentheses.
[a] Liver biopsy was not performed in 11 patients.

ing previously gone negative. This reappearance could be observed only using real-time PCR, and all patients who showed the phenomenon relapsed after the completion of the therapy.

3. Study design

Between January 2006 and March 2008, a total of 156 patients with chronic HCV genotype 1b infection underwent antiviral combination therapy with PEG-IFN and ribavirin at our institution. Among these patients, 148 had pretreatment HCV RNA concentrations >100,000 IU/mL as assayed by quantitative Amplicor Monitor assay (AMPLICOR HCV MONITOR Test, version 2.0; Roche Molecular Systems, Pleasanton, CA). No patients with HCV genotype 1a were included because this type is not found in the general Japanese population. In this study, we included the 144 of these 148 patients who agreed to store serum samples and to have them used in the study. Table 1 shows the baseline characteristics of the 144 study patients. Although 35 patients had a history of previous antiviral monotherapy with conventional IFN or combination therapy with conventional IFN and ribavirin (retreatment cases), no patients had a history of combination therapy with PEG-IFN and ribavirin. Of 133 patients who underwent a pretreatment liver biopsy, the grade of liver fibrosis according to the METAVIR score[19] was F0 in 5 patients (3.8%), F1 in 79 patients (59.4%), F2 in 33 patients (24.8%), and F3 in 16 patients (12.0%), respectively. No patients were coinfected with hepatitis B virus or human immunodeficiency virus. No patients had histories of alcohol abuse or intravenous drug use. For combination therapy with PEG-IFN and ribavirin, all patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough) daily. The dose of PEG-IFN and ribavirin were adjusted by patient body weight. Patients weighing ≤45 kg were given 60 μg of PEG-IFN alpha-2b once a week, those weighing >45 kg and ≤60 kg were given 80 μg, those weighing >60 kg and ≤75 kg were given 100 μg, those weighing >75 kg and ≤90 kg were given 120 μg, and those weighing >90 kg were given 150 μg. Patients weighing ≤60 kg were given 600 mg of ribavirin per day, those weighing >60 kg and ≤80 kg were given 800 mg of ribavirin per day, and those weighing >80 kg were given 1000 mg of ribavirin per day. Dose modification or discontinuation of PEG-IFN or ribavirin was based on the manufacturer's recommendations. During the therapy, 40 patients (27.8%) had their PEG-IFN doses reduced and 71 patients (49.3%) had their ribavirin doses reduced. No patients discontinued the therapy. SVR was defined as undetectable serum HCV RNA throughout 24 weeks after the end of therapy. Relapse was defined as positive serum HCV RNA during the period between the end of treatment and 24 weeks thereafter, despite the disappearance of serum HCV RNA by the end of treatment. As for responses during the therapy, rapid virologic response (RVR) was defined as negative serum HCV RNA at 4 weeks after the start of the therapy. Complete early virologic response (cEVR) was defined as negative serum HCV RNA at 12 weeks after the start of the therapy.[20] Slow virologic response was defined as the disappearance of serum HCV RNA between 12 and 24 weeks after the start of the therapy. Non-response was defined as failure to clear serum HCV RNA until 24 weeks after the start of the therapy (null-response or partial response).[1] End-of-treatment response (ETR) was defined as negative serum HCV RNA at the end of the therapy.[1] HCV RNA in the serum was measured by the qualitative Amplicor Monitor HCV RNA assay (AMPLICOR Hepatitis C Virus (HCV) Test, version 2.0, Roche Molecular Systems)[21] to confirm the undetectability of serum HCV RNA, when it was unquantifiable (under the detection limit) by the quantitative Amplicor Monitor assay. Patients who showed slow virologic response were recommended to elongate the treatment duration from 48 to 72 weeks according to previously published reports.[22,23]

Table 2
Responses to combination therapy with peginterferon and ribavirin evaluated by Amplicor and TaqMan assay.

	Evaluation by Amplicor	Evaluation by TaqMan
Rapid virologic response	10 (6.9)	9 (6.3)
Complete early virologic response[a]	70 (48.6)	54 (37.5)
Slow virologic response	34 (23.6)	39 (27.1)
Non-response[b]	40 (27.8)	51 (35.4)
End-of-treatment response	104 (72.2)	104 (72.2)
Sustained virologic response	63 (43.8)	63 (43.8)
Relapse	41 (28.5)	41 (28.5)

Amplicor, measured by AMPLICOR HCV MONITOR Test, version 2.0; TaqMan, measured by COBAS AmpliPrep/COBAS TaqMan HCV Test.
[a] Includes patients with rapid virologic response.
[b] Patients with null-response and those with partial response.

After a patient gave consent, serum samples were obtained at the patient's regular visit to the hospital just prior to beginning treatment, and at every 4 weeks during the treatment and during the 24-week follow-up period after the treatment. Serum samples were stored at −80 °C. We measured the HCV RNA levels in these stored serum samples using a real-time PCR-based quantitation method for HCV (COBAS AmpliPrep/COBAS TaqMan HCV Test, Roche Molecular Systems), and compared the results with those from the Amplicor Monitor assays. When serum HCV RNA level was low and unquantifiable, the detection of HCV RNA was tested repeatedly and the presence or absence of serum HCV RNA was confirmed.

Quantitative values are reported as mean ± SD. Between-group differences were analyzed by Chi-square test. The study protocol was approved by the institutional review board and was in compliance with the Helsinki Declaration. Written informed consent was obtained from all patients prior to the study for use of the clinical data and serum samples.

4. Results

4.1. Response of HCV RNA during treatment and final outcomes

All patients completed the therapy. Table 2 shows the responses to the therapy evaluated by the Amplicor Monitor assay and by the TaqMan PCR assay. Based on the evaluation of serum HCV RNA by the Amplicor Monitor assay during the treatment, 10 patients (6.9%) showed RVR, 70 (48.6%) showed cEVR (including the 10 with RVR), and 34 (23.6%) showed slow virologic response. The elonga-

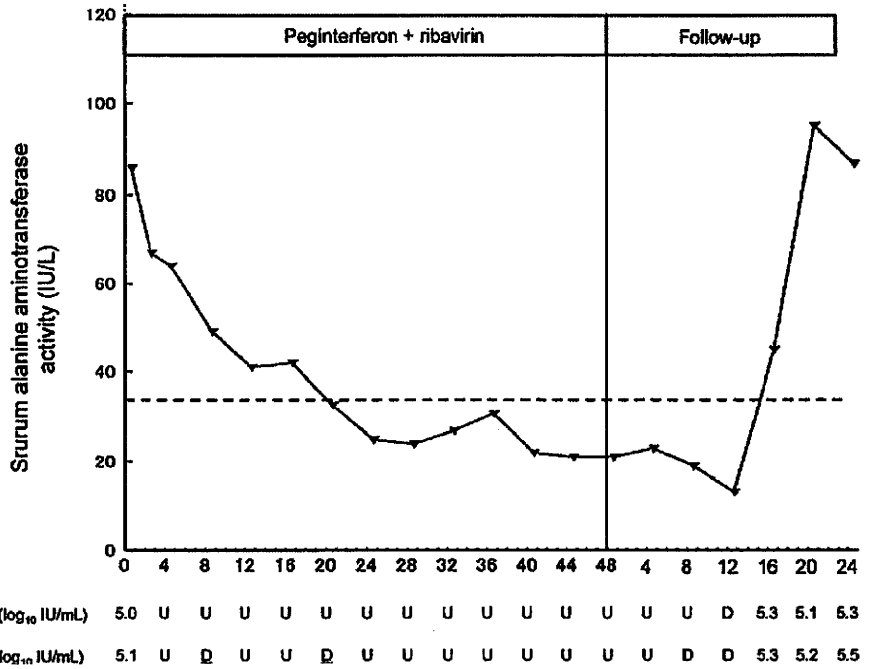

Fig. 1. Changes in serum alanine aminotransferase activity and serum HCV RNA concentration as detected by the Amplicor Monitor and TaqMan PCR assays during treatment and follow-up period (24 weeks) in a patient in whom serum HCV reappeared transiently during treatment after it had previously disappeared (patient 6). Pretreatment serum HCV RNA concentration was 5.05 $\log_{10}$ IU/mL. The Amplicor Monitor assay showed that serum HCV RNA disappeared at 4 weeks after the start of the therapy; therefore, the patient was classified as rapid virologic response. He relapsed after the end of the treatment with an increase in serum HCV RNA concentration and alanine aminotransferase activity. The TaqMan PCR assay also showed a disappearance of serum HCV RNA at 4 weeks, but this assay further showed that it reappeared transiently at 8 and 20 weeks after the start of the therapy (underline). Without the measurement of HCV RNA at 8 and 20 weeks this patient would be classified as rapid virologic response by the TaqMan PCR assay as well. If only the measurement at 20 weeks was omitted, he would be classified as complete early virologic response by the TaqMan PCR assay. "D" at Amplicor lane means that HCV RNA was under detection limit of quantitative Amplicor Monitor assay (3.70 $\log_{10}$ IU/mL) but was detected by qualitative Amplicor Monitor assay. "D" at TaqMan lane means that HCV RNA was under quantitation limit of TaqMan assay but was detected. "U" at Amplicor lane and TaqMan lane mean undetectable.

tion of the treatment duration from 48 weeks to 72 weeks was recommended for patients with slow virologic response; 17 of 34 patients (50.0%) followed the recommendation. As the final outcomes, 63 patients (43.8%) showed SVR and 41 (28.5%) relapsed. Among the 34 patients who showed the slow virologic response, the SVR rate was 5.9% (1 of 17) in the patients without the elongated treatment duration, and 41.2% (7 of 17) in the patients with it ($p = 0.0432$).

Under evaluation with the TaqMan PCR assay, 9 patients (6.3%) showed RVR, 54 (37.5%) showed cEVR (including 9 patients with RVR), and 39 (27.1%) showed slow virologic response. For 16 patients, the Amplicor Monitor assay gave the cEVR result, while the TaqMan PCR assay gave the slow virologic response result; elongation of treatment duration was not recommended for these patients because only the Amplicor Monitor assay was used during the treatment. At 24 weeks after the start of therapy, serum HCV RNA was detectable by the TaqMan PCR assay (non-response) in 11 patients for whom the Amplicor Monitor assay had given a result of slow virologic response.

4.2. Detection by TaqMan PCR assay of transient reappearance of serum HCV RNA during treatment

Using the TaqMan PCR assay, serum HCV RNA was detected again in 9 (6.3%) patients after having previously disappeared from the serum. Table 3 summarizes the data from these 9 patients. Patients 2, 5, 6, 7, 8, and 9 were categorized as cEVR by the Amplicor Monitor assay during treatment and underwent 48-week treatment. Patient 6 showed RVR during treatment (Fig. 1). Patient 1 was categorized as cEVR during treatment but strongly desired the elongation of the treatment duration and underwent 72-week treatment (Fig. 2). Patients 3 and 4 showed slow virologic response and underwent 72-week treatment. Under reanalysis of the serum samples with the TaqMan PCR assay, patients 1, 2, 6, 7, 8, and 9 remained cEVR, patient 6 remained RVR, and patients 3 and 4 remained classified as slow virologic response, when the responses were determined by the first disappearance of serum HCV RNA. However, when the reappearance was considered, patients 1, 2, 5, 6, 7, 8, and 9 actually had a slow virologic response, and serum HCV RNA remained detectable at 24 weeks after the start of the therapy (non-response) in patients 3 and 4.

Reappearance of serum HCV RNA was found at only one measurement point in 7 patients and at 2 points in the remaining 2 patients. In all case, the level of reappeared HCV RNA was low and unquantifiable despite detection. Although patients 1, 4, and 9 experienced the reduction of ribavirin dose, the reduction was not concomitant with the reappearance of serum HCV RNA. HCV RNA reappeared transiently at these points and disappeared again thereafter. In 8 patients, the reappearance was observed at the measurement point just after the first disappearance of serum HCV RNA (i.e., 4 weeks after the previous measurement). In patient 9, HCV RNA reappearance was observed at 8 weeks after the initial disappearance. In patient 6, HCV RNA first disappeared at 4 weeks after the start of the therapy but reappeared at 8 weeks. It became negative again at 12 and 16 weeks, but reappeared again at 20 weeks. In the final outcome, all 9 patients relapsed after the end of treatment regardless of treatment duration. The prevalence of relapse in patients who experienced transient reappearance of serum HCV RNA were significantly higher than those without it, by the evaluation in patients with cEVR (100% vs. 2.1%, $p < 0.0001$), in those with cEVR and slow virolgic response (100% vs. 25.0%, $p < 0.0001$), and in those with ETR (100% vs. 33.7%, $p = 0.0004$).

Table 3
Patients in whom serum HCV reappeared transiently during treatment after having previously disappeared.

	Age	Sex	History of IFN therapy	Pretreatment HCV RNA[a] (log_{10} IU/mL)	HCV RNA disappearance by Amplicor	HCV RNA disappearance by TaqMan	HCV RNA reappearance by TaqMan[b]	ALT flare during therapy	Treatment duration	Outcome
1	29	F	No	5.89	12W	12W	16W	No	72W	Relapse
2	57	F	No	6.46	12W	12W	16W	No	48W	Relapse
3	51	F	No	6.26	16W	20W	24W	No	72W	Relapse
4	65	M	No	5.66	20W	20W	24W	No	72W	Relapse
5	58	M	Yes	6.51	12W	16W	20W	No	48W	Relapse
6	65	M	Yes	5.05	4W	4W	8W and 20W	No	48W	Relapse
7	58	M	No	6.97	12W	12W	16W	No	48W	Relapse
8	61	F	No	5.99	12W	12W	16W and 20W	No	48W	Relapse
9	68	F	No	6.09	8W	12W	20W	No	48W	Relapse

M, male; F, female; W, weeks; Amplicor, measured by AMPLICOR HCV MONITOR Test, version 2.0; TaqMan, measured by COBAS AmpliPrep/COBAS TaqMan HCV Test.
[a] Measured by COBAS AmpliPrep/COBAS TaqMan HCV Test.
[b] HCV RNA reappeared transiently only at these measurement points and again disappeared thereafter.

5. Discussion

Measurement of HCV RNA with the real-time PCR-based TaqMan PCR assay has been reported to be superior to previous methods for the prediction of treatment outcome and the selection of a response-guided therapy regimen.[24,25] In the present study, we used the PCR-based TaqMan assay to reanalyze the changes in serum HCV RNA in patients who underwent antiviral combination therapy with PEG-IFN and ribavirin under the guidance of the Amplicor Monitor assay, in order to evaluate the usefulness of the newer technique. We found the TaqMan PCR assay to be a more sensitive detector of serum HCV RNA; it detected HCV RNA at 12 weeks after the start of the therapy in 24.3% of patients showing a cEVR result by the Amplicor Monitor assay. Umder the guidance of the TaqMan PCR assay, elongation of the treatment duration would have been recommended for these patients and their rate of SVR would presumably have increased.

More importantly, only by measurement with the TaqMan PCR assay did we observed the transient reappearance of serum HCV RNA after it had previously disappeared. Because this phenomenon was not accompanied by a flare of alanine aminotransferase and because serum HCV RNA continued to be negative by the Amplicor assay, it was missed during treatment. Breakthrough of HCV RNA during treatment is usually accompanied by an increase in serum HCV RNA concentration and a serum ALT flare, and is not transient. The phenomenon that we observed was, therefore, different from the typical breakthrough.

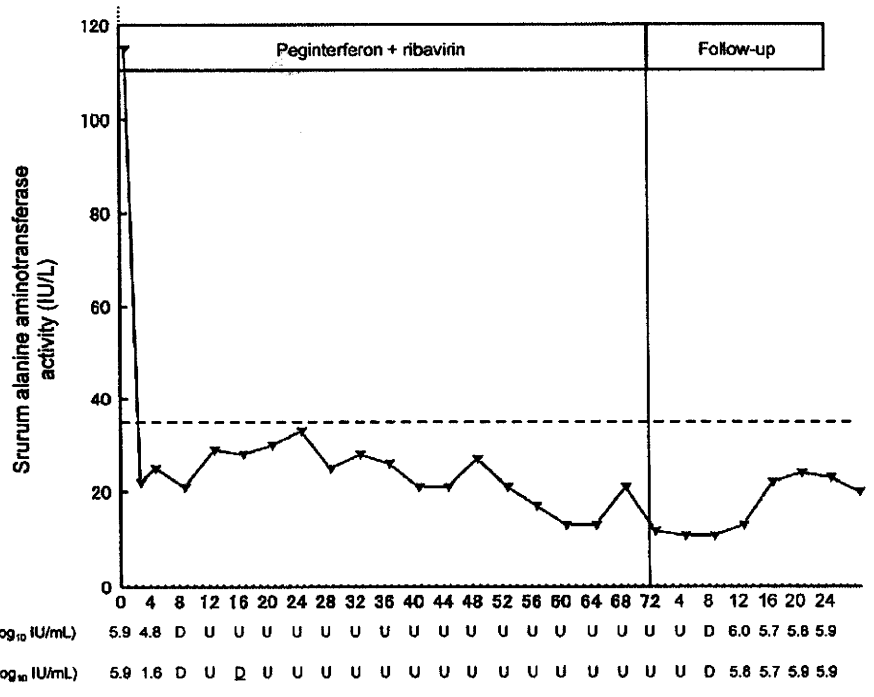

Fig. 2. Changes in serum alanine aminotransferase activity and serum HCV RNA concentration as measured by the Amplicor Monitor and TaqMan PCR assays during the treatment and follow-up period (24 weeks) in a patient in whom serum HCV reappeared transiently during treatment after having previously disappeared (patient 1). Her pretreatment serum HCV RNA concentration was 5.89 log_{10} IU/mL. The Amplicor Monitor assay showed that her serum HCV RNA disappeared at 12 weeks after the start of the therapy; therefore, she was classified as complete early virologic response. She strongly desired to elongate the treatment duration, and completed 72 weeks treatment. However, she relapsed after the end of the treatment, showing an increase in serum HCV RNA concentration. The TaqMan PCR assay also showed the disappearance of serum HCV RNA at 12 weeks, but this assay further showed that it reappeared transiently at 16 weeks after the start of the therapy (underline). "D" at Amplicor lane means that HCV RNA was under detection limit of quantitative Amplicor Monitor assay (3.70 log_{10} IU/mL) but was detected by qualitative Amplicor Monitor assay. "D" at TaqMan lane means that HCV RNA was under quantitation limit of TaqMan assay but was detected. "U" at Amplicor lane and TaqMan lane mean undetectable.

Because the phenomenon is transient, it is likely to be missed even under monitoring by the TaqMan PCR assay unless the measurement is frequently performed. In the 9 of our study patients who showed this phenomenon, reappearance of HCV RNA was found at 8, 16, or 20 weeks after the start of the therapy. Serum HCV RNA is usually measured at 4, 12, and 24 weeks after the start of the therapy[1]; therefore, any transient reappearance would be missed, unless the measurement was performed every 4 weeks. Patients 1, 2, 3, 8, and 9 would have remained cEVR and patient 6 would have remained RVR, even under measurement with the TaqMan PCR assay, if the measurement was performed only at the standard 4, 12, and 24 weeks after the start of the therapy.

In the final outcome, all these 9 patients relapsed. It is unclear why they all relapsed and no patient achieved SVR. Patients 2, 5, 6, 7, 8, and 9 would have been classified as slow virologic responders if the reappearance of HCV RNA had been detected; in these cases, the elongation of the treatment duration to 72 weeks might have resulted in SVR. In patients 3 and 4, HCV RNA was positive at 24 weeks after the start of therapy; this could explain the lack of SVR even with their 72-week treatment duration. Otherwise, a very low level of serum HCV RNA, close to the detection threshold for the TaqMan PCR assay was present throughout the treatment period, causing redetection of HCV RNA in the serum by this assay. For example, one of a few minor HCV strain that are resistant to PEG-IFN and ribavirin therapy could have been present throughout the treatment period. Further improvement of the sensitivity of the detection of serum HCV RNA will explain the results.

In conclusion, using the TaqMan PCR assay we observed a transient reappearance of serum HCV RNA after it had previously disappeared in patients with HCV genotype 1b undergoing antiviral combination therapy with PEG-IFN and ribavirin. This phenomenon is likely to be missed without frequent measurements of serum HCV RNA by sensitive detection method, and it may indicate a high likelihood of relapse after treatment even if the treatment duration is elongated. The possibility of this phenomenon should be considered during treatment in order to select the appropriate response-guided therapy. In addition, large-scale prospective studies will be needed to clarify the biological significance and clinical impact of this phenomenon.

Conflict of interest

There is no conflict of interest and there is no grant support and other assistance on this study.

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Original Article

Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development

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MicroRNAs (miRNAs) belong to a class of the endogenously expressed non-coding small RNAs which primarily function as gene regulators. Growing evidence suggests that miRNAs have a significant role in tumor development and may constitute robust biomarkers for cancer diagnosis and prognosis. The *miR-17-92* cluster especially is markedly overexpressed in several cancers, and is associated with the cancer development and progression. In this study, we have demonstrated that miR-92a is highly expressed in hepatocellular carcinoma (HCC). In addition, the proliferation of HCC-derived cell lines was enhanced by miR-92a and inhibited by the anti-miR-92a antagomir. On the other hand, we have found that the relative amount of miR-92a in the plasmas from HCC patients is decreased compared with that from the healthy donors. Interestingly, the amount of miR-92a was elevated after surgical treatment. Thus, although the physiological significance of the decrease of miR-92a in plasma is still unknown, deregulation of miR-92 expression in cells and plasma should be implicated in the development of HCC.

Key words: hepatocellular carcinoma, microRNA, miR-638, miR-92a, plasma

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MicroRNAs (miRNAs) are small endogenous non-coding RNAs that regulate gene expression and have a critical role in many biological and pathological processes.[1] Recent studies have shown that deregulation of miRNA expression contributes to the multistep processes of carcinogenesis, and have shown promise as tissue-based markers for cancer classification and prognostication.[2,3] However, biological roles of only a small fraction of known miRNAs have been elucidated to date.

The miR-17-92 cluster at 13q31.3 is consists of six miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1, and plays an important role for development of lung cancer,[4] B-cell lymphomas,[5] chronic myeloid leukemia,[6] medulloblastomas,[7] colon cancer[8] and hepatocellular carcinoma (HCC).[9] In addition, mice deficient in the miR-17-92 cluster died shortly after birth with lung hypoplasia, and B-cell development was impaired in the mice.[10] It has been reported, however, that miR-92a increases cell proliferation by negative regulation of an isoform of the cell-cycle regulator p63.[11] Furthermore, miR-92a regulates angiogenesis.[12] Thus, it is clear that the miR-92a has some oncogenic characteristics. However, the specific biological role of miR-92a in the processes of human cancer development has remained unclear.

Here, we have revealed that miR-92a is implicated in human HCC development. Furthermore, we have demonstrated that miR-92a in human blood has the potential to be a noninvasive molecular marker for diagnosis of human HCC.

MATERIALS AND METHODS

In situ hybridaization of miR-92a

Locked nucleic acid (LNA)-modified probes for miR-92a and negative control (miRCURY-LNA detection probe, Exiqon, Vedbaek, Denmark) were used. The probe sequences were as follows; *miR-92a*, 5′-ACAGGCCGGGACAAGTGCAATA-3′; and a scrambled oligonucleotides used for negative control, 5′-GTGTAACACGTCTATACGCCCA-3′. *In situ* hybridization was performed using the RiboMap *in situ* hybridization kit (Ventana Medical Systems, Tucson, AZ, USA) on the Ventana Discovery automated *in situ* hybridization instrument (Ventana Medical Systems). The *in situ* hybridization steps were performed as previously described.[13] Staining was evaluated by two investigators and graded as follows: negative (–), no or occasional (<5%) staining of tumor cells; positive (+), mild to strong (>5%) staining of tumor cells. Paraffin–embedded tissue samples of hepatocellular carcinoma (HCC) and adjacent non-tumorous liver cirrhosis (LC) were obtained from HCC patients at Ogaki Municipal Hospital (Ogaki, Japan). Details of the clinical data are provided in Table 1.

Plasma collection, RNA isolation and quantitative RT-PCR

Whole blood samples were collected from healthy donors and the patients with HCC at Ogaki Municipal Hospital. This study was approved by the institutional review board (IRB) of Tokyo Medical University, and all subjects provided written informed consent under the institutional review board. Details of clinical data are provided in Table 1. Diagnoses were confirmed using the post-operated tissues. Blood samples of the patients (Cases 1–10) were collected one day before the operation and then properly stored. One week after operation, blood samples of the patients were collected again. Whole blood was separated into plasma and cellular fractions by centrifugation at 1600 *g* for 15 min. Total RNA in the

Table 1 Summary of clinical details of hepatocellular carcinoma (HCC)used for in situ hybridaization and serum analysis

	Year	Sex	Virus type	Histologic type	Stage	Child-Pugh	miR-92a
Case 1	53	Male	HBV	Poorly	I	A	+
Case 2	59	Male	HBV	Moderate	II	A	+
Case 3	79	Male	NBNC	Moderate	III	A	+
Case 4	73	Male	HCV	Well	I	A	+
Case 5	76	Female	HCV	Moderate	IV-A	A	+
Case 6	59	Male	HCV	Moderate	II	A	+
Case 7	69	Female	HCV	Moderate	I	A	+
Case 8	71	Male	HCV	Moderate	I	A	+
Case 9	59	Female	HBV	Well	I	A	–
Case 10	69	Male	NBNC	Moderate	IV-A	A	–
Case 11	61	Female	HBV	Poorly	IV-A	B	+
Case 12	73	Male	NBNC	Moderate	II	A	+
Case 13	67	Male	NBNC	Moderate	IV-A	A	+
Case 14	61	Male	NBNC	Moderate	III	A	+
Case 15	45	Male	HBV	Moderate	I	A	+
Case 16	68	Female	HCV	Moderate	III	A	+
Case 17	70	Male	NBNC	Poorly	II	A	+
Case 18	59	Male	HCV	Moderate	III	A	+
Case 19	43	Male	HBV	Moderate	II	A	+
Case 20	69	Male	HCV	Moderate	II	A	–
Case 21	76	Male	HCV	Moderate	III	A	–
Case 22	53	Male	HCV	Moderate	II	A	–

HCV, hepatitis C virus; HBV, hepatitis B virus; NBNC, non-B non-C virus.

Table 2 Summary of clinical details of hepatocellular carcinoma (HCC) used for qPCR analysis

Code no.	Year	Sex	Virus type	Histologic type	Non-tumorous tissue	AFP	PIVKA-II
91	53	Male	HCV	Moderate	LC	5	0.06
160	59	Male	HCV	Moderate	LC	NI	NI
O89	68	Male	HCV	Moderate	LC	8	25
O90	70	Male	HCV	Moderate	LC	686	962
K89	51	Male	HCV	Moderate	LC	NI	NI

LC, liver cirrhosis; HCV, hepatitis C virus; NI, no information.

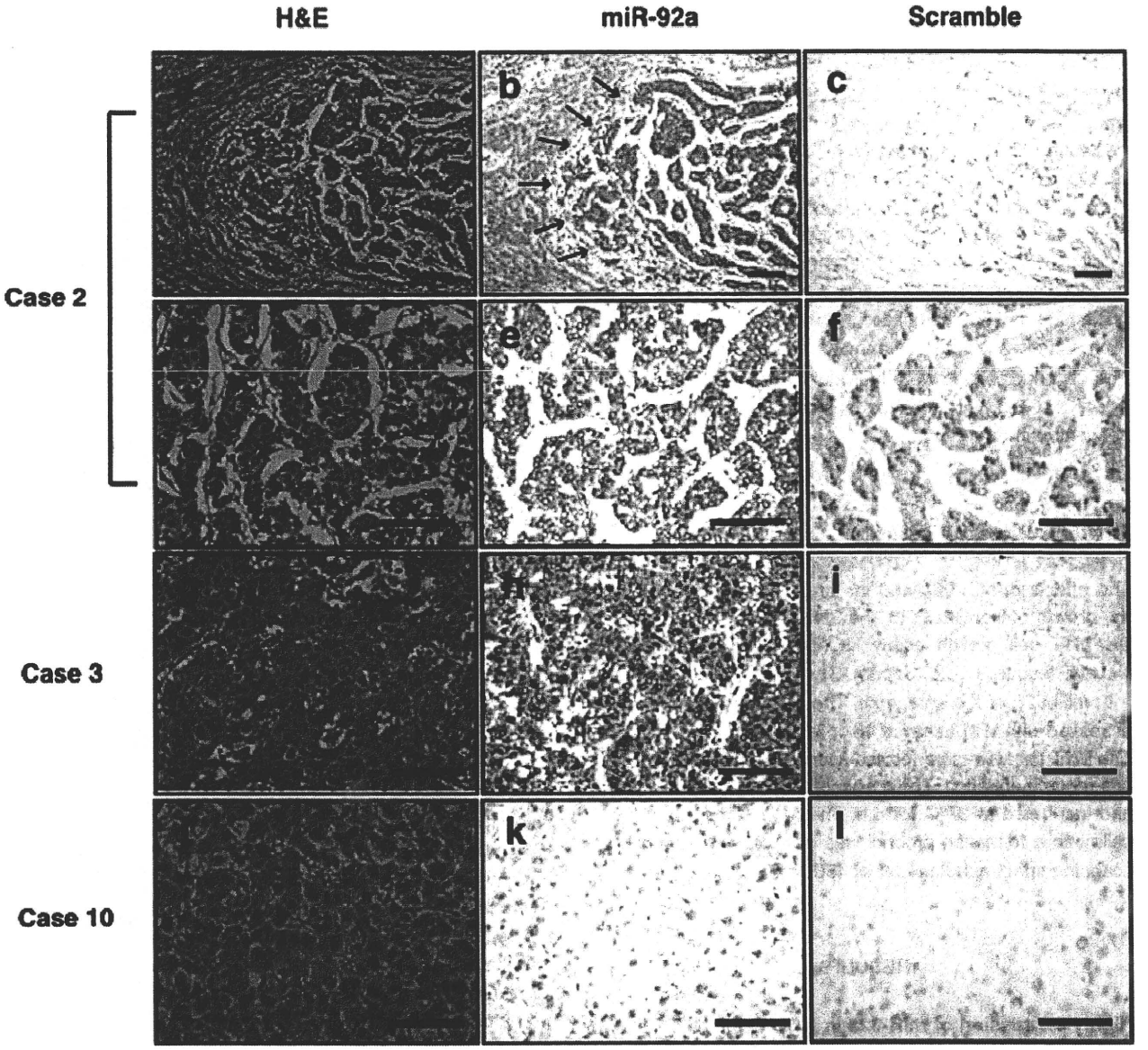

Figure 1 MiRNA expression in hepatocellular carcinoma (HCC). *In situ* hybridization was performed using Locked nucleic acid (LNA)-modified probes for miR-92a and negative control. Case 2 and Case 3 were positive cases for miR-92a. Case 10 was a negative case for miR-92a. (**a–c**) Low power field of boundary of HCC and non-tumor lesion. Arrowheads indicated a border. Only HCC regions were positive for miR-92a. (**d–l**) High power field of HCC. Blue signals represent positive for miR-92a. Bars indicate 100 μm.

plasma was isolated using Isogen-LS (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. The RNA sample was suspended in 20 μL of nuclease free water. In general, we obtained 400 ng of RNA from 1 mL of plasma. MiRNAs were quantified using TaqMan MiRNA Assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) as previously described.[13]

For miR-92a quantification in tissue samples, five pairs of fresh HCC and non-tumorous LC samples were surgically resected from HCC patients (Table 2). All the patients or their guardians provided written informed consent, and the Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine approved all aspects of this study. The amounts of miR-92a were normalized to RNU48 that is one of rRNAs (Applied Biosystems).

Cell culture and transfection

Hepatocellular carcinoma (HCC) cell lines HepG2, OR6 and SN1a were cultured in Dulbecco's modified Eagle's medium

(DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS). OR6 and SN1a are derived from the Huh7 HCC cell line and maintain hepatitis C virus (HCV) replicon.[14–16] The miR-92a oligonucleotide used in the transfection experiments is a synthetic double-strand 19 nucleotide RNA oligonucleotide (5′-UUGCACUUGUCCC GGCCUG-3′) purchased from B-Bridge International (Tokyo, Japan). The scrambled oligonucleotide represents a mix of two different frames of the miR-92 sequence (5′-UAUUGC ACUUGUCCCGGCCUGUCCCGGCC-3′ and 5′-AUUGCAC UUGUCCCGGCCUTT-3′). Locked nucleic acid (LNA) oligonucleotide miR-92 knockdown (antagomir) was obtained from Exiqon (Vedbaek, Denmark, http://www.exiqon.com). The oligonucleotides were individually transfected by Hiper-Fect (QIAGEN K. K., Tokyo, Japan) into the cells at a final concentration of 100 nM.

In vitro proliferation assays

The effects of miR-92a and the anti-miR-92a antagomir on the growth of HepG2, OR6 and SN1a were evaluated using the MTT cell growth assay kit (Cell Count Reagent SF, Nacalai tesque, Kyoto, Japan). The cells were transfected with miR-92a or the antagomir. The cell numbers were then assessed with MTT assay at 48 or 72 h after the transfection. The MTT assay was performed according to the manufacturer's recommendation. The reagents were added to each well and incubated at 37°C for 4 h. The MTT reduced by living cells into a formazan product was assayed with a multiwell scanning spectrophotometer at 450 nm.

RESULTS

Highly expression of miR-92a in HCC cells

We first examined whether or not miR-92a is expressed in hepatocellular carcinoma (HCC). We performed *in situ* hybridization using locked nucleic acid (LNA)-modified probes digoxigenin (DIG) labelled. We found that miR-92a was strongly expressed in cancer cells of 17 out of 22 HCC cases (Table 1 and Fig. 1). No significant differences were observed in age, sex, virus type, clinical stage and tumor differentiation of the clinical samples. In contrast, we did not detect miR-92a expression in non-cancerous hepatocytes around the HCCs.

Furthermore, we quantified miR-92a levels in HCC sections ($n = 5$) and their adjacent non-tumorous liver cirrhosis (LC) sections ($n = 5$) by *Taq*Man qRT-PCR (Table 2 and Fig. 2). The levels of miR-92a expression in HCC sections were higher than that in adjacent LC sections (Fig. 2).

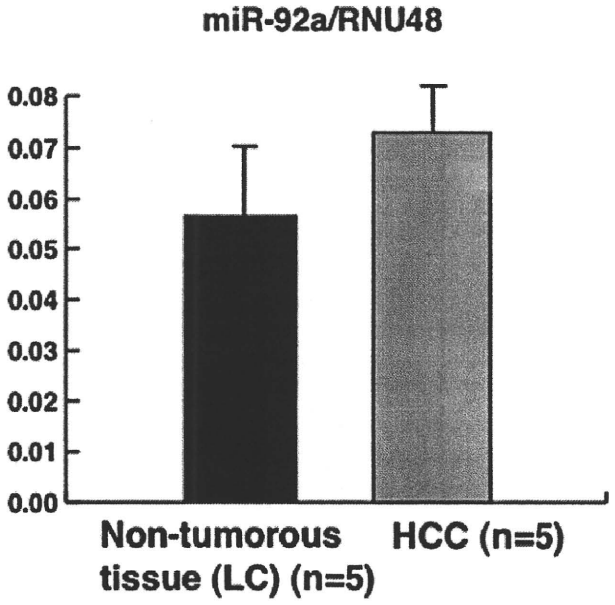

Figure 2 Quantification of miR-92a expression in hepatocellular carcinoma (HCC) tissue samples. The ratios of miR-92a to RNU48 in HCC tissues and their adjacent non-tumorous liver cirrhosis (LC) tissues were analyzed by *Taq*Man qRT-PCR. *Bars*, s.d.

Effects of miR-92a on a Hepatoma cell lines HepG2, OR6 and SN1a

Next, we investigated whether miR-92a affects cell proliferation of human HCC cell lines, HepG2, OR6 and SN1a. We transiently transfected either miR-92a or the anti-miR-92a antagomir into the cells. Antagomirs are single-stranded RNAs that are complementary to a specific miRNA and cause the depletion of the miRNA.[17] After the transfection, we found that all of the cells transfected with the anti-miR-92a antagomir showed lower proliferation rate than the cells transfected with a control RNA oligonucleotide (Fig. 3a). In contrast, the cells except for HepG2 showed increased proliferation rate when miR-92a was transfected (Fig. 3a). We also confirmed the amounts of miR-92a in the cells by quantitative real time PCR (Fig. 3b).

The ratio of miR-92a to miR-638 serves as a biomarker for HCC

Finally, we sought to determine whether the expression level of miR-92a in blood sera could discriminate HCC patients from healthy individuals. Previously, we have revealed that miR-92a is dramatically reduced in the plasmas of acute leukemia patients although in leukemic cells it is strongly expressed.[13] We analyzed the miR-92a levels in the plasma samples from normal individuals ($n = 10$) and HCC patients

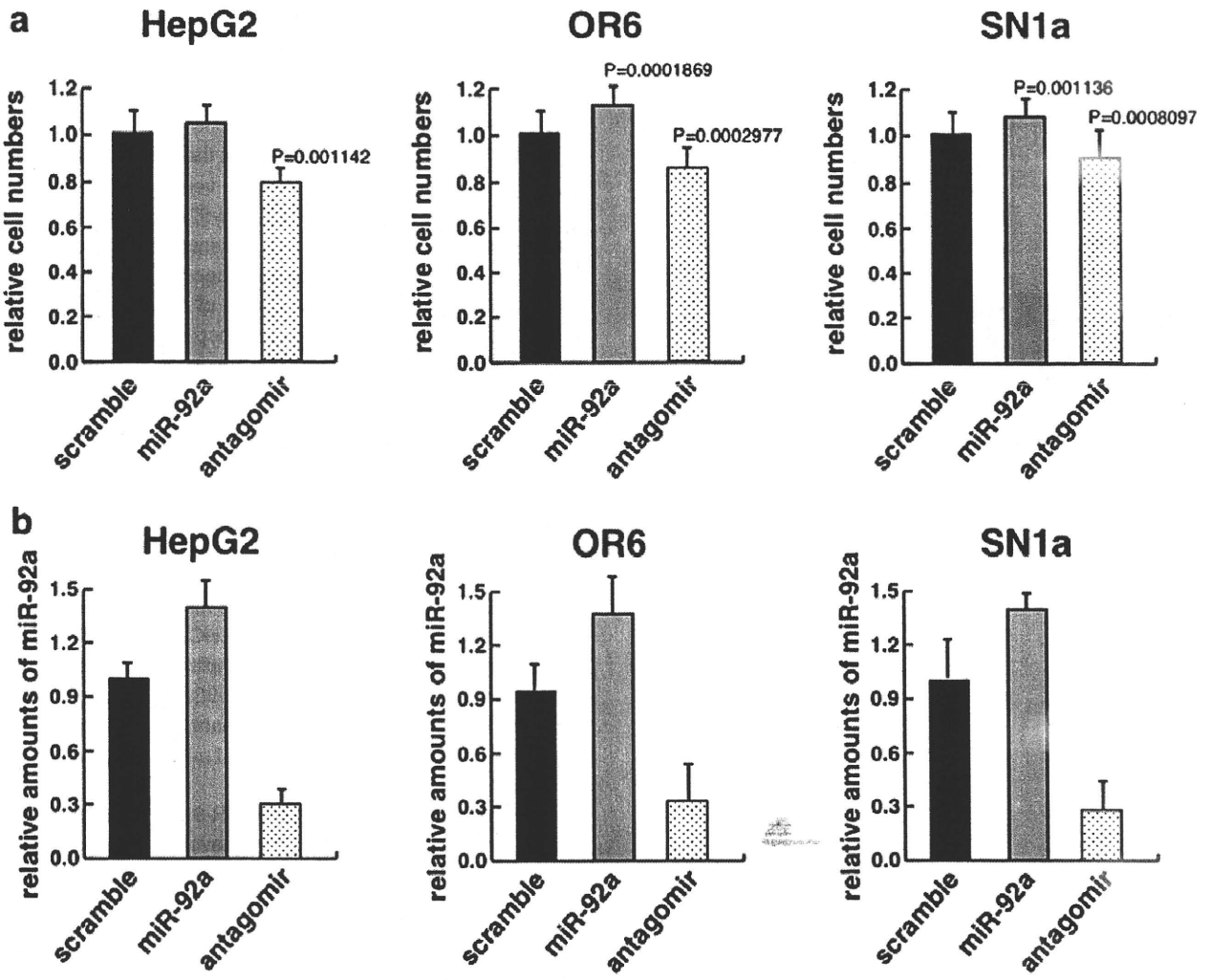

Figure 3 miR-92a modulates proliferation of HepG2, OR6 and SN1a cells. (**a**) Cell numbers of the HepG2, OR6 and SN1a cells transfected with synthetic miR-92a, anti-miR-92a antagomir, or scrambled control oligonucleotide were analyzed by MTT assays at 48 h for OR6 and SN1a and 72 h for HepG2 after transfection. *Bars*, s.d. (**b**) qRT-PCR analysis of miR-92a amounts in the cells transfected with miR-92a, anti-miR-92a antagomir or scrambled control at 48 h for OR6 and SN1a and 72 h for HepG2 after the transfection.

(n = 10) by *Taq*Man qRT-PCR. Because miR-638 is stably present in human plasmas,[13] we used miR-638 as the standard to improve the precision of the data. The ratio of miR-92a to miR-638 in the plasma samples from the HCC patients were decreased compared with that from the normal donors (Fig. 4a). Then, we further examined the ratio from the patients after surgical resection. Interestingly, the miR-92a/miR-638 levels were significantly higher than that in the plasmas from the patients before surgical resection (Fig. 4b).

DISCUSSION

In this study, we found that miR-92a was highly expressed in HCC (Figs 1,2). In addition, we demonstrated that the expression level of miR-92a affects the proliferation of hepatoma cell lines, HepG2, OR6 and SN1a (Fig. 3). These results suggest that miR-92a may play an important role in tumor progression of hepatocyte. We do not know why, but addition of miR-92a did not significantly increase the proliferation of HepG2 cells. It may be possible that HepG2 cells themselves already contain enough miR-92a to promote cancer cell proliferation. In addition, miR-92a is a part of the miR-17-92 cluster, which is actively involved in the development and progression of various cancers.[4–10] However, the molecular function of miR-92a is still unknown, and its mRNA targets have not been identified. Recently, it has been shown that one of the molecular mechanisms through which miR-92a increases cell proliferation is by negative regulation of an isoform of the cell-cycle regulator p63.[11] Thus, we examined

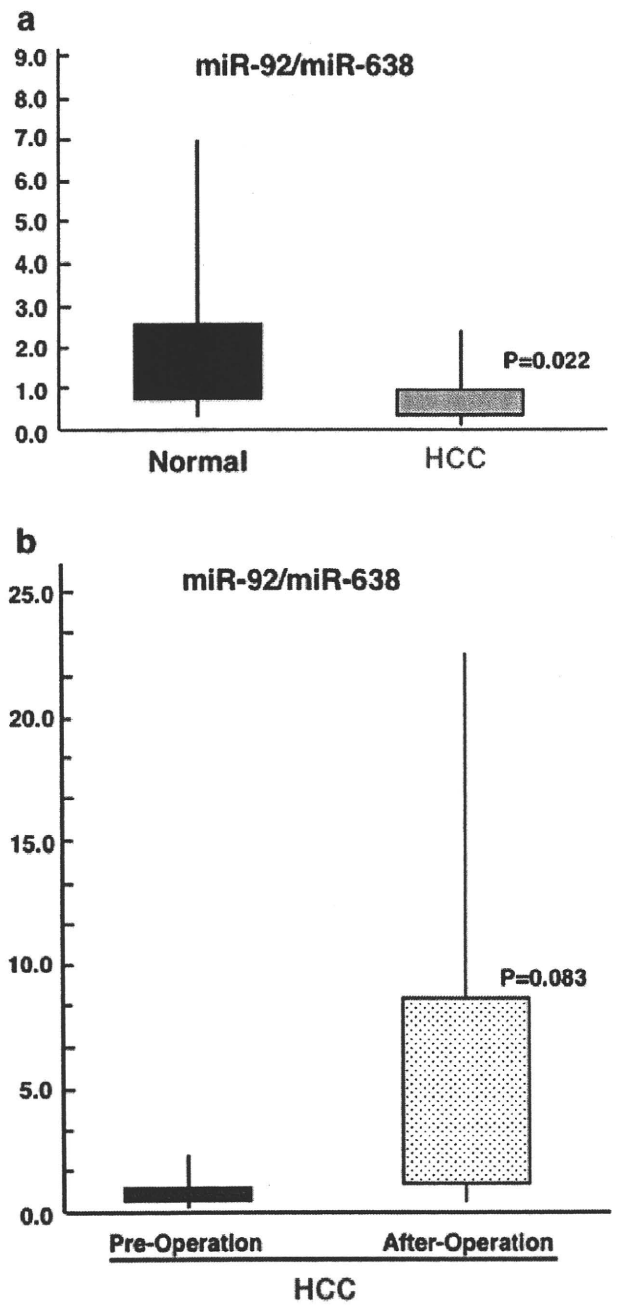

Figure 4 Comparison of miR-92a levels in the plasmas from normal individuals and hepatocellular carcinoma (HCC) patients. (**a**) The ratios of miR-92a to miR-638 in the plasmas from normal donors and HCC patients were analyzed by *Taq*Man qRT-PCR. Student's *t*-test was used to determine statistical significance. (**b**) The ratios of miR-92a to miR-638 in the plasmas from HCC patients before and after tumor resection were analyzed by *Taq*Man qRT-PCR.

the expression of p63 in HCC by immunohistochemistry. However, we could not find the positive nuclear staining both in HCC and normal hepatocyte (data not shown). On the other hand, the miRanda software found 300 different genes that have putative miR-92a binding sites conserved among *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus* at the 3′-UTR regions of their transcripts. Therefore, at least in HCC, there may be novel miR-92a targets that are involved in cancer cell proliferation.

In this report, we have revealed that the value of miR-92a/miR-638 in plasma has potential as a very sensitive marker for HCC. We found that the ratio of miR-92a to miR-638 in the plasma samples from the HCC patients were decreased compared with that from the normal donors (Fig. 4a). We did not find any differences in the values of the ratios between hepatitis B virus (HBV) infection and hepatitis C virus (HCV) infection (data not shown). On the other hand, we recently observed decrease of miR-92a in plasma samples of acute leukemia.[13] These results suggest that the decrease of the miR-92a/miR-638 level in human plasma may serve as a valuable diagnostic marker for not only acute leukemia but also solid tumors such as HCC. Moreover, we observed increase of miR-92a/miR-638 levels in the plasmas from the HCC patients after tumor resection (Fig. 4b). Thus, the miR-92a/miR-638 levels in human plasmas may also be a potential noninvasive follow up marker of HCC. To confirm this notion, a large number of plasma samples should be examined. Nevertheless, the levels of miR-92a/miR-638 promise to be an effective biomarker for malignant tumors. The physiological significance of the decrease of miR-92a in plasma is still unknown.

In summary, we have shown that miR-92a may be involved in HCC development. In addition, we have demonstrated that the ratio of miR-92a/miR-638 in blood is expected to be useful for diagnosis of HCC patients. This study may also provide useful information for further investigations of functional association between miRNAs and HCC.

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HEPATOLOGY

Association between HCV amino acid substitutions and outcome of peginterferon and ribavirin combination therapy in HCV genotype 1b and high viral load

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Key words
amino acid substitution, chronic hepatitis C, hepatitis C virus, peginterferon and ribavirin therapy, resistance to interferon.

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Abstract

Background and Aim: We prospectively compared the sensitivity to interferon (IFN) and the efficacy of antiviral combination therapy with peginterferon (PEG-IFN) and ribavirin for chronic hepatitis C virus (HCV) genotype 1b infection according to the amino acid sequences of the HCV core, E1, and NS5A regions reported to be associated with the outcome of antiviral therapy.

Methods: A total of 107 patients with HCV genotype 1b were investigated. All patients received combination therapy with PEG-IFN alpha-2b and ribavirin. Amino acids 70 and 91 (core), 139 (E1), and 2209–2248 (NS5A) of HCV were analyzed by direct nucleotide sequencing.

Results: The reduction in HCV RNA concentration at 24 h after a single administration of conventional IFN-alpha and after the start of combination therapy was significantly less marked, and rates of complete early virologic response, end-of-treatment response, and sustained virologic response (SVR) were significantly lower (all $P < 0.0001$) in patients with glutamine at amino acid 70 ($n = 29$) than in those with arginine at that position ($n = 70$). We found no differences associated with the other amino acid positions. Amino acid 70 was an independent factor for the responses to the therapy in multivariate analysis.

Conclusion: The identity of amino acid 70 of the HCV core region affected the sensitivity to IFN; patients with glutamine at amino acid 70 of HCV showed resistance to IFN. Consequently, it strongly affected the outcome of combination therapy with PEG-IFN and ribavirin in Japanese patients with HCV genotype 1b.

Introduction

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin.[1] The rate of sustained virologic response (SVR), which indicates the eradication of the hepatitis C virus (HCV) is around 50% in patients infected with HCV genotype 1, which is more resistant to this therapy than genotypes 2 or 3; the recommended duration of the PEG-IFN/ribavirin treatment period differs between patients with HCV genotype 1 and those with genotype 2 or 3.[1,2]

Many studies have been performed to elucidate the viral factor determining the sensitivity or resistance to the IFN-based antiviral therapy, especially for HCV genotype 1. Several amino acid substitutions have been reported to be associated with the efficacy of IFN-based antiviral therapy in patients infected with HCV genotype 1b.[3–9] In the late 1990s, Enomoto *et al.* reported that mutations in the amino acids at positions 2209–2248 of the NS5A region of HCV were closely associated with the efficacy of IFN monotherapy;[3] however, these results proved controversial.[10–22] Very recently, a few studies have reported an association between other amino acid substitutions and the rate of SVR by the PEG-IFN/ribavirin therapy in patients with HCV genotype 1b.[7,8] However, the influence of these amino acid substitutions on the sensitivity to IFN or the outcome of PEG-IFN/ribavirin combination therapy has not been fully established.

In the present study, the authors investigated the association of four HCV amino acid substitutions (70 and 91 of the core region, 139 of the E1 region, and 2209–2248 of the NS5A region), with IFN sensitivity. Its association with the combination therapy PEG-IFN and ribavirin was also investigated in Japanese patients chronically infected with HCV genotype 1b, and having high pretreatment HCV RNA concentration.

Patients and methods

Patients

A total of 148 patients with chronic hepatitis C and without cirrhosis received antiviral combination therapy with PEG-IFN and ribavirin at the Ogaki Municipal Hospital, Ogaki, Japan, between July 2005 and June 2007. Among them, 109 patients had been infected with HCV genotype 1b and had pretreatment HCV RNA concentration $> 100 \times 10^3$ IU/mL, as assessed by quantitative polymerase chain reaction (PCR) assay (Amplicor HCV Monitor Test, version 2.0; Roche Molecular Systems, Pleasanton, CA, USA). Of those 109 patients, 107 patients were enrolled in the study (two patients declined to enroll). The clinical characteristics of study patients are listed in Table 1. The patient group was comprised of 52 males (48.6%) and 55 females (51.4%), with a mean age of 58.9 ± 9.0 years. Twenty-two patients (20.6%) had previously received blood transfusion. Although 32 patients (29.9%) had a history of previous antiviral therapy by monotherapy with conventional IFN or combination therapy with conventional IFN and ribavirin, no patients had a history of the combination therapy with PEG-IFN and ribavirin. The average pretreatment HCV RNA concentration was $1760 \pm 1139 \times 10^3$ IU/mL. In 102 patients who underwent pretreatment liver biopsy, the grade of liver fibrosis according to the METAVIR score[23] was F0 in 5 patients (4.9%), F1 in 61 patients (59.8%), F2 in 24 patients (23.5%), and F3 in 12 patients (11.8%), respectively. No patients had co-infection with hepatitis B virus or human immunodeficiency virus. No patients were alcohol abusers or intravenous drug users.

Table 1 Clinical characteristics of study patients ($n = 107$)

Age (years)	58.9 ± 9.0
Sex (female/male)	55 (51.4)/52 (48.6)
Body weight (kg)	59.1 ± 10.2
History of interferon therapy (naive/retreatment)	75 (70.1)/32 (29.9)
History of transfusion (–/+)	85 (79.4)/22 (20.6)
Alanine aminotransferase (IU/L)	65.8 ± 64.9
Aspartate aminotransferase (IU/L)	55.9 ± 44.3
Gamma-glutamyl transpeptidase (IU)	53.7 ± 53.6
Alkaline phosphatase (IU/L)	265.2 ± 86.4
Albumin (g/dL)	4.14 ± 0.35
Total bilirubin (mg/dL)	0.69 ± 0.28
White blood cell count (/μL)	5201 ± 1197
Hemoglobin (g/dL)	14.0 ± 1.4
Platelet count ($\times 10^3$/μL)	166 ± 51
Liver histology-activity (A0/A1/A2/A3)†	2 (2.0)/55 (53.9)/36 (35.3)/9 (8.8)
Liver histology-fibrosis (F0/F1/F2/F3)†	5 (4.9)/61 (59.8)/24 (23.5)/12 (11.8)
HCV RNA concentration ($\times 10^3$ IU/mL)	1760 ± 1139
Reduction of peginterferon dose	29 (27.1)
Reduction of ribavirin dose	49 (45.8)
Response (SVR/relapse/NR)	39 (36.5)/38 (35.5)/30 (28.0)

Percentages are shown in parentheses.
†Liver biopsy was not performed in five patients.
HCV, hepatitis C virus; NR, no response; SVR, sustained virologic response.

Single administration test of conventional interferon alpha to evaluate sensitivity to interferon

All patients were underwent a single administration test of conventional IFN alpha more than 2 weeks before the start of the combination therapy to evaluate the sensitivity of HCV to IFN in each patient. They received intramuscular administration of 6 mega-units of standard IFN alpha-2b (Intron A; Schering-Plough, Tokyo, Japan). The concentration of HCV RNA was measured before and 24 h after the single administration test and the reduction of serum HCV RNA was calculated.

Combination therapy with peginterferon and ribavirin

For combination therapy with PEG-IFN and ribavirin, all patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough) weekly and ribavirin (Rebetol, Schering-Plough) daily according to the manufacturer's recommendations. The dose of PEG-IFN and ribavirin were adjusted by patient body weight. Patients weighing ≤ 45 kg were given 60 μg of PEG-IFN alpha-2b once a week, those weighing > 45 kg and ≤60 kg were given 80 μg, those weighing > 60 kg and ≤75 kg were given 100 μg, those weighing > 75 kg and ≤90 kg were given 120 μg, and those weighing > 90 kg were given 150 μg. Patients weighing ≤ 60 kg were given 600 mg of ribavirin per day, those weighing > 60 kg and ≤80 kg were given 800 mg of ribavirin per day, and those weighing > 80 kg were given 1000 mg of ribavirin per day. All patients were scheduled to undergo 48 weeks of treatment; longer durations were not considered in this study. Serum HCV RNA concentration was measured every 4 weeks on an outpatient basis. The presence of HCV RNA in the serum was measured by the qualitative Amplicor Monitor HCV RNA assay (AMPLICOR Hepatitis C Virus (HCV) Test, version 2.0, Roche Molecular Systems; detection limit, 50 IU/mL) to confirm the undetectability of serum HCV RNA, when it was unquantifiable (under the detection limit) by the quantitative Amplicor Monitor assay (detection limit, 615 IU/mL). Patients were classified into categories as follows: rapid virologic response (RVR) was defined as undetectable serum HCV RNA at 4 weeks from the start of the combination therapy. Complete early virologic response (cEVR) was defined as undetectable serum HCV RNA within 12 weeks of the start of the therapy. End-of-treatment response (ETR) was defined as undetectable serum HCV RNA at the end of the treatment period (i.e. 48 weeks after the start of the therapy). Sustained virologic response (SVR) was defined as undetectable serum HCV RNA at 24 weeks after the end of therapy. Relapse was defined as positive serum HCV RNA during the period between the end of treatment and 24 weeks thereafter, following ETR. Null-response (NR) was defined as positive serum HCV RNA throughout the treatment period and thereafter.