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Prospective Risk Assessment for Hepatocellular Carcinoma Development in Patients with Chronic Hepatitis C by Transient Elastography

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Liver stiffness, noninvasively measured by transient elastography, correlates well with liver fibrosis stage. The aim of this prospective study was to evaluate the liver stiffness measurement (LSM) as a predictor of hepatocellular carcinoma (HCC) development among patients with chronic hepatitis C. Between December 2004 and June 2005, a total of 984 HCV-RNA positive patients, without HCC or a past history of it, visited the University of Tokyo Hospital. LSM was performed successfully in 866 patients, who gave informed consent. During the follow-up period (mean, 3.0 years), HCC developed in 77 patients (2.9% per 1 person-year). The cumulative incidence rates of HCC at 1, 2, and 3 years were 2.4%, 6.0%, and 8.9%, respectively. Adjusting for other significant factors for HCC development, patients with higher LSM were revealed to be at a significantly higher risk, with a hazard ratio, as compared to LSM ≤10 kPa, of 16.7 (95% confidence interval [CI], 3.71-75.2; $P < 0.001$) when LSM 10.1-15 kPa, 20.9 (95% CI, 4.43-98.8; $P < 0.001$) when LSM 15.1-20 kPa, 25.6 (95%CI, 5.21-126.1; $P < 0.001$) when LSM 20.1-25 kPa, and 45.5 (95% CI, 9.75-212.3; $P < 0.001$) when LSM >25 kPa. *Conclusions:* This prospective study has shown the association between LSM and the risk of HCC development in patients with hepatitis C. The utility of LSM is not limited to a surrogate for liver biopsy but can be applied as an indicator of the wide range of the risk of HCC development. (HEPATOLOGY 2009;49:1954-1961.)

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Hepatocellular carcinoma (HCC) is a common malignancy worldwide,[1] currently showing an increasing incidence in the United States and elsewhere.[2-4] HCC usually develops in liver already suffering from chronic liver diseases. In particular, hepatitis C virus (HCV)-related cirrhosis is associated with an extremely high risk of HCC development, with a reported annual incidence ranging between 3% and 8%.[5-7] The prognosis of HCC is deemed poor unless the cancer is detected and treated at an early stage.[8-10] Thus, the assessment of risk for HCC development is essential in the management of patients with chronic liver diseases.

Chronic hepatitis C is an endemic disease affecting millions of individuals globally.[11-14] HCV infection is typically accompanied by no conspicuous symptoms and may result in cirrhosis unnoticed over a couple of decades. The risk factors for hepatic carcinogenesis in patients with chronic hepatitis C have been vigorously studied,[5,6,15,16] and the degree of liver fibrosis is known to be the strongest.[6] Until recently, however, the degree of liver fibrosis could be reliably assessed only with liver biopsy, an invasive procedure with the possibility of life-threatening complications.[17]

Recently, liver stiffness measured noninvasively by transient elastography has been reported to be well correlated with histologically assessed liver fibrosis stage.[18-22] Both routine and specific biomarkers, together with a combination thereof, have been proposed as noninvasive indicators of the degree of liver fibrosis.[23-25] Among them, Fibrotest is accepted as a promising noninvasive marker to

Abbreviations: AFP, alpha fetoprotein; AIC, Akaike information criterion; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; CT, computed tomography; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVPG, hepatic venous pressure gradient; HR, hazard ratio; LSM, liver stiffness measurement; SVR, sustained virological response.

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assess liver fibrosis stage and also reported as a useful prognostic factor for patients with hepatitis C.[26] A previous cross-sectional study reported that transient elastography surpassed Fibrotest in the diagnosis of cirrhosis.[18] However, the accuracy of transient elastography in assessing the prognosis of patients with hepatitis C has not been validated in prospective studies. We previously reported in a cross-sectional setting that liver stiffness measurement (LSM) was strongly associated with the probability of the presence of HCC among patients with hepatitis C.[27] Besides its noninvasiveness, LSM has a possible advantage over liver biopsy of being less prone to sampling errors and intra- and interobserver variability.[28,29]

Portal hypertension is a direct consequence of the fibrotic transformation of liver and a progressive complication of cirrhosis. Therefore, the management of patients with cirrhosis and portal hypertensive gastrointestinal bleeding depends on the phase of portal hypertension. Measurement of the hepatic venous pressure gradient (HVPG) is currently employed for the evaluation of portal hypertension.[30] Vizzutti et al.[31] reported that LSM was well correlated with HVPG.

Not only the presence of cirrhosis but also the degree of fibrosis in noncirrhotic liver, as expressed in fibrosis stages, is known to be correlated with the risk of HCC. The correlation can be more rigorously analyzed by using LSM, which is expressed in kPa as a continuous variable. Moreover, LSM has a wide dynamic range within the cirrhotic stage, from the cutoff level from noncirrhosis (15-17 kPa) to the upper measurement limit of the present device (75 kPa). It is of interest to know whether the risk of HCC can be differentiated further among cirrhotic patients according to their LSM.

We conducted the present study to prospectively evaluate the efficacy of LSM by transient elastography as a predictor of HCC development among a cohort of patients with hepatitis C with various degrees of liver fibrosis.

Patients and Methods

Patients. Between December 2004 and June 2005, a total of 984 HCV-RNA positive patients, excluding those with HCC or a past history of it, visited the liver clinic of Department of Gastroenterology, the University of Tokyo Hospital. LSM was performed on those patients who gave informed consent. All patients were positive for serum HCV-RNA and showed at least a transiently elevated serum alanine aminotransferase (ALT) level. We excluded from this study patients with concomitant hepatitis B virus surface antigen positivity, patients with uncontrollable ascites, patients on interferon (IFN) therapy, and patients who visited only for consultation purposes. We also examined the history of IFN therapies and responses during the follow-up period. A sustained virological response (SVR) was defined as undetectable HCV-RNA at least 24 weeks after the end of therapy. Diagnosis of cirrhosis was based on the presence of clinical and laboratory features of portal hypertension (the presence of esophageal varices and/or collateral circulation at endoscopy and ultrasonography). The study protocol conformed to the ethical guidelines of the 1975 Helsinki Declaration and was approved by the Institutional Review Board. All blood tests were performed at the time of LSM.

Patient Follow-up and Diagnosis of HCC. Each patient was screened for HCC with ultrasonography at or immediately after the first visit and those in whom HCC was detected were not included in this study. Afterwards, patients were followed up at the outpatient clinic with blood tests including tumor markers and ultrasonography every 3 to 6 months. Contrast-enhanced computed tomography (CT) was performed when serum alpha fetoprotein (AFP) levels showed an abnormal rise and/or tumors were detected as possible HCC on ultrasonography.[32] HCC was diagnosed by dynamic CT, considering hyperattenuation in the arterial phase with washout in the late phase as the definite sign of HCC.[33,34] When the diagnosis of HCC was not clear, ultrasound-guided tumor biopsy was performed and pathological diagnosis was made based on Edmondson-Steiner criteria.[35] The last observation analyzed in this study was May 31, 2008.

Transient Elastography. LSM was performed using Fibroscan (Echosens, Paris, France), a new medical device based on elastometry.[21] The investigators had undergone a previous training period in which each had performed at least 50 measurements. The procedure is totally noninvasive and performed on the right lobe of the liver through the intercostal space. LSM was performed within 1 week after laboratory tests were obtained. Only LSM obtained in at least eight successful acquisitions with a success rate of at least 60% were considered valid.

Statistical Analysis. Data are expressed as the mean ± standard deviation (SD) and range in parentheses unless otherwise indicated. The categorical variables were compared by chi-square tests, whereas continuous variables were compared with unpaired Student's t test (parametric) or Mann-Whitney U test (nonparametric). A P-value < 0.05 on two-tailed test was considered significant. Annual incidence of hepatocarcinogenesis was assessed with the person-year method. Patients were censored at the time of death without HCC development, the last visit when lost to follow-up, or the end of the study period. Cumulative incidence of HCC was estimated using the Kaplan-Meier method. In the analysis of

risk factors for hepatocarcinogenesis, we tested the following variables obtained at the time of entry in univariate and multivariate Cox proportional hazard regression analysis: age, gender, body mass index (BMI), heavy alcohol drinking, liver stiffness, clinical cirrhosis, serum albumin concentration, total bilirubin concentration, ALT levels, aspartate aminotransferase (AST) levels prothrombin activity, platelet counts, and AFP concentration. Stepwise variable selection with Akaike information criterion (AIC) was used to find the best model in multivariate analysis. Multichotomous categorical variables were represented by corresponding binary dummy variables. Subgroup analyses using a Cox proportional hazard model was applied to estimate the hazard ratios (HRs) of higher LSM (>15 kPa) versus lower LSM (≤15 kPa), with their two-tailed *P*-values, for explanatory variables. The explanatory variables used for HR estimation were: age, gender, serum albumin concentration, BMI, AST level, ALT level, platelet counts, AFP concentration, clinical cirrhosis, and IFN therapy (with or without history of therapies and SVR or non-SVR during the follow-up period). The median value was chosen as each cutoff level. Processing and analysis were performed using S-PLUS 2000 (MathSoft, Seattle, WA).

Results

Patients' Profile. Between December 2004 and June 2005, a total of 876 patients underwent LSM. Ten patients were excluded because of unsuccessful measurements, mostly due to obesity (four patients had less than eight valid measurements and six had a success rate lower than 60%). Thus, 866 patients were included in the current analysis. Their characteristics at the time of LSM are summarized in Table 1. There were 398 men and 468 women, with a mean age of 62.2 ± 11.4 years. Heavy alcohol consumption was noted in 33 (3.8%). There were 109 patients whose AFP level exceeded 20 ng/mL.

Incidence of HCC. The mean follow-up period was 3.0 years, constituting 2,627 person-years overall. During the follow-up period a total of 35 (4.0%) patients had been lost to follow-up and censored at the time of last visit. Six patients died without HCC and they were censored at the time of death. The remaining patients were censored at the end of the study observation period (May 31, 2008). By the end of the follow-up, HCC developed in 77 patients (2.9% per 1 person-year). The cumulative incidence rates of HCC at 1, 2, and 3 years estimated by the Kaplan-Meier method were 2.4%, 6.0%, and 8.9%, respectively. The baseline characteristics of patients who developed HCC and those who did not are shown in Table 2.

Table 1. Baseline Characteristics of Patients (n = 866)

Variables	n = 866
Age (years) *	62.2 ± 11.3 (17-89)
Male, n (%)	398 (46.0)
Alcohol consumption > 80 g/day, n (%)	33 (3.8)
BMI (kg/m^2)*	22.5 ± 3.1 (14.4-36.9)
Serum albumin (g/dL)*	4.0 ± 0.4 (2.5-5.0)
Total bilirubin (mg/dL)*	0.8 ± 0.4 (0.3-4.6)
AST (IU/L)*	51 ± 34.2 (9-286)
ALT (IU/L)*	54 ± 46.9 (2-503)
Prothrombin time activity (%)*	84.9 ± 14.9 (38.9-100)
Platelet count (10^9/L)*	160 ± 67 (21-436)
AFP (ng/mL)*	14.9 ± 44.6 (0.8-591.8)
Liver stiffness (kPa)*	11.9 ± 9.7 (2.5-75)
IQR	2.4 ± 1.9 (0.5-12.0)
Success rate	78.5 ± 12.5 (60-100)
Patients who received IFN, n (%)	173 (20.0)
Patients who achieved SVR, n (%)	83 (9.6)

*Expressed as mean ± SD (range).

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; IQR, interquartile range; IFN, interferon; LSM, liver stiffness measurement; SVR, sustained virologic response.

Cause of Death. Two patients died of HCC. Two patients died of liver failure without HCC development. Four patients died of liver-unrelated causes. None received liver transplantation.

Incidence of HCC Stratified by LSM. Cumulative incidence rates at 1, 2, and 3 years in each group were 0.4%, 0.4%, and 0.4% (0.11% per 1 person year) in the patients with LSM ≤10 kPa; 1.4%, 5.5%, and 11.7% (2.9% per 1 person-year) in the patients with LSM 10.1-15 kPa; 3.8%, 12.0%, and 19.2% (5.0% per 1 person-year) in the patients with LSM 15.1-20 kPa; and 8.7%, 15.7%, and 25.2% (8.3% per 1 person-year) in the patients with LSM 20.1-25 kPa, 11.5%, 30.4%, and 38.5% (14.4% per 1 person-year) in the patients with LSM >25 kPa, respectively (Fig. 1). The incidence rates differed significantly among the five groups ($P < 0.001$ by the log-rank test), increasing in accordance with liver stiffness.

The number of patients who developed HCC and those who did not in each rank of LSM is shown in Table 3, together with a summary of some baseline characteristics. Patients who developed HCC tended to be older and have a higher AFP level at the time of entry than those in the same rank of LSM who did not develop cancer.

Risk Analyses. Univariate analyses showed that the risk of HCC increased in accordance with LSM (Table 4). Other significant risk factors for HCC included older age, male gender, clinical cirrhosis, heavy alcohol intake, lower serum albumin level, higher total bilirubin level, higher ALT and AST levels, lower prothrombin time activity, lower platelet counts, higher BMI level, AFP over 10 ng/mL, no treatment of IFN, and without SVR.

Table 2. Baseline Characteristics of Patients According to HCC Development

Variables	HCC Development (+), n = 77	HCC Development (−), n = 789	P Value
Age (years) *	68.2 ± 8.0 (50-89)	61.6 ± 11.5 (17-88)	<0.001
Male, n (%)	41 (53.2)	357 (45.2)	0.19
Alcohol consumption > 80 g/day, n (%)	7 (9.1)	26 (3.2)	0.02
BMI (kg/m^2)*	23.3 ± 3.1 (16.8-29.7)	22.4 ± 3.1 (14.4-36.9)	0.02
Serum albumin (g/dL)*	3.6 ± 0.4 (2.7-4.5)	4.0 ± 0.4 (2.5-5.0)	<0.001
Total bilirubin (mg/dL)*	1.1 ± 0.6 (0.4-3.3)	0.9 ± 0.4 (0.3-4.6)	<0.001
AST (IU/L)*	70 ± 33 (29-217)	49 ± 34 (9-286)	<0.001
ALT (IU/L)*	66 ± 42 (19-231)	53 ± 47 (2-503)	0.019
Prothrombin time activity (%)*	73.6 ± 10.9 (50-100)	86.1 ± 14.8 (38.9-100)	<0.001
Platelet count (10^9/L)*	104 ± 44 (36-246)	166 ± 66 (21-436)	<0.001
AFP (ng/mL)*	53.4 ± 111 (2.0-591.8)	11.1 ± 28.8 (0.8-339.4)	<0.001
Liver stiffness (kPa)*	26.0 ± 13.8 (8.9-69.1)	10.5 ± 8.0 (2.5-75)	<0.001
Clinical cirrhosis, n (%)	57 (74.0)	139 (17.6)	<0.001

*Expressed as mean ± SD (range).

Abbreviations: AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index.

Stepwise variable selection with AIC was used to find the best model in multivariate analysis (Table 5). Patients with higher LSM were revealed to be at a significantly higher risk, with an HR of 16.7 (95% confidential interval [CI], 3.71-75.2; $P < 0.001$) with LSM 10.1-15 kPa, 20.9 (95% CI, 4.43-98.8; $P < 0.001$) with 15.1-20 kPa, 25.6 (95%CI, 5.21-126.1; $P < 0.001$) with 20.1-25 kPa, and 45.5 (95% CI, 9.75-212.3; $P < 0.001$) with >25 kPa, as compared to LSM ≤10 kPa. The presence of clinical cirrhosis is also found to be a significant risk factor for HCC development. LSM is thought to represent the degree of live fibrosis, whereas clinical cirrhosis is based not directly on fibrosis but on the degree of liver dysfunction and portal hypertension. Thus, these two factors are mutually related but not identical, and may be complementary in evaluating the risk of HCC. The other risk factors considered significant are older age, male gender, and serum albumin level.

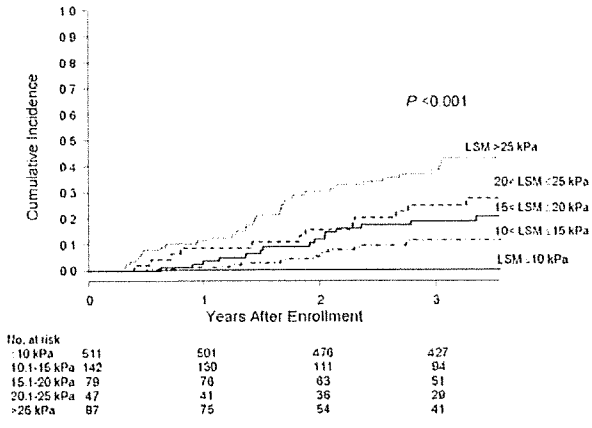

Fig. 1. Cumulative incidence of HCC development stratified based on LSM (N = 866). LSM, liver stiffness measurement.

The effects of LSM on the risk of HCC development were also evaluated in subgroup analyses to check whether higher LSM was a significant risk factor over strata (Fig. 2). Indeed, higher LSM was found to be a significant risk factor for HCC development in almost every subgroup. Interestingly, the HR attributed to higher LSM (>15 kPa) was greater in the subgroups unlikely to develop HCC, such as those with higher platelet count, absence of clinical cirrhosis, or lower AFP, than in the alternative subgroups. Higher LSM was a significant risk factor in both IFN-treated and IFN-untreated patients. Higher LSM may indicate a risk of HCC also among IFN-treated patients who achieved SVR, although statistical significance was not reached because of the small number of events among the subgroup (n = 2).

Discussion

Liver fibrosis is the strongest prognostic indicator of chronic hepatitis, which is currently best evaluated by liver biopsy.[6,17,36,37] However, liver biopsy has several disadvantages, including poor patient compliance, sampling errors, limited usefulness for dynamic follow-up, and a risk of complications. LSM has been confirmed to be well correlated with histological fibrosis stage in the literature.[18-22] We have previously shown the relationship between LSM and hepatocarcinogenesis in a cross-sectional study.[27] However, the results remained to be confirmed prospectively.

Various risk factors have been reported for HCC development among patients with HCV: older age,[6] male sex,[6] heavy alcohol intake,[38] high BMI,[39] cirrhosis,[6,16] lower platelet count, high serum AFP level,[40] low serum albumin level,[38] and high serum ALT level.[40] Our results were consistent with these findings. In the present cohort

Table 3. Characteristics of Patients in Each Rank of LSM

	Patients Who Developed HCC (n = 77)	Patients Who Did Not Develop HCC (n = 789)	P Value
LSM ≤ 10 kPa, n (%)	2 (0.4)	509 (99.6)	
Gender, male/female	0/2	231/278	0.50
Age, years*	74.5 ± 0.1	60.0 ± 11.8	0.08
AFP (ng/mL)*	11.2 ± 8.7	4.3 ± 4.8	0.07
Platelet count (per 10^9/L)*	141 ± 60	191 ± 60	0.24
LSM 10.1-15, n (%)	14 (10.0)	128 (90.0)	
Gender, male/female	11/3	68/60	0.12
Age, years*	68.7 ± 8.8	63.7 ± 11.0	0.10
AFP (ng/mL)*	12.3 ± 9.2	12.5 ± 9.2	0.06
Platelet count (per 10^9/L)*	114 ± 57	138 ± 54	0.13
LSM 15.1-20, n (%)	15 (19.0)	64 (81.0)	
Gender, male/female	9/6	25/39	0.23
Age, years*	69.3 ± 7.2	65.7 ± 9.5	0.17
AFP (ng/mL)*	31.8 ± 57.7	19.0 ± 25.3	0.59
Platelet count (per 10^9/L)*	117 ± 40	114 ± 48	0.84
LSM 20.4-25, n (%)	12 (25.5)	35 (74.5)	
Gender, male/female	7/5	8/27	0.05
Age, years*	70.0 ± 8.3	67.9 ± 9.0	0.46
AFP (ng/mL)*	17.2 ± 22.7	47.8 ± 79.0	0.14
Platelet count (per 10^9/L)*	119 ± 31	102 ± 38	0.18
LSM >25, n (%)	34 (39.1)	53 (61.9)	
Gender, male/female	14/20	25/28	0.74
Age, years*	66.4 ± 8.1	63.0 ± 9.9	0.09
AFP (ng/mL)*	95.1 ± 153.5	39.6 ± 60.5	0.005
Platelet count (per 10^9/L)*	87 ± 39	103 ± 55	0.15

*Expressed as mean ± SD.

study we have shown that LSM is also a significant risk factor of HCC development independent of these factors. Transient elastography can be considered as a surrogate marker for liver fibrosis. Although it is not clear whether liver fibrosis plays a direct role in hepatocarcinogenesis, the degree of fibrosis may be a surrogate for the accumulated DNA damage as a consequence of long-term necroinflammation and regeneration. A distinct advantage of LSM over liver biopsy is the wider dynamic range in the evaluation of liver cirrhosis. In the METAVIR[41] and Desmet et al.[42] scoring systems, cirrhosis is represented by a single category, F4. However, the degree of fibrosis may vary widely among patients in this category, and the risk of HCC may not be uniform. In the Ishak scoring system, incomplete cirrhosis scores 5 and complete cirrhosis scores 6 in fibrosis staging. Complete cirrhosis is not further divided. These histopathologic scoring systems are

Table 4. Risk Factors for HCC Development: Univariate Analysis

Variables	Hazard Ratio (95%CI)	P Value
Age (per 1 year old)	1.06 (1.04-1.09)	<0.001
Male	1.36 (0.87-2.01)	0.17
BMI (per 1 kg/m^2)	1.08 (1.01-1.16)	0.02
Alcohol consumption > 80g/day	1.81 (1.04-3.34)	0.03
Clinical cirrhosis	12.3 (7.3-20.6)	<0.001
LSM (kPa)		
≤10	1.00	
10.1-15	28.8 (6.55-126.8)	<0.001
15.1-20	54.7 (12.5-239.1)	<0.001
20.1-25	76.3 (17.1-340.7)	<0.001
>25	135.6 (32.6-564.8)	<0.001
Serum albumin (per 1.0 g/dL)	0.11 (0.07-0.17)	<0.001
Total bilirubin (per 1.0 mg/dL)	2.04 (1.53-2.70)	0.005
AST (per 1 IU/L)	1.01 (1.01-1.01)	<0.001
ALT (per 1 IU/L)	1.00 (1.00-1.01)	<0.001
Prothrombin time activity (per 1%)	0.94 (0.94-0.95)	<0.001
Platelet count (per 10^9/L)	0.84 (0.80-0.88)	<0.001
Patients treated by IFN	0.46 (0.22-0.95)	0.036
Patients with SVR	0.24 (0.059-0.97)	0.0045
AFP > 10 ng/mL	2.58 (2.05-3.25)	<0.001

Abbreviations: AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; LSM, liver stiffness measurement.

Table 5. Risk Factors for HCC Development: Multivariate Analysis

Variables	Hazard Ratio (95%CI)	P Value
Age (per 1 year old)	1.04 (1.01-1.07)	<0.001
Male	1.62 (1.03-2.56)	<0.001
Clinical cirrhosis	2.11 (1.15-3.89)	<0.001
LSM (kPa)		
≤10	1.00	
10.1-15	16.7 (3.71-75.2)	<0.001
15.1-20	20.9 (4.43-98.8)	<0.001
20.1-25	25.6 (5.21-126.1)	<0.001
>25	45.5 (9.75-212.3)	<0.001
Serum albumin (per 1.0 g/dL)	0.52 (0.28-0.96)	<0.001

LSM, liver stiffness measurement.

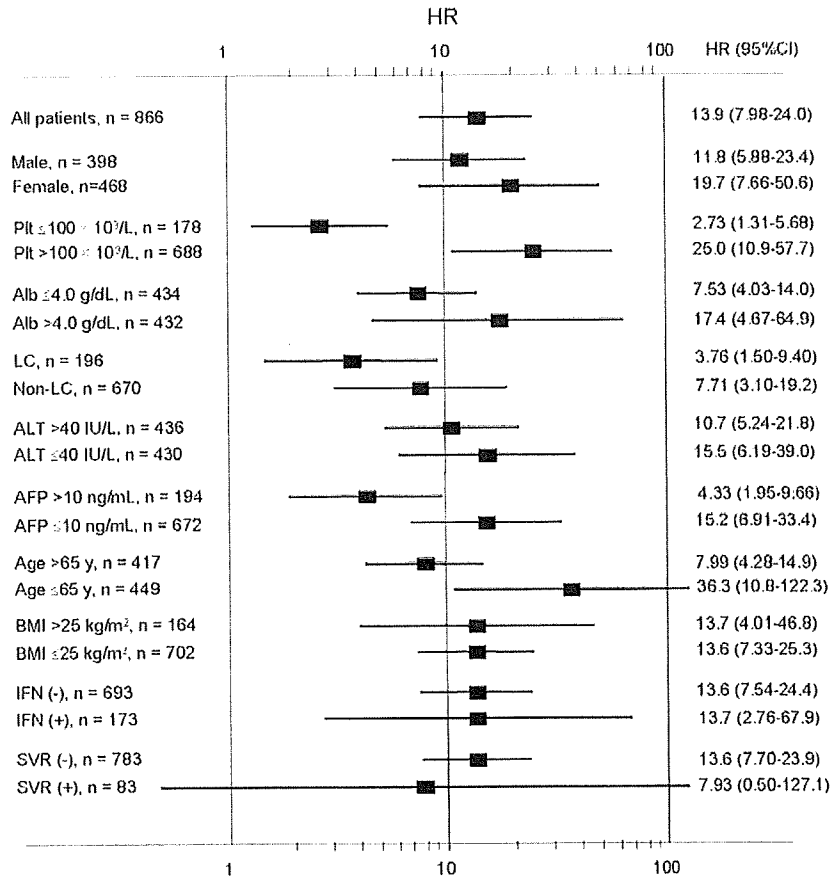

Fig. 2. Sensitivity analyses divided by risk factors. The risk of HCC development was evaluated in subgroups to check whether higher LSM was a significant risk factor over strata, which was confirmed in most subgroups. The HR attributed to higher LSM (>15 kPa) was greater in the subgroups less likely to develop HCC, such as those with higher platelet count, absence of clinical cirrhosis, or lower AFP, than in the alternative subgroups. HR, hazard ratio; LC, liver cirrhosis; ALT, alanine aminotransferase; AFP, alpha fetoprotein; BMI, body mass index; IFN, interferon; SVR, sustained virological response.

defined by qualitative characters, and thus do not constitute an interval scale.

Foucher et al.[19] reported that the LSM in cirrhotic patients ranged from 17.6 to 75 kPa (maximum measurable value) and correlated well with clinical parameters indicating severity of cirrhosis. They established the cutoff values for complications of cirrhosis with a negative predictive value of greater than 90%. The cutoff was 27.5 kPa for the presence of esophageal varices stage 2/3, 37.5 kPa for liver function Child B or C, 49.1 kPa for a past history of ascites, 53.7 kPa for HCC, and 62.7 kPa for esophageal variceal bleeding. In other words, cirrhosis can be further stratified with clinical relevance based on LSM. In the current study the risk of HCC development increased in accordance with LSM, even within the range of cirrhosis, reemphasizing the importance of further stratification of cirrhosis.

In clinical practice, surveillance is intensively performed on patients at high risk of development of HCC. Our data suggested that LSM may sometimes be high even in patients without other risk factors for HCC such as low platelet count, low albumin level, or high bilirubin level. Such patients are nevertheless at a high risk of HCC, which indicates that transient elastography complements other laboratory tests in identifying high-risk patients. Indeed, among 77 patients who developed HCC, 19 patients were diagnosed as noncirrhosis by clinical parameters. However, LSM was higher than 15 kPa in eight of these 19 patients (data not shown). Subgroup analyses also suggest that even in those patients who are unlikely to develop HCC, i.e., female, young, with high platelet count, low BMI, low transaminase level, and low AFP level, a high LSM indicates a significant risk of HCC development.

One of the limitations of the present study is that this cohort was constructed based on a split-sample technique. Although validation in an independent study population will be of greater value, the number of patients is currently not large enough for that. Another limitation is the fact that about 20% of the patients underwent IFN therapy after enrollment, possibly affecting disease progression and hepatocarcinogenesis. Among those who underwent IFN therapy, 83 patients achieved SVR and two among them developed HCC during the follow-up period. LSM

was 12 kPa and 21.8 kPa, respectively, in these two patients. This result suggests that patients with a high LSM need attention for the development of HCC even after achieving SVR by IFN therapy. The changes in LSM after IFN therapy, especially after achieving SVR, together with the changes in the risk of HCC development, is to be elucidated in future studies.

In the present study, HCC rarely developed in patients with LSM $\leq$10 kPa. The two patients who did develop HCC in spite of low LSM had F3 liver fibrosis at the time of HCC development and one of them had esophageal varices on endoscopy. Moreover, those patients who developed HCC in spite of lower LSM tended to be of older age and had higher baseline AFP levels and lower platelet counts than those patients in the same range of LSM who did not develop cancer. Thus, patients with clinical cirrhosis or other risk factors need proper attention for HCC development even if the LSM is low.

In conclusion, this prospective cohort study has shown the association between LSM and the risk of HCC development in chronic HCV patients. The utility of LSM is not limited to a surrogate for liver biopsy but can be applied as a dynamic indicator of the risk of HCC development.

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ORIGINAL ARTICLE

Quantification of hepatitis C amino acid substitutions 70 and 91 in the core coding region by real-time amplification refractory mutation system reverse transcription-polymerase chain reaction

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Abstract

Objective. The effects of hepatitis C virus (HCV) sequence variations on the success of antiviral therapy or the development of hepatocellular carcinoma (HCC) are complex for many reasons. Recently, there have been several reports on the effects of genotype 1b HCV core amino acid substitutions 70 and/or 91 on the outcome of antiviral therapies and the clinical course. The purpose of this study was to establish real-time amplification refractory mutation system (ARMS) reverse transcription (RT)-polymerase chain reaction (PCR) assays for easy detection of these HCV mutations. ***Material and methods.*** Plasmids p-core-W, including the wild-type HCV core coding region (70R and 91L), and p-core-M, including the mutant-type HCV core (70Q and 91M), were constructed by cloning and PCR-based mutagenesis for control vector of the wild-type core and that of the mutant core, respectively. Using serially diluted forms of these vectors, SyBr Green-based real-time ARMS RT-PCR detection with each of the specific primer pairs was performed. ***Results.*** Each primer could clearly distinguish the difference between p-core-W and p-core-M at the same copy numbers. Concerning substitution 70, the ratios 100:1, 10:1, 1:1, 1:10, and 1:100 of p-core-W versus p-core-M could be distinguished, while for substitution 91, the ratios 100:1, 10:1, 1:1, 1:10, 1:100, and 1:1000 could be distinguished, confirming the sensitivity and specificity of the assay. ***Conclusions.*** This method could be a useful alternative for the detection of genotype 1b HCV core amino acid substitutions 70 and 91 and be reliably applied for rapid screening.

Key Words: *ARMS, core, HCV, interferon response, real-time PCR*

Introduction

More than 170 million people world-wide are chronically infected with hepatitis C virus (HCV), which can lead to hepatic cirrhosis and hepatocellular carcinoma (HCC) [1]. Treatment with peginterferon and ribavirin for 24–48 weeks can result in a sustained loss of serum HCV-RNA (termed a sustained virological response (SVR)), with resolution of chronic hepatitis in approximately half of the patients [2]. Several new, potent HCV protease and polymerase inhibitors have been described recently, but none of them are available for therapeutic use.

The genomic region encoding the HCV core protein is located between amino acids 1 and 191 and is likely to be the first gene product synthesized due to its localization at the 5′ end of the HCV polyprotein transcript [3]. The core protein has an ability to interact with the viral genomic region to form nucleocapsids [4], and the presence of a putative DNA-binding motif, nuclear localization signals, phosphorylation sites, and a nucleocytoplasmic localization of the core protein suggest its possible function as a gene regulatory protein [3,5]. In many previous studies it has been suggested that the HCV core protein may be important in hepatocarcinogenesis and interferon signaling [3,6–8].

HCV genotype 1b is a major genotype (~70%) in Japan. HCV genotype 1 is one of the most refractory to interferon treatment with or without ribavirin. It has been reported that its response to interferon

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monotherapy is affected by HCV NS5A gene diversity [9]. Thus, sequence diversity may predict the response to the combination therapy of peginterferon and ribavirin. Furthermore, ribavirin has different antiviral effects from those of interferon [10]. An approach to the prediction of treatment against hepatitis C in patients who do not have SVR is urgently needed. Several reports suggest that HCV amino acid substitutions 70 and 91 in the core coding region affect the results of combination therapies of interferon and ribavirin [11–13], but most of these studies were retrospective, and we do not know whether these substitutions already existed before treatment or were selected by the treatment. A sensitive, real-time polymerase chain reaction (PCR)-based assay for the detection of these mutations in the presence of high levels of wild-type virus is described here. The method is based on the amplification refractory mutation system (ARMS) reverse transcription (RT)-PCR for detection of single base mutations [14,15].

Material and methods

Plasmid DNA controls

Plasmids carrying HCV genotype 1 b core wild-type and mutant clones were made as described previously [16,17] and are summarized in Table I. Plasmid DNA was purified using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Plasmids were serially diluted 1:10 in EASY dilution (for real-time PCR) (Takara, Ohtsu, Shiga, Japan) to give a dilution range of 1–1 $\times 10^9$ copies for controls of real-time PCR.

Extraction of HCV-RNA from serum

Serum samples (100 µl) were extracted using the high pure viral RNA kit (Roche Diagnostics, Indianapolis, Ind., USA) according to the manufacturer's protocol. The RNA was eluted in RNase-free water. Written informed consent was obtained from each patient included in this study.

cDNA synthesis and SyBr Green real-time PCR

Reverse transcription was carried out using random hexamers to make HCV cDNA by superscript cDNA synthesis kit (Invitrogen, Carlsbad, Calif., USA). ARMS primers were designed so that the 3′ base matched either the wild-type or mutant sequence [18] (Table II). Each 25-µl reaction contained 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Tokyo, Japan), 2.5 pmol of each primer (Table II). Reactions were run on the Step One real-time PCR system (Applied Biosystems). Cycling conditions were: denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 s and 60°C for 1 min, followed by a melting curve analysis, confirming their specificity. A plasmid DNA standard was included in each run.

Cloning of clinical HCV sequences and site-directed mutagenesis

To make the plasmid p-core-mutant, PCR products were cloned into pCR-TOPO2.1 vector (Invitrogen). To make the plasmid p-core-wild, PCR-based *in vitro* site-directed mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, Calif., USA). DNA sequences of clones were confirmed by direct sequencing.

Results

Optimization of real-time PCR

For this study, real-time PCR using the SYBR Green I detection system (Applied Biosystems) was implemented to detect the HCV amplicon. ARMS PCR specificity is conferred by direct placement of the 3′ end of one of the primers (Figure 1). Cross-reactivity was tested to ensure that the primer sets specifically bound their targets.

When 10^8 copies of the CAA (codon c70) template were amplified using the primer with a base mismatch, approximately 15 cycles were required before the crossing threshold was reached. This compares with 8 cycles for the matching primer. On the other hand, when 10^8 copies of the CGA (codon c70) template were amplified using the primer with a base mismatch, approximately 16 cycles were required before the crossing threshold was reached. This compares with 6 cycles for the matching primer (Figure 1A and B).

When 10^8 copies of the ATG (codon c91) template were amplified using the primer with a base mismatch, approximately 25 cycles were required before the crossing threshold was reached. This compares

Table I. Plasmid DNA used as standard in this study.

Plasmid	Amino acid c70	Codon c70	Amino acid c91	Codon c91
p-core-wild	Arginine	CGA	Leucine	CTG
p-core-mutant	Glutamine	CAA	Methionine	ATG

Table II. Primers used for detection of substitutions at residues c70 (A) and c91 (B).

A.

Primers for detection of substitution at c70		
Primer common to all reactions		
c70 sense primers HCV-c-reverse: 5′-CGGGGTGACAGGAGCCATCC-3′	Codon	Amino acids
HCV 70W: 5′-TATCCCCAAGGCTCGCCG-3′	CGN	Arg
HCV 71M: 5′-TATCCCCAAGGCTCGCCA-3′	CAN	Gln, His

N = A, G, T, or C; Arg = arginine; Gln = glutamine; His = histidine.

B.

Primers for detection of substitution at c91		
Primer common to all reactions		
c91 reverse primers HCV-c-sense: 5′-TCGCAACCTCGTGGAAGGC-3′	Codon	Amino acids
HCV 91W: 5′-CATCCTGCCCACCCCAR-3′	TTG or CTG	Leu
HCV 91M: 5′-CATCCTGCCCACCCCAT-3′	ATG	Met

R = A, G; Met = methionine; Leu = leucine.
HCV sequences are identical to AJ238799. Ref. [11].

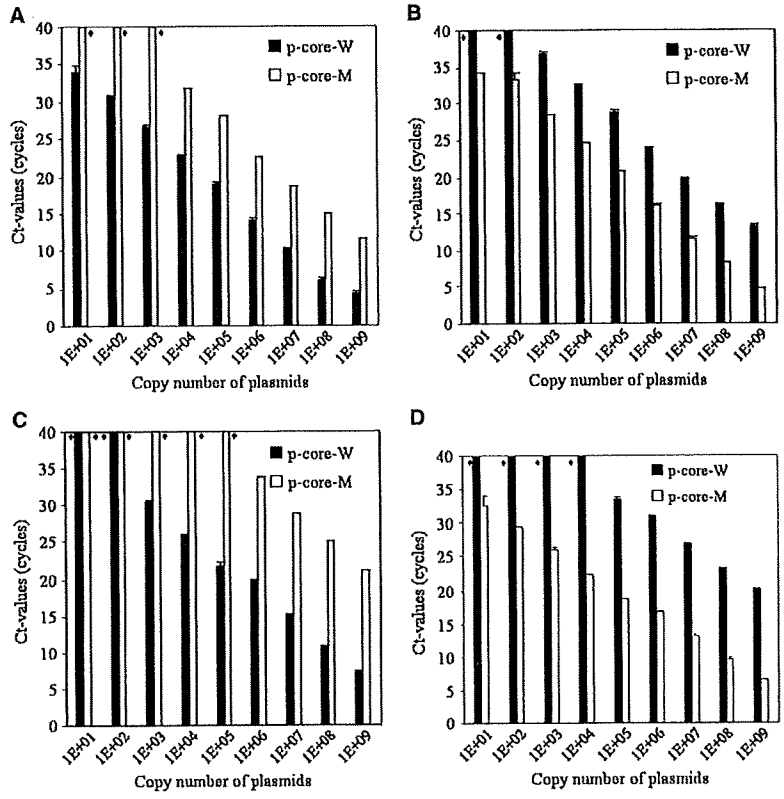

Figure 1. Quantitation of a 10-fold dilution of plasmid p-core-W or p-core-M with wild- or mutant-type primers. Cycle numbers were plotted against the logarithmic concentration of serial dilutions. A. c70-wild primer sets (HCV-70W and HCV-c-reverse). B. c70-mutant primer sets (HCV-70M and HCV-c-reverse). C. c91-wild primer sets (HCV-c-sense and HCV-91W). D. c91-mutant primer sets (HCV-c-sense and HCV-91M). *Unable to detect any signals by 40 cycles.

with 10 cycles for the matching primer. On the other hand, when 10^8 copies of the CTG (codon c91) template were amplified using the primer with a base mismatch, approximately 23 cycles were required before the crossing threshold was reached. This compares with 10 cycles for the matching primer (Figure 1C and D).

The detection limits of these methods were at least 10 copies, 10 copies, 1000 copies, and 10 copies of c70-wild primer sets (HCV-70W and HCV-c-reverse), c70-mutant primer sets (HCV-70M and HCV-c-reverse), c91-wild primer sets (HCV-c-sense and HCV-91W), and c91-mutant primer sets (HCV-c-sense and HCV-91M), respectively (Figure 1).

Selectivity of ARMS assay

Using the plasmid mixture containing the wild-type (p-core-W) and the mutant-type (p-core-M) as a template, real-time ARMS PCR was performed to establish the concentration at which the c70-wild primer sets (HCV-70W and HCV-c-reverse) would detect the wild-type DNA (codon c70). In Table IIIA we present the results of these primer sets showing that, when the wild DNA was 10^9 copies/tube, from 10^5 to 10^9 copies of mutant templates did not affect the results. When the mutant DNA was 10^9 copies/tube, from 10^9 to 10^7 copies of the wild templates could be detected. Similarly, each primer could clearly distinguish the difference between p-core-W and p-core-M at the same copy numbers. Concerning substitution 70, the ratios 100:1, 10:1, 1:1, 1:10, and 1:100 of p-core-W versus p-core-M could be distinguished (Table IIIA and B). However, for substitution 91, the ratios 100:1, 10:1, 1:1, 1:10, 1:100, and 1:1000 could be distinguished, confirming the sensitivity and specificity of the assay [16] (Table IIIC and D).

Hepatitis C core substitutions in serum by real-time ARMS RT-PCR

Quantitative ARMS assays were carried out in parallel reactions, one with a primer matching the variant at the 3′ end, and the other with the primer matching the wild-type variant. We measured the HCV core substitutions at residues c70 and c91 in two patients who did not respond to combination peginterferon and ribavirin therapy after 12 weeks and finally did not become SVRs (Table IV). In patient no. 1, we could detect the minority, wild-type at c70 (4% at 4 weeks). This became diminished at 12 weeks after treatment. In both patients, we could not detect any wild-type template at 12 weeks after treatment.

Comparison of real-time ARMS RT-PCR and conventional sequencing

The real-time ARMS RT-PCR method was compared to direct sequencing in patients treated with peginterferon and ribavirin. In patient no. 1, the minority, wild-type at c70 at 4 weeks could not be detected by direct sequencing (Table IV). In patient no. 2, there were some discrepancies between the results of direct sequencing and those of real-time ARMS RT-PCR (Table IV).

Table III. A mixture of the dilution series of mutants with fixed concentration of wild-type DNA or mutant-type DNA was assayed with each primer to establish the concentration at which the primers would detect each DNA by real-time ARMS PCR. Copy number: copies/tube; template W: p-core-W; template M: p-core-M.

Copy number of template (W:M)	Ct (cycle number)
A. c70-wild primer sets (HCV-70W and HCV-c-reverse).	
10^5:10^9	12.66±0.050
10^6:10^9	12.47±0.099
10^7:10^9	11.46±0.036
10^8:10^9	8.87±0.279
10^9:10^9	5.29±0.018
10^9:10^8	5.24±0.075
10^9:10^7	5.24±0.070
10^9:10^6	5.15±0.091
10^9:10^5	5.13±0.014
B. c70-mutant primer sets (HCV-70M and HCV-c-reverse).	
10^9:10^5	14.44±0.026
10^9:10^6	14.18±0.017
10^9:10^7	12.66±0.044
10^9:10^8	9.68±0.041
10^9:10^9	6.00±0.126
10^8:10^9	5.72±0.10
10^7:10^9	5.57±0.028
10^6:10^9	5.90±0.072
10^5:10^9	5.77±0.063
C. c91-wild primer sets (HCV-c-sense and HCV-91W).	
10^5:10^9	22.77±0.197
10^6:10^9	20.99±0.182
10^7:10^9	17.46±0.0457
10^8:10^9	13.36±0.10
10^9:10^9	9.30±0.053
10^9:10^8	9.29±0.12
10^9:10^7	9.19±0.043
10^9:10^6	9.14±0.060
10^9:10^5	9.23±0.0011
D. c91-mutant primer sets (HCV-c-sense and HCV-91M).	
10^9:10^5	20.89±0.056
10^9:10^6	18.52±0.351
10^9:10^7	14.89±0.016
10^9:10^8	11.53±0.033
10^9:10^9	7.99±0.023
10^8:10^9	7.82±0.0040
10^7:10^9	7.80±0.0098
10^6:10^9	7.86±0.044
10^5:10^9	7.82±0.0025

Table IV. HCV core substitutions at residues c70 and c91 detected by real-time ARMS RT-PCR and direct sequencing.

Patients No.	Study Week	ALT (IU/L)	HCV-RNA (log copies/ml)	c70 W:M	c91 W:M	Direct sequencing c-70/c-91
1.	0	31	6.6	0:100	0:100	M/M
	4	26	6.3	4:96	0:100	M/M
	12	24	5.8	0:100	0:100	M/M
2.	0	53	6.3	0:100	ND	Mix/M
	4	25	6.0	0:100	0:100	M/M
	12	14	5.3	0:100	0:100	M/M

Abbreviations: ARMS = amplification refractory mutation system; ALT = alanine aminotransferase; W = wild-type; M = mutant-type; Mix = mixed-type; ND = not determined.
"Study Week" = weeks after administration of peginterferon and ribavirin.

Discussion

In this article we describe a rapid and sensitive method for the quantitative detection and monitoring of the core amino acid substitutions of HCV genotype 1b. SyBr Green real-time PCR and specific ARMS primers were used to quantify viral RNAs carrying particular sequences, HCV amino acid substitutions 70 and 91 in the core coding region. The specificity of the ARMS primers results in large differences in PCR crossing thresholds being observed between matching and mismatched targets.

For the current standard treatment with peginterferon alpha and ribavirin in patients with chronic hepatitis C, infection with HCV genotypes 2 and 3, lower baseline viral load, Asian and Caucasian ethnicity, younger age, low γ-GTP levels, absence of advanced fibrosis/cirrhosis, and absence of steatosis in the liver have been identified as independent pretreatment predictors of SVR [19]. Early virological response (EVR), defined as a $\geq$2-log reduction in HCV-RNA or undetectable HCV-RNA at 12 weeks, is associated with a favorable virological response. EVR is reached in only ~70% of patients infected with genotype 1 treated with combination therapy [20,21].

Recently, it was reported that core residues Arg70 and Leu91 were associated with response therapy in Japanese genotype 1b patients [11,13]. Donlin et al. [12] reported a similar association of Arg70 with a marked response for genotype 1b but not 1a; however, Met91 was highly dominant in both the marked- and poor-responder sequences, but few other studies have examined the role of diversity in the core in the outcome of therapy. Concerning hepatocarcinogenesis associated with HCV genotype 1b, Akuta et al. [22] reported that cumulative hepatocarcinogenesis rates in double wild-type (Arg70 and Leu91) of the HCV core region were significantly lower than those in non-double wild-type. Direct sequencing [11,13] and nested-RT-PCR using ARMS primers with gel electrophoresis [12,22] were performed in these studies. Higher sensitivity assays may be more useful for predicting the outcomes of therapy and hepatocarcinogenesis [23]. The real-time ARMS RT-PCR described here does not require restriction enzyme digestion, gel-electrophoresis or sequence analysis of PCR products, and it can quantify the core substitution proportions more quickly.

Hepatitis C core substitutions in serum detected by real-time ARMS RT-PCR showed mutant c70 and mutant c91 at 12 weeks in two non-EVRs (Table IV). Most non-SVR rates result from non-EVR. It was reported that the 72-week regimen significantly improved the SVR rates in non-EVRs with Arg70 and/or Leu91 of core [24]. Peginterferon plus ribavirin treatment is costly and has several side effects, possibly reducing its attractiveness for patients. If we were able to identify these HCV core substitutions at 12 weeks, we would know whether to stop or continue treating patients. This could prevent patients from serious side effects or bring about a better treatment outcome by the resulting shorter regimens. Moreover, if direct viral enzyme inhibitors such as protease inhibitor and polymerase inhibitor, which potently suppress viral replication, could be used, the predictability of outcome would be even more important. Recently, it was also reported that maintenance or prolonged peginterferon did not reduce the incidence of HCC in advanced chronic hepatitis C patients [1,25]. We are now focusing on a larger study, and real-time ARMS RT-PCR is expected to be useful for the important prediction of peginterferon plus ribavirin treatment outcomes or that of hepatocarcinogenesis in hepatitis C patients.

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Impact of early viral kinetics on pegylated interferon alpha 2b plus ribavirin therapy in Japanese patients with genotype 2 chronic hepatitis C

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SUMMARY. The recommended therapy for genotype-2 chronic hepatitis C is a regimen of pegylated interferon alpha (peginterferon) plus ribavirin. This study was conducted to determine the value of early viral kinetics as a predictive factor for sustained virologic responder (SVR). Peginterferon alpha 2b (1.5 μg/kg/week) plus weight-based ribavirin (600–1000 mg/day) was administered to 51 patients with chronic HCV genotype 2 for 24 weeks. The HCV-RNA loads were measured at the baseline, hour 24, and week 1. The rebound index (RI, an index obtained from the viral load of week 1 divided by that of hour 24) was calculated. Compared with the baseline, the viral load at hour 24 for SVR was reduced by more than 1-log; it continued to decline thereafter, and at week 1 it was significantly lower than at hour 24 ($P < 0.05$). The viral load for non-SVR increased again between hour 24 and week 1. The SVR of patients with RI ≤1.0 was 100% (39/39). The SVR conversion for rapid virologic responders was 92% (35/38). The RI (≤1.0) was the only significant independent factor for SVR by multiple logistic regression analysis and is the first predictive factor in 24-week peginterferon plus ribavirin therapy for patients infected with genotype 2.

Keywords: chronic hepatitis C, early viral kinetics, genotype 2, pegylated interferon plus ribavirin, rebound index.

INTRODUCTION

The pegylated interferon alpha 2b (peginterferon) plus ribavirin combination therapy is recommended to treat genotype 2 chronic hepatitis C [1,2]. The two major predictive factors for a sustained virologic response (SVR) to interferon therapy are hepatitis C virus (HCV) genotype and viral load [3–7]. In Japan, the major genotypes include types 1 and 2 [8]. Compared with the former, the therapeutic efficacy of IFN is higher with the latter [8,9]. The duration of peginterferon plus ribavirin therapy for chronic hepatitis C is defined as 48 weeks for genotype 1 and 24 weeks for genotype 2 [10,11]. Attempts have been made to shorten the duration of the peginterferon plus ribavirin therapy for genotype 2 from 24 weeks to 12 or 16 weeks for rapid virologic responders (RVR; undetectable HCV-RNA at week 4) [12–18]. When peginterferon plus ribavirin is administered for 24 weeks, the rate of SVR is about 80% with relapse occurring in about 20%. It is believed that RVR is the primary predictive factor for SVR in the treatment of peginterferon plus ribavirin for genotype 2.

This study focused on early viral kinetics and RVR as predictive factors for SVR in the treatment of HCV patients with genotype 2 with peginterferon plus ribavirin. It was determined that rebound index (RI), a new index computed from early viral kinetics, is the first predictive factor for SVR and a substitute for RVR.

Abbreviations: EVR, early virologic response; NVR, nonvirologic response; RI, rebound index; RVR, rapid virologic response; SVR, sustained virologic responder.

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PATIENTS AND METHODS

Chronic HCV genotype 2 infected patients were eligible for enrollment if they fulfilled the following pretreatment criteria: baseline elevated serum alanine aminotransferase (ALT) levels, detectable serum HCV RNA via nucleic acid testing, HCV genotype 2, viral loads ≥5.30 log IU/mL, age ≥30 years, and a liver biopsy in the past 3 months consistent with chronic hepatitis (F1–F3) diagnosed based on the scoring system of Desmet *et al.* [19]. Fifty-one patients were treated with subcutaneous peginterferon alpha 2b (1.5 μg/kg/week) (PegIntron; Schering-Plough, Osaka, Japan) and

oral ribavirin (600 mg/day based on weight: <60 kg, 800 mg; 60–80 kg, 1000 mg; >80 kg) (Rebetol; Schering-Plough) for 24 weeks.

The 51 patients who participated in this study consisted of 28 males and 23 females ranging in age from 30 to 71 years, with a mean age of 52.1 years. Treatment was interrupted in three patients due to the development of adverse events. The remaining 48 patients completed 24 weeks of treatment. For all 48 patients, the total dosage of peginterferon or ribavirin exceeded 80% of the planned total dosage.

Peginterferon was administered at 9:00 in the morning at the initial, second, and third dosing points. The HCV loads were tested immediately before the start of treatment, at hour 24, and in weeks 1 and 2. The coefficient derived by dividing the viral load of week 1 by that of hour 24 was defined as the RI. The patients were grouped into the following 3 groups based on the RI and viral load in week 1: group A, RI >1.0; group B, RI ≤1.0 and viral load ≥3.70 log IU/mL in week 1; group C, RI ≤1.0 and viral load <3.70 log IU/mL in week 1.

The qualitative test for HCV-RNA was conducted five times (at weeks 4, 8, and 12, at the completion of treatment, and at week 24 after the completion of therapy). Patients showing the absence of HCV-RNA by week four were designated as RVR; and those with viral negativity between weeks 5 and 12, early virologic responders (EVR). The patients who remained HCV-RNA-negative until week 24 after the therapy was completed were defined as SVR and all other patients were designated non-SVR. Those who failed to achieve HCV-RNA negativity by the end of the treatment were designated nonvirologic responders (NVR).

Frozen sera were collected from the patients before and during IFN treatment, and the viral loads were measured by employing a quantitative HCV-RNA PCR assay (COBAS Amplicor HCV Monitor Test version 2.0 using a 10-fold dilution method, Roche Diagnosis, Tokyo, Japan), which has a lower threshold of quantification of 3.70 log IU/mL and an outer limit of quantification of 6.71 log IU/mL. A quantitative test for serum HCV-RNA was performed by using an Amplicor-HCV kit version 2.0 (Roche Diagnosis) and the results were labelled as positive or negative. The lower limit of detection was 1.70 log IU/mL. The preserved serum that produced a negative result for qualitative analysis of HCV-RNA was later re-examined by using the COBAS TaqMan HCV (AUTO) (Roche Diagnosis). If both tests produced negative results, the sample was judged to be HCV-RNA-negative. All testing was performed at a single reference laboratory. The HCV genotype was determined by a type-specific primer from the core region of the HCV genome. The protocol for genotyping was carried out as previously described.

The criteria for exclusion were: (i) clinical or biochemical evidence of hepatic decomposition; and advanced cirrhosis identified by ascites, encephalopathy, or hepatocellular carcinoma; (ii) white blood cell count of less than 3000/mm^3 and platelet count of less than 50 000/mm^3; (iii) concomitant liver disease other than hepatitis C (hepatitis B surface antigen- or human immunodeficiency virus-positive); (iv) excessive active alcohol consumption exceeding 60 g/day or drug abuse; (v) severe psychiatric disease; and (vi) antiviral or corticosteroid therapy within the 12 months prior to enrollment. Both peginterferon alpha-2b and ribavirin were discontinued if the haemoglobin level, white blood cell count, or platelet count fell below 8.5 g/dL, 1000/mm^3 and 25 000/mm^3, respectively. The treatment was also discontinued if severe general fatigue, hyperthyroidism, interstitial pneumonia or severe haemolytic problems developed, continuation of treatment was judged not to be possible by the attending physician, or the patient no longer desired to continue treatment.

This study was conducted at the Shin-Kokura Hospital between December 2004 and June 2007. The study protocol was approved by the institutional ethics committee of Shin-Kokura Hospital and all patients gave informed consent to participate in this study, which was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and International Conference on Harmonization Guidelines for Good Clinical Practice.

Sustained virologic responder was analysed on an intention-to-treat basis. Differences between viral loads among groups were analysed using the Student's *t*-test and Mann–Whitney rank-sum test. Multivariate logistic regression analysis was used to determine predictive factors for SVR. We also calculated odds ratios and 95% confidence intervals. Predictive factors associated with SVR included: age, sex, body mass index (BMI), HCV-RNA loads, ALT levels, platelet counts, haemoglobin levels, RI, and the time HCV-RNA became undetectable.

All statistical analyses were conducted on a Macintosh computer using STATVIEW 5.0 (Abacus Concepts, Berkeley, CA, USA). Values for $P < 0.05$ were considered to be statistically significant.

RESULTS

Patient population

Of 51 patients, 48 patients completed the 24-week regimen. Of these 48 patients, 40 achieved SVR, resulting in an SVR rate of 83.3% (40/48). Seven patients remained HCV-RNA negative until week 24 of treatment but became positive again after the completion of the treatment. One patient failed to achieve HCV-RNA negativity by the end of the treatment. Of the three patients who interrupted treatment, two patients dropped out in weeks 17 and 19 due to general malaise and the other patient suffered from systemic eczema in week 5, necessitating the interruption of medication. Two of these three patients were SVR and one was non-SVR. The intention to treat analysis yielded a figure of 82.4% (42/51).

Table 1 Baseline characteristics of patients by SVR and non-SVR

	SVR $n = 42$	Non-SVR $n = 9$	Total $n = 51$
Age (years)	50.8 (12.7)	57.5 (12.7)	51.7 (12.6)
Male (%)	23 (55%)	5 (55%)	28 (55%)
Laboratory			
ALT (IU/L)	115 (111)*	56 (16)*	108 (106)
Haemoglobin (g/dL)	14.6 (1.6)	15.1 (1.3)	14.6 (1.5)
Platelet count ($\times 10^4/mm^3$)	19.1 (6.3)†	16.2 (6.2)†	18.7 (6.2)
HCV RNA loads (log IU/mL)	5.95 (0.47)‡	6.45 (0.33)‡	6.01 (0.48)
BMI (kg/m^2)	22.7 (2.8)	22.2 (3.6)	22.6 (2.8)

Values represent means with standard deviation in parentheses or as absolute values with percentages in parentheses.
*$P < 0.01$ for SVR *vs* non-SVR.
†$P < 0.001$ for SVR *vs* non-SVR.
‡$P < 0.05$ for SVR *vs* non-SVR.
SVR, sustained virologic responder; ALT, alanine aminotransferase; BMI, body mass index.

The baseline characteristics of these 51 patients by SVR and non-SVR are shown in Table 1. The mean age was not significantly different between SVR at 50.8 years and non-SVR at 57.4 years. The ALT level and platelet counts were significantly higher ($P < 0.01$ and $P < 0.001$, respectively) while the HCV-RNA load was significantly lower ($P < 0.05$) in patients with SVR.

Early viral kinetics and rebound index in relation to SVR and non-SVR

Early viral kinetics and the RI in relation to SVR and non-SVR are shown in Table 2 and Fig. 1. HCV-RNA load for all SVR patients was reduced by 1-log in hour 24. The viral load thereafter (in week 1) was significantly reduced in contrast to that of hour 24 ($P < 0.05$). Furthermore, in week 2, the viral load was significantly reduced compared with that of week 1 ($P < 0.001$). With the exception of one patient, none of the patients with SVR showed a rise in the viral load in week 1. Compared with the baseline, the viral load in week 1 was reduced by more than 1-log in all SVR patients. The viral load for non-SVR was reduced by 1-log in hour 24 but a 1-log reduction was not achieved with NVR. Thereafter, the viral load rose again in week 1, then was reduced in week 2. The viral loads of all nine patients exhibited an increase in week 1, and the viral load of three of these patients in week 1 failed to be reduced from the baseline by 1-log. Among these three patients, HCV-RNA became negative in week 12 in two patients but reverted to positive 1 month after the completion of the treatment. The RI

Table 2 Kinetics of HCV RNA and RI during the first 2 weeks of treatment relative to SVR and non-SVR.

	SVR ($n = 42$)	Non-SVR ($n = 9$)	Total ($n = 51$)
HCV loads (log IU/mL)			
Before	5.95 (0.47)*	6.45 (0.33)*	6.01 (0.48)
Hour 24	4.56 (0.75)†	4.97 (1.23)	4.68 (0.86)
Week 1	4.02 (0.69)†,‡	5.49 (0.90)	4.30 (0.95)
Week 2	3.77 (0.45)‡	4.14 (1.15)	3.97 (0.85)
RI	0.63 (0.09)*	2.13 (0.33)*	0.92 (0.12)

Values represent means with standard deviation in parentheses. SVR, sustained virologic responder; SD, standard deviation.
*$P < 0.05$ for SVR *vs* non-SVR.
†$P < 0.05$ for hour 24 *vs* week 1 in SVR.
‡$P < 0.001$ for week 1 *vs* week 2 in SVR.

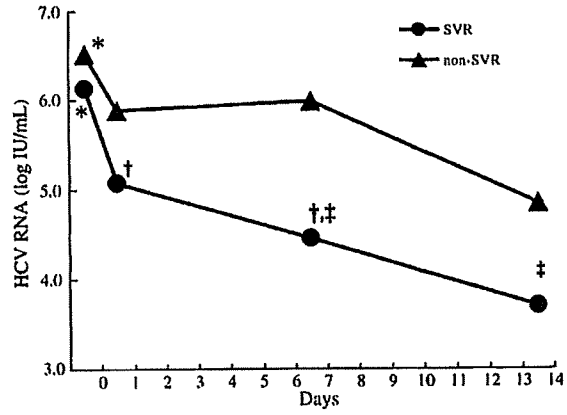

Fig. 1 Kinetics of HCV-RNA during the first 2 weeks of therapy relative to SVR (black circle) and non-SVR (black triangle). *$P < 0.05$ for SVR *vs* non-SVR, †$P < 0.05$ for hour 24 *vs* week 1 in SVR, ‡$P < 0.001$ for week 1 *vs* week 2 in SVR. SVR, sustained virologic response.

(0.43) of SVR patients was significantly lower than that of non-SVR (4.13) ($P < 0.05$).

SVR and non-SVR in relation to the timing of HCV-RNA negativity in groups A, B and C

Sustained virologic responder and non-SVR for groups A, B and C stratified by the timing of HCV-RNA negativity are shown in Table 3. Among the 48 patients, there were 38 RVR (79.2%), 9 EVR (18.7%), and 1 NVR (2.1%). The percentages for achieving SVR by RVR, EVR, and NVR were 92.1%, 55.6%, and 0.0%, respectively. The percentages of achieving SVR in groups A, B and C were 11%, 100%, and 100%, respectively. In groups B and C with the

Table 3 SVR and non-SVR in relation to the timing of HCV-RNA negativity in group A, B and C

	RVR $n = 38$	EVR $n = 9$	NVR $n = 1$	Total $n = 48$
RI>1.0 (Group A, $n = 9$)				
SVR (%)	1 (25)	0 (0)	0 (0)	1 (11)
Non-SVR (%)	3 (75)	4 (100)	1 (100)	8 (89)
RI ≤1.0, ≥3.7 log IU/mL* (Group B, $n = 23$)				
SVR (%)	18 (100)	5 (100)	0 (0)	23 (100)
Non-SVR (%)	0 (0)	0 (0)	0 (0)	0 (0)
RI ≤1.0, <3.7 log IU/mL* (Group C, $n = 16$)				
SVR (%)	16 (100)	0 (0)	0 (0)	16 (100)
Non-SVR (%)	0 (0)	0 (0)	0 (0)	0 (0)
Total ($n = 48$)				
SVR (%)	35 (92)	5 (56)	0 (0)	40 (83)
Non-SVR (%)	3 (8)	4 (44)	1 (100)	8 (17)

*HCV RNA loads at week 1.
SVR, sustained virologic responder; RVR, rapid virologic responder; EVR, early virologic responder; NVR, non-virologic responder; RI, rebound index.

RI below 1.0, all became HCV-RNA-negative within 8 weeks, thus achieving SVR status. The 24-week peginterferon plus ribavirin treatment for genotype 2 required the RI to be less than 1.0. Among the patients with a RI of ≤1.0, 16 had a viral load of less than 3.7 log IU/mL (Group C) at week 1. These patients were considered to be super-high responders to peginterferon. The early viral kinetics of these patients are shown in Table 4. The group included nine males and seven females with a mean age of 47.1 years. The mean age for men was 50.6 years, which was higher than 42.8 years for women but the difference was not statistically significant. The viral load of these 16 patients before treatment was 5.90 log IU/mL, which was significantly lower than 6.22 log IU/mL, viral load for other SVR ($P < 0.01$). Of these 16, the viral load up to hour 24 was less than 3.70 log IU/mL in six patients. HCV-RNA was negative in week 2 in five of these six patients.

The viral loads of SVR patients in group A at baseline (RI >1.0), hour 24, weeks 1 and 2 were 6.08, 3.95, 4.40, and <3.70 log IU/mL, respectively. Compared with the viral load immediately before treatment, that at hour 24 was reduced by more than 2-log10.

Three patients interrupted treatment

Among the 51 patients who participated in the study, treatment was interrupted in three due to the development of adverse effects. These patients dropped out in weeks 5, 17, and 19. In these three patients, HCV-RNA became negative in week 4 and their RI was below 1.0. The patient who was discontinued in week 5 showed a relapse of HCV-RNA during a subsequent observation. The viral load for the two patients who dropped out in weeks 17 and 19 was less than 3.70 log IU/mL in week 1, and HCV-RNA continued to be negative 24 weeks after drug withdrawal. These two patients were judged to be SVR.

Predictive factors of SVR by multivariate analysis

Rebound index (≤1.0) was the only significant independent factor for SVR by multiple logistic regression analysis (Table 5). All other factors were not significant.

Table 4 Early viral kinetics of patients in super-high responder group (Group C)

			HCV loads (log IU/mL)			HCV-RNA	
Number	Age	Sex	Before	Hour 24	Week 1	Week 2	Week4
1	30	M	6.23	<3.70	<3.70	Negative	Negative
2	42	M	5.37	<3.70	<3.70	Negative	Negative
3	45	M	5.98	<3.70	<3.70	Negative	Negative
4	56	M	6.20	<3.70	<3.70	Negative	Negative
5	32	F	5.40	<3.70	<3.70	Negative	Negative
6	66	F	5.54	<3.70	<3.70	Positive	Negative
7	42	M	5.41	4.64	<3.70		Negative
8	48	M	6.08	4.28	<3.70		Negative
9	59	M	6.08	4.46	<3.70		Negative
10	66	M	6.28	4.23	<3.70		Negative
11	67	M	5.70	4.80	<3.70		Negative
12	30	F	6.18	4.41	<3.70		Negative
13	31	F	5.89	4.51	<3.70		Negative
14	32	F	5.94	4.08	<3.70		Negative
15	42	F	5.40	4.34	<3.70		Negative
16	67	F	5.70	4.63	<3.70		Negative

Table 5 Predictive factors of SVR by multivariate analysis

Factor	Category	Odds ratio	95% CI	*P*-value
Age	≥50 years	0.622	0.035–11.114	0.746
	<50 years	1		
Sex	Male	1.972	0.109–35.799	0.646
	Female	1		
BMI	<22.5	1.251	0.085–18.462	0.871
	≥22.5	1		
HCV load	<6.0 logIU/mL	0.98	0.061–15.788	0.988
	≥6.0 logIU/mL	1		
ALT	<50 IU/L	0.757	0.038–15.240	0.856
	≥50 IU/L	1		
Platelet count	≥18 × 10^4/mm^3	1.795	0.104–31.019	0.687
	<18× 10^4/mm^3	1		
Haemoglobin level	<14 mg/dL	0.398	0.012–12.7171	0.602
	≥14 mg/dL	1		
RI	≤1.0	689.586	4.214–>999.999	0.012
	>1.0	1		
Time to HCV RNA negativity(–)	≤Week 4	1.612	0.050–51.632	0.787
	>Week4	1		

BMI, body mass index; ALT, alanine aminotransferase.

DISCUSSION

The early viral kinetics in association with the peginterferon plus ribavirin treatment for genotype 1 have been reported [20,21]; but reports on early viral kinetics are scarce when the same combination is applied to genotype 2. This is the first investigation of early viral kinetics during peginterferon plus ribavirin therapy for genotype 2 chronic hepatitis C patients with high viral loads. We found that the RI (a new index) that is computed from the early viral kinetics is the first predictive factor for SVR as a substitute for RVR as a result of multiple analysis data. Patients with a RI of less than 1.0 and a viral load of less than 3.7 log IU/mL in week 1 were also identified as super-high responders to peginterferon plus ribavirin therapy.

The serum concentration of peginterferon alpha 2b peaked around 24 h, followed by a gradual decrease thereafter [22,23]. Thus the earlier studies on viral kinetics in association with peginterferon plus ribavirin for genotype 1 reported that the HCV load declines in hour 24 and increases again in week 1 [20,21]. In the responder group, the HCV load continues to decline every week thereafter [21]. A similar pattern is also seen in peginterferon monotherapy [22]. However, there are few reports on early viral kinetics involving genotype 2. In this study, the early viral kinetics of genotype 2 was investigated. Noting this increase in week 1, the viral load in week 1 was divided by that of hour 24 and the resultant coefficient was defined as the RI. In this study, the SVR rate was 100% for groups B and C, with the RI being less than 1.0. In these groups, HCV-RNA was eliminated by week 12 in all patients. On the other hand, re-emergence of virus was noted in 8% among the RVR. These findings suggested that the RI is the first predictive factor for SVR as a substitute for RVR in 24-week peginterferon plus ribavirin therapy for genotype 2. For those patients with a RI of >1.0, treatment lasting more than 24 weeks appeared necessary.

Peginterferon plus ribavirin therapy results in SVR exceeding 80% in genotype 2 patients when treatment lasts for 24 weeks [1,2,9]. Because these patients are high responders to peginterferon plus ribavirin therapy, attempts have been made to shorten the duration of treatment [12–18]. Earlier, RVR patients have been treated for shorter periods (e.g. 12, 14 and 16 weeks) and it was reported that there was no difference in the SVR rate compared with the treatment duration of 24 weeks [12–15] According to a recent randomized study, the SVR rate is high even in RVR patients when treated for 24 weeks [16]. It has been reported that shortening of the treatment period results in economic advantages and reductions in the development of side effects [17]. Thus it becomes necessary to evaluate the super-high responder group to peginterferon plus ribavirin therapy who do not show reductions in the SVR rate even when the duration of treatment is reduced. Among those with genotype 2, about 80% or more convert to RVR but RVR alone does not sufficiently explain the state of super-high responders. In interferon therapy of genotype 2 patients, peginterferon alone produces therapeutic effects [2]. An HCV load below 3.0 log IU/mL on day 7 and undetectable HCV-RNA on day 29 were predictive of successful short-term treatment [18]. It is essential to identify super-high responders to peginterferon by using the early viral kinetics during the first 2 weeks of therapy. In this study, the viral load was investigated in week 1 following the