alcoholic liver diseases (3, 8). On the other hand, several recent large-scale studies have indicated that coffee drinking suppressed the progression of liver fibrosis and inhibited the development of hepatocellular carcinoma (18, 19).

The fact that liver cirrhosis is not a necessary condition for hepatocellular carcinoma occurrence was already known, not only from clinical findings but also from genetic findings. Among hepatocellular carcinoma cases with HBV, a part of the HBV genome has been shown to be integrated into the host's intracellular DNA, thereby causing hepatocellular carcinoma (20). Among hepatocellular carcinoma cases with HCV, the HCV core protein seems to directly contribute to the mechanism of carcinogenesis by elevating oxidative stress (21). In light of the aforementioned findings, for the purpose of determining independent risk factors for hepatocellular carcinoma, careful analyses are needed controlling for severity of liver fibrosis, as well as for viral etiologic factors.

With the aim of determining whether HBV or HCV infections, alcohol consumption, smoking, coffee drinking, BMI, and diabetes mellitus are independent risk factors for hepatocellular carcinoma, and how the effects of these factors might change after adjusting for severity of liver fibrosis, we conducted a nested case-control study among the Adult Health Study longitudinal cohort using stored sera. We also evaluated whether viral etiology and increase of BMI exert synergistic effects on the risk for hepatocellular carcinoma.

Materials and Methods

Cohorts. The Atomic Bomb Casualty Commission and its successor, the Radiation Effects Research Foundation, established the Adult Health Study longitudinal cohort in 1958, in which 20,000 age-, gender-, and city-matched proximal and distal atomic bomb survivors and persons not present in the cities at the time of bombings have been examined biennially in outpatient clinics in Hiroshima and Nagasaki.

Study Population. Serum samples obtained from the study participants on each occasion of visiting outpatient clinics have been collected and stored systematically since 1969 (22). Incident cancer cases were identified through the Hiroshima Tumor and Tissue Registry and Nagasaki Cancer Registry, supplemented by additional cases detected via pathologic review of related diseases (23). There were 359 primary hepatocellular carcinoma cases among Adult Health Study participants diagnosed between 1970 and 2002, who visited our outpatient clinics before their diagnosis. Of these, 130 cases were excluded because of nonavailability of stored serum or having only one stored sample. The other 229 cases had serum samples obtained within 6 years before hepatocellular carcinoma diagnosis. After excluding five cases with inadequate stored serum, 224 cases remained for our study. For each case, three controls were selected from the cohort in nested case-control fashion. Nested control selection was random among those who matched the case on gender, age (±2 years), city, time of serum storage (±2 years), and method of serum storage, and countermatched on radiation exposure (24). Although the total number of potential matched control serum samples is 672, because of occasional lack of subjects with stored sera who met the matching and countermatching criteria, the total number of control serum samples actually used was 644.

Laboratory Tests. HBV surface antigen and antibody to hepatitis B core antigen were measured by enzyme immunoassay, and anti-HCV antibody was measured by second-generation enzyme immunoassay as previously described (22, 25). Qualitative detection of HCV RNA among anti-HCV–positive samples was done using a thermocycler (Whatman Biometra) with two sets of PCR primers corresponding to the 5′-untranslated region, as previously described (25). Qualitative detection of HCV RNA was conducted at least twice. HBV infection (HBV+) status was defined as positive for HBV surface antigen or having a high titer of the antibody to hepatitis B core antigen. HCV infection (HCV+) status was defined as positive for HCV RNA (25). Hyaluronic acid and type IV collagen as liver fibrosis markers were measured using an autoanalyzer (Hitachi 7180, Hitachi, Ltd.) and latex agglutination–turbidimetric immunoassay (Fujirebio, Inc., Daiichi Pure Chemicals Co. Ltd.). Ferritin was measured using an autoanalyzer (Hitachi 7180, Hitachi) and colloidal gold immunoassay (Alfresa Pharma Corporation). Platelet count was measured using an automatic blood cell counter at the time of serum storage.

Information on Covariates. Self-administered questionnaires on various lifestyle factors were given to participants in 1965 during attendance at the Adult Health Study examination and in 1978 by mail survey. Information from the 1978 survey was obtained before hepatocellular carcinoma diagnosis for all but 19 (15%) of the cases. Information on alcohol consumption was obtained from the 1965 questionnaire when available, with missing data complemented using the 1978 survey. Alcohol consumption per volume of each type of alcoholic beverage was quantified as previously described (26), and mean ethanol amounts were calculated as grams per day. Information on smoking habits was obtained from the 1965 questionnaire; subjects were divided into the following categories: never, prior, and current smoker. Information on coffee drinking was obtained from the 1978 survey; subjects were divided into the following categories of frequency of coffee consumption: never, 1 day per week, 2 to 4 days per week, and almost daily. Disease diagnoses were based on the International Classification of Diseases (ICD) codes: diabetes mellitus was defined by ICD-7 code 260, ICD-8 code 250, ICD-9 code 250, and ICD-10 codes E10 through E14. BMI (kg/m^2) was calculated from height and weight measured at the Adult Health Study examination.

Subjects were classified based on BMI quintiles with cut points of 19.5, 21.2, 22.9, and 25.0. The number of hepatocellular carcinoma cases with BMI of >30.0 kg/m^2 was too small to be analyzed in detail. Following the recommendations for Asian people by the WHO, the International Association for the Study of Obesity, and the International Obesity Task Force (27), 21.3 to 22.9 kg/m^2 was considered as normal, 23 to 25 kg/m^2 as overweight, and >25.0 kg/m^2 as obese in the present study. We used information on diabetes mellitus and BMI obtained 10 years before the time of hepatocellular

Table 1. Characteristics of hepatocellular carcinoma cases and controls

Study variables	Hepatocellular carcinoma cases (n = 224)			Controls (n = 644)		
	Complete data (%)	n (%)	Mean (SD)	Complete data (%)	n (%)	Mean (SD)
Matched variables						
Gender	100			100		
Male		136 (60.7)			387 (60.1)	
Female		88 (39.3)			257 (39.9)	
Age at hepatocellular carcinoma diagnosis (y)	100		67.6 (10.1)	—		—
City	100			100		
Hiroshima		155 (69.2)			444 (68.9)	
Nagasaki		69 (30.8)			200 (31.1)	
Age at serum storage (y)	100		66.4 (10.2)	100		63.7 (9.8)
Unmatched variables						
Etiology (HBV/HCV status)	94.2			99.4		
HBV−/HCV−		45 (21.3)			579 (90.5)	
HBV+/HCV−		29 (13.7)			18 (2.8)	
HBV−/HCV+		132 (62.6)			41 (6.4)	
HBV+/HCV+		5 (2.4)			2 (0.3)	
Fibrosis markers	94.2			99.4		
Hyaluronic acid (ng/mL)			288.6 (284.6)			69.1 (108.3)
Type IV collagen (ng/mL)			245.2 (136.9)			148.8 (122.1)
Platelet count ($\times 10^4/\mu L$)	67.4		13.0 (6.0)	70.0		22.4 (6.2)
Ferritin (ng/mL)	92.0		250.5 (278.6)	98.6		136.7 (151.0)
Alcohol consumption (g of ethanol per day)	88.8			89.6		
>0 and <20		37 (18.6)			130 (22.5)	
≥20 and <40		20 (10.1)			64 (11.1)	
≥40		45 (22.6)			68 (11.8)	
Current smoking		107 (53.8)			262 (45.3)	
Prior smoking	88.8	12 (6.0)		89.8	33 (5.7)	
Daily coffee drinking	62.1	38 (27.3)		73.3	175 (37.1)	
BMI (kg/m^2) 10 y before diagnosis	93.8			98.3		
≤19.5		38 (18.1)			122 (19.3)	
19.6-21.2		33 (15.7)			136 (21.5)	
21.3-22.9		36 (17.2)			142 (22.4)	
23-25		49 (23.3)			124 (19.6)	
>25		54 (25.7)			109 (17.2)	
Diabetes 10 y before diagnosis	100	18 (8.0)		100	33 (5.1)	
Radiation dose to the liver (Gy)	91.1		0.46 (0.69)	94.1		0.34 (0.56)

carcinoma diagnosis or control matching because these conditions are subject to change because of disease progression in the later stages before diagnosis of hepatocellular carcinoma. Atomic bomb radiation dose was estimated for each subject according to the Dosimetry System DS02 (28).

Ethical Consideration. This nested case-control study was based on RERF Research Protocol 1-04 and approved by the Human Investigation Committee of Radiation Effects Research Foundation.

Statistical Analyses. The nested case-control design is analyzed using a partial likelihood method analogous to that used for cohort follow-up studies (29), which is, in practice, the same as the conditional binary data likelihood for matched case-control studies (30) except that the subjects (cases and controls) in the study are not completely independent because of the possibility of repeated selection. All factors other than radiation were analyzed using relative risks estimated by a log-linear model. The population attributable fraction was estimated for individual factors that increased the risk for hepatocellular carcinoma in the present study. Population attributable fraction was calculated as $pd \times [(mRR - 1) / mRR]$, where mRR is the multivariate adjusted relative risk for the covariates and pd is the proportion of cases exposed to the risk factor. Statistical interaction between viral infection and BMI was tested by adding the product of the two factors to the log-linear model, which tests departure from a multiplicative relationship. Reported P values and confidence limits are based on Wald statistics. Although radiation exposure could have been adjusted by matching on radiation dose as an additional matching factor in the control selection (31), in addition to assessing effects of lifestyle factors and viral hepatitis, another purpose of the present study was to examine effects of radiation exposure after adjustment for possible confounding and interaction by these factors, so matching on radiation, which prevents analysis of radiation risk, was not desirable; rather, we countermatched on radiation (29, 32). Radiation risk was analyzed by using an excess relative risk model as has been done previously (33).

Results

Characteristics of Study Population. Characteristics of the 224 hepatocellular carcinoma cases and 644 comparison subjects are shown in Table 1. The mean age of the cases was 67.6 years, and 61% were men. Cases and controls were comparable with respect to gender, age, city, time of serum storage, and method of serum storage by design. Virological and biochemical assays were done on 211 case and 640 control sera because 13 case samples and 4 control samples had insufficient stored sera for these assays. Hepatocellular carcinoma

case sera evidenced a higher prevalence of HBV or HCV infection status, higher values of fibrosis markers and ferritin, and lower platelet counts compared with control sera. Greater proportions of hepatocellular carcinoma cases had a history of alcohol consumption of ≥40 g of ethanol per day, were current smokers, were obese, had diabetes mellitus, and received high radiation doses compared with the controls. In addition, hepatocellular carcinoma cases were less likely than controls to be daily coffee drinkers. There were no important differences in characteristics such as gender, age at hepatocellular carcinoma diagnosis, city, or BMI between hepatocellular carcinoma cases excluded because of nonavailability of stored serum and those included in this study.

Risk Factors for Hepatocellular Carcinoma Development. Table 2 shows the results of univariate and multivariate analyses using HBV and HCV infection status, alcohol consumption, smoking habit, coffee drinking, BMI, diabetes mellitus, and radiation dose. Strong association was found between hepatocellular carcinoma and hepatitis virus infection, resulting in unadjusted relative risks of 33.7 [95% confidence interval (95% CI), 12.7-89.6] for HBV+/HCV− status and 64.5 (95% CI, 29.1-143) for HBV−/HCV+ status. As expected, the risk for hepatocellular carcinoma for alcohol consumption was significant, with an unadjusted relative risk of 1.34 (95% CI, 1.12-1.60) per 20 g of ethanol per day using continuous alcohol consumption and 2.66 (95% CI, 1.55-4.55) at ≥40 g of ethanol per day using grouped alcohol consumption. Although the grouped results suggest that a simple log-linear model in continuous alcohol consumption may not be adequate, a quadratic term did not significantly improve the model (data not shown). Current smoking was significantly associated with hepatocellular carcinoma risk, with an unadjusted relative risk of 1.87 (95% CI, 1.14-3.07). Daily coffee drinking was associated with decreased risk for hepatocellular carcinoma, with an unadjusted relative risk of 0.51 (95% CI, 0.29-0.90). The presence of obesity and diabetes mellitus 10 years before diagnosis were statistically associated with increased risk for hepatocellular carcinoma, resulting in unadjusted relative risks of 1.88 (95% CI, 1.13-3.13) and 1.88 (95% CI, 1.01-3.50), respectively. The relative risk for a 1-unit difference in BMI was 1.04 (95% CI, 0.99-1.09). Radiation exposure was marginally significantly associated with increased risk for hepatocellular carcinoma ($P = 0.055$).

The risks for viral infection in multivariate analysis did not meaningfully differ from those obtained in the univariate analysis. Alcohol consumption of ≥40 g of ethanol per day and obesity remained significant risk factors for hepatocellular carcinoma even after adjusting for viral infection status and the other factors, whereas the effects of current smoking and diabetes mellitus became nonsignificant after adjustment. Daily coffee drinking was marginally significantly associated with decreased risk for hepatocellular carcinoma after adjustment for viral infection and the other factors. The adjusted relative risk for a one unit difference in BMI, 1.12 (95% CI, 1.03-1.22), was statistically significant, but a quadratic term was not significant.

Table 3 shows the estimated population attributable fraction based on the multivariate adjusted relative risks in the present study. The proportion of hepatocellular

Table 2. Relative risks of hepatocellular carcinoma for individual factors

Variables	Unadjusted		Multivariate adjusted	
	RR (95% CI)	*P*	RR (95% CI)*	*P*
Etiology (HBV/HCV status)				
HBV−/HCV−	1	—	1	—
HBV+/HCV−	33.7 (12.7-89.6)	<0.001	45.8 (15.2-138)	<0.001
HBV−/HCV+	64.5 (29.1-143)	<0.001	101 (38.7-263)	<0.001
HBV+/HCV+	42.4 (6.2-291)	<0.001	70.7 (8.3-601)	<0.001
Alcohol consumption (g of ethanol per day)				
Never	1	—	1	—
>0 and <20	1.11 (0.69-1.78)	>0.5	1.27 (0.56-2.87)	>0.5
≥20 and <40	1.07 (0.57-1.99)	>0.5	1.02 (0.34-3.05)	>0.5
≥40	2.66 (1.55-4.55)	<0.001	4.36 (1.48-13.0)	0.008
Continuous (per 20-g ethanol per day)	1.34 (1.12-1.60)	<0.001	1.73 (1.19-2.52)	0.004
Smoking habit				
Never	1	—	1	—
Current smoking	1.87 (1.14-3.07)	0.014	2.03 (0.82-4.98)	0.13
Prior smoking	1.80 (0.81-3.99)	0.15	1.12 (0.25-5.07)	>0.5
Coffee drinking				
Never	1	—	—	—
Daily	0.51 (0.29-0.90)	0.016	0.40 (0.16-1.02)	0.055
BMI (kg/m^2) 10 y before diagnosis				
≤19.5	1.24 (0.73-2.11)	0.43	1.31 (0.51-3.34)	>0.5
19.6-21.2	0.97 (0.55-1.70)	>0.5	1.24 (0.43-3.54)	>0.5
21.3-22.9	1	—	1	—
23-25	1.61 (0.96-2.70)	0.074	2.51 (0.99-6.37)	0.053
>25	1.88 (1.13-3.13)	0.016	4.57 (1.85-11.3)	<0.001
Continuous (+1 kg/m^2 difference)	1.04 (0.99-1.09)	0.087	1.12 (1.03-1.22)	0.010
Diabetes 10 y before diagnosis	1.88 (1.01-3.50)	0.047	1.98 (0.63-6.27)	0.24

Abbreviation: RR, relative risk.
*Adjusted for hepatitis virus infection, continuous alcohol consumption, smoking habit, coffee drinking, BMI, diabetes mellitus, and radiation dose to the liver.

Table 3. Estimated population attributable fraction of hepatocellular carcinoma for risk factors in this study population

Variables*	Proportion of cases exposed (%)	Multivariate-adjusted RR	Population attributable fraction (%)
Etiology (HBV/HCV status)			
HBV+/HCV−	13.7	45.8	13.4
HBV−/HCV+	62.6	101	62.0
HBV+/HCV+	2.4	70.7	2.4
Alcohol consumption			
≥40-g ethanol per day	22.6	4.36	17.4
BMI 10 y before diagnosis			
>25 kg/m^2	25.7	4.57	20.1

*Population attributable fraction was estimated only for the significant hepatocellular carcinoma risk factors.

carcinoma cases that is attributable to HBV+/HCV−, HBV−/HCV+, HBV+/HCV+, alcohol consumption of ≥40 g of ethanol per day, and obesity were 13.4%, 62.0%, 2.4%, 17.4%, and 20.1%, respectively. These values are not mutually exclusive because some cases were exposed to more than one risk factor.

Analyses with Adjustment for Variables Associated with Severity of Liver Fibrosis. Table 4 shows results for univariate analyses incorporating biomarkers associated with progression of liver fibrosis, such as hyaluronic acid and type IV collagen of fibrosis markers, platelet count, and ferritin. Large statistically significant differences in the mean values of these variables were observed between hepatocellular carcinoma cases and controls. Figure 1 shows a comparison of multivariate analysis results with or without adjustment for ln(type IV collagen) and platelet count using HBV and HCV infection status, alcohol consumption, smoking habit, coffee drinking, BMI, diabetes mellitus, and radiation dose as adjustment variables. We evaluated type IV collagen and platelet count as surrogate markers associated with severity of liver fibrosis. Hepatocellular carcinoma risk for hepatitis virus infection status after adjusting for liver fibrosis meaningfully decreased compared with the results indicated in the previous multivariate analysis, with relative risks of 20.8 (95% CI, 4.8-90.3) and 37.8 (95% CI, 12.4-115) for HBV+/HCV− status and HBV−/HCV+ status, respectively (Fig. 1A). Effects of ≥40 g of ethanol per day and daily coffee drinking decreased and disappeared, respectively, so that adjustment for liver fibrosis decreased the effect of these factors on risk for hepatocellular carcinoma. Current smoking became marginally significantly associated with increased risk for hepatocellular carcinoma after adjusting for liver fibrosis. Obesity remained a significant risk factor independent of adjustment for severity of liver fibrosis, and the relative risk for diabetes mellitus did not meaningfully differ from that without such adjustment (Fig. 1B).

Interaction between Hepatitis Virus Infection Status and Increase of BMI. Table 5 shows the joint effects of hepatitis virus infection status and BMI, with adjustment for alcohol consumption, smoking habit, coffee drinking, diabetes mellitus, and radiation dose. Although being obese was clearly a risk factor for hepatocellular carcinoma subjects with adjustment for viral factors, it was not a significant risk factor in those with HBV−/HCV− status. However, despite the appearance of a trend with BMI, only 15 hepatocellular carcinoma cases were identified among HBV−/HCV− individuals with obesity. Among hepatocellular carcinoma subjects with HBV−/HCV+ status, the relative risk increased dramatically with increasing BMI. Linear (P = 0.003) and quadratic (P = 0.013) terms in continuous BMI were

Table 4. Relative risks of hepatocellular carcinoma for variables associated with severity of liver fibrosis: unadjusted relative risk and 95% CI

Variables	Hepatocellular carcinoma cases/controls	Unadjusted	
		RR (95% CI)	P
Liver fibrosis markers	211/640		
Hyaluronic acid (+per 10 ng/mL)		1.10 (1.08-1.12)	<0.001
ln(hyaluronic acid) (+per 1 unit)		5.43 (4.04-7.30)	<0.001
Type IV collagen (+per 10 ng/mL)		1.14 (1.10-1.17)	<0.001
ln(type IV collagen) (+per 1 unit)		80.9 (35.8-183)	<0.001
Platelet count	151/448		
+Per 10^4/μL		0.75 (0.71-0.80)	<0.001
≥25.0 (×10^4/μL)	4/133	1	
20.0-24.9 (×10^4/μL)	19/163	4.5 (1.3-1.6)	0.02
15.0-19.9 (×10^4/μL)	26/105	11.8 (3.2-43)	<0.001
10.0-14.9 (×10^4/μL)	52/42	61 (16-232)	<0.001
<10.0 (×10^4/μL)	50/5	822 (125-5400)	<0.001
Ferritin	206/635		
+ Per 10 ng/mL		1.03 (1.02-1.04)	<0.001
ln(ferritin) (+per 1 unit)		1.51 (1.25-1.82)	<0.001

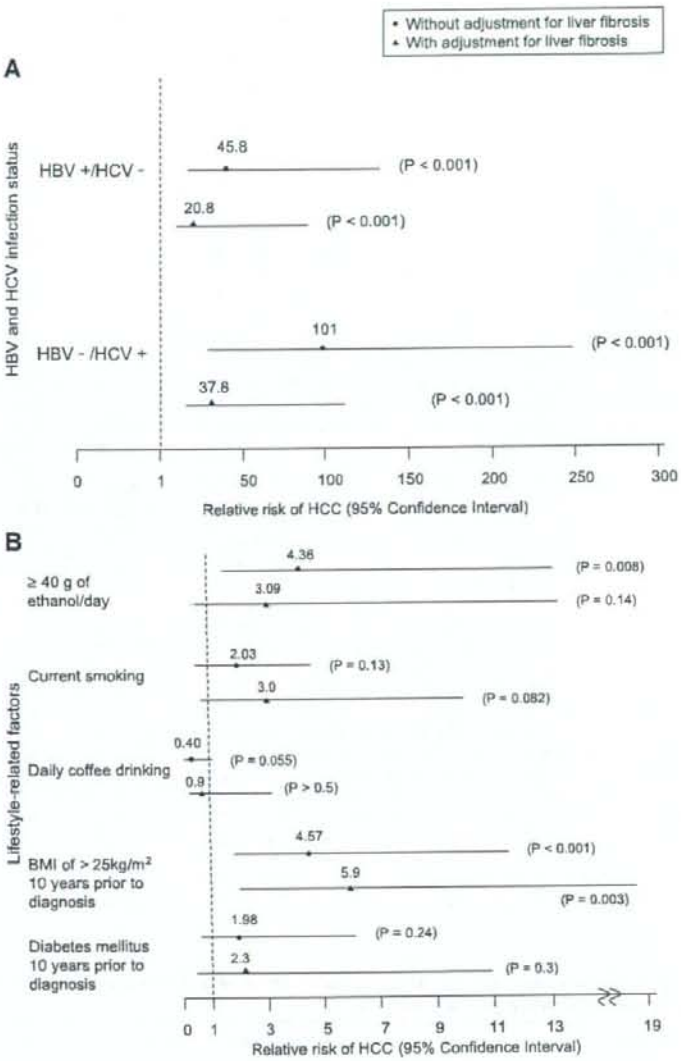

Figure 1. Multivariate relative risk for hepatocellular carcinoma for individual risk factors, with and without adjustment for variables associated with severity of liver fibrosis. Each relative risk was analyzed with and without adjustment for ln(type IV collagen) and platelet count, using HBV and HCV infection status, continuous alcohol consumption, smoking habit, coffee drinking, BMI, diabetes mellitus, and radiation dose as adjustment variables. **A.** HBV and HCV infection status. **B.** Lifestyle-related factors. *HCC*, hepatocellular carcinoma.

significant among HBV−/HCV+ individuals. Among hepatocellular carcinoma subjects with HBV+/HCV− status, the relative risk for hepatocellular carcinoma did not show evidence of an increase with increased BMI, although the examination of a joint effect of HBV infection and BMI was based on only one hepatocellular carcinoma case out of three subjects who were HBV+/HCV− and obese. The reason for the relatively small unadjusted relative risk for obesity (Table 2) might have been due to the small number of cases and controls with HBV+/HCV− status, which apparently offset the increase observed in HBV−/HCV+ status individuals.

Discussion

This nested case-control study indicated that HBV and HCV infection, alcohol consumption of ≥40 g of ethanol per day, and obesity 10 years before hepatocellular carcinoma diagnosis were independent risk factors for hepatocellular carcinoma, and that obesity as well as hepatitis virus infection remained independent risk factors for hepatocellular carcinoma after taking into account the severity of liver fibrosis. Furthermore, significant multiplicative interaction in hepatocellular carcinoma risk between viral etiology and increased BMI was observed in HCV-infected individuals. The

population attributable fraction of 62.0% for hepatocellular carcinoma cases with HCV infection was highest, and hepatocellular carcinoma cases with HBV infection, alcohol consumption of ≥40 g of ethanol per day, or obesity had population attributable fractions in the range of 13.4% to 20.1%. These are only approximate estimates of the potential for reducing hepatocellular carcinoma occurrence, as we do not know what effect removal of one risk factor would have on the distribution of the other risk factors.

Multivariate analysis after adjusting for severity of liver fibrosis indicated that hepatocellular carcinoma risk for HBV and HCV infections significantly decreased, which is consistent with the existing notion that hepatocellular carcinoma risk increases with progression from chronic hepatitis B and C to liver cirrhosis. A large-scale meta-analysis (34) and a case-control study (35) showed a combined effect of HBV and HCV infections on hepatocellular carcinoma risk, whereas our study did not detect similar effects among those with HBV+/HCV+ status. This difference may be partly attributable to the extremely limited number of coinfected subjects with HBV and HCV among our study population. It may be also partly because most past epidemiologic studies have defined chronic HCV infection by either anti-HCV antibody positivity or by HCV RNA positivity in serum (34, 35).

Several epidemiologic studies and clinical trials revealed an association between obesity and hepatocellular carcinoma risk (9-12), but few population-based cohort studies have been conducted with precise adjustment for HBV and HCV infection status, the major risk factors for hepatocellular carcinoma. Obesity was recently found to be one of the etiologic factors for nonalcoholic steatohepatitis, which is considered a non-B, non-C liver disease, and it has been shown to be a risk factor for hepatocellular carcinoma (12, 16). Although many clinical studies showed that, among chronic hepatitis C patients, obesity was associated with progression of inflammation, insulin resistance, hepatic steatosis, and liver fibrosis (17, 36), a study by Nair et al. (12) reported that obesity was not an independent risk factor for hepatocellular carcinoma among liver cirrhosis patients with HBV and HCV. On the other hand, a recent Western cohort study showed that being overweight (BMI, 25 to <30 kg/m^2) or obese (BMI, ≥30 kg/m^2) was an independent risk factor for hepatocellular carcinoma (37).

In the present study, we adjusted for potentially confounding factors including hepatitis virus infection and also found that being obese 10 years before hepatocellular carcinoma diagnosis was associated with a 4.57-fold increase in hepatocellular carcinoma risk. Furthermore, we observed a statistically significant, positive, multiplicative interaction between HCV infection and increased BMI on the risk for hepatocellular carcinoma, which indicates decisively that the joint effect of the two factors is greater than additive.

Obesity contributes to a high rate of visceral fat storage, accelerating production of tumor necrosis factor-α, interleukin 6, resistin, and leptin, and decreasing production of adiponectin (16). These cytokines presumably foster insulin resistance (16), cause hepatic steatosis and oxidative stress, and eventually promote hepatocellular carcinoma occurrence. A large number of studies pointed out association between progression of liver fibrosis and insulin resistance or hepatic steatosis (15-17), but authenticity of any connection is now being questioned (8, 36). Interestingly, in this study, obesity remained an independent risk factor for hepatocellular carcinoma even after adjusting for all confounding factors including severity of liver fibrosis. The following are the possible reasons why obesity increases hepatocellular carcinoma risk irrespective of severity of liver fibrosis: Several animal experiments showed that liver tumors were not always accompanied by advanced fibrosis among a variety of genetically engineered mouse models with steatohepatitis (38), and some reports indicated several nonalcoholic steatohepatitis–derived human cancer cases without significant liver fibrosis (39). The findings suggest that significant liver fibrosis is not essential for the carcinogenic process, but that steatohepatitis itself is a state conferring a risk for high carcinogenicity. With regard to the proven relationship between obesity and such malignant tumors as colon, breast, and ovarian cancers (10), the cell proliferation activity of insulin due to hyperinsulinemia is believed to play a role in a common carcinogenic mechanism (5).

It is well documented that obesity induces insulin resistance, with a tendency to cause diabetes mellitus. In the case of hepatic cirrhosis accompanied by highly advanced liver fibrosis, glucose intolerance tends to lead to diabetes mellitus. A recent animal experiment showed that HCV contributed to progression of insulin resistance, resulting in diabetes mellitus (40). The present study failed to show that diabetes mellitus 10 years before hepatocellular carcinoma diagnosis was an independent risk factor for hepatocellular carcinoma, but an adjustment for all factors, except alcohol consumption and BMI, brought about a 30% increase in the effect of diabetes on hepatocellular carcinoma risk (data not shown). Such findings suggest a relationship between diabetes mellitus and alcohol consumption, as well as BMI. Therefore, by taking into account the proven association between alcohol consumption, obesity, and increased risk for hepatocellular carcinoma, our results will not likely refute an association between diabetes mellitus and hepatocellular carcinoma risk.

A large number of epidemiologic studies showed that heavy alcohol consumption was an independent risk factor for hepatocellular carcinoma and that there was

Table 5. Interaction between hepatitis virus infection status and increase of BMI on hepatocellular carcinoma risk (joint hepatitis virus/BMI)

Viral etiology	BMI (kg/m^2)	RR* (95% CI)†	Likelihood ratio P†
HBV−/HCV−	+1	1.05 (0.95-1.17)	0.33
HBV+/HCV−	+1	0.89 (0.64-1.23)	0.50
HBV−/HCV+	+1	1.39 (1.11-1.83)	0.003‡
HBV+/HCV+	+1	—§	—§

*Adjusted for continuous alcohol consumption, smoking habit, coffee drinking, diabetes mellitus, and radiation dose to the liver.
†Likelihood bounds and P values for relative risks estimated separately within each BMI/hepatitis virus category.
‡A quadratic term was also significant for HBV−/HCV+ individuals (P = 0.013). However, only the relative risk for the linear model in continuous BMI is shown because it is not possible to express the risk as a single value with a two-parameter linear-quadratic model.
§Neither could the joint effect of obesity and simultaneous HBV+/HCV+ status be estimated because of small numbers of jointly affected cases and controls.

correlation between increased risk for hepatocellular carcinoma and amount of alcohol consumed (3, 9, 13, 14). In addition, in some case-control studies of hepatocellular carcinoma risk, synergistic interactions between alcohol consumption and hepatitis virus infection, or between obesity and diabetes mellitus, have been observed (9, 13, 14). In the present study, after adjusting for other factors such as hepatitis virus infection and BMI, alcohol consumption of ≥40 g of ethanol produced a 4.36-fold increase in hepatocellular carcinoma risk. A few recent case-control studies suggested that ethanol consumption of <50 to 60 g/d (41, 42) or alcohol exposure <1,500 gram-years (9) had protective effects on the progression of liver fibrosis and risk for developing hepatocellular carcinoma. Reasons for such discrepancy between our result and former reports are unclear, but factors such as gender, age, race (43), hereditary predisposition, and etiology of liver disease presumably affect the severity of alcohol-related liver diseases. Our study also showed that effects of alcohol consumption of ≥40 g of ethanol per day on hepatocellular carcinoma risk were reduced after adjusting for all confounding factors including severity of liver fibrosis. The finding suggests that alcohol consumption may contribute to hepatic carcinogenesis by enhancing oxidative stress and aggravating liver fibrosis.

As a result of recent assessments by the IARC, hepatocellular carcinoma has been positioned as a smoking-related malignant disease (44). However, it has yet to be determined whether smoking itself has direct hepatic carcinogenic effects or whether smoking contributes to hepatic carcinogenesis by way of progression of liver fibrosis. A case-control study showed that 4-aminobiphenyl DNA adducts contained in tobacco smoke are a liver carcinogen (45). In the present study, we adjusted for potential confounding factors including hepatitis virus infection and failed to detect significant smoking effects on hepatocellular carcinoma risk; however, a multivariate analysis that excluded hepatitis virus infection showed significant effects of smoking (data not shown). With adjustment for all factors including severity of liver fibrosis, effects of smoking on hepatocellular carcinoma risk were found to be marginally significant. These findings suggest the possibility that smoking, in conjunction with hepatitis virus infection, further enhances the risk for hepatocellular carcinoma and might directly contribute to the mechanism of liver carcinogenesis.

Several epidemiologic studies indicated the involvement of coffee in decreased alanine aminotransferase activity and γ-glutamyltransferase level, suppression of progression to liver cirrhosis, and inhibited development of hepatocellular carcinoma (18, 19). Such oxidation inhibitors as caffeine, coffee diterpenes, and chlorogenic acid are among candidate substances in coffee that potentially reduce the risk for hepatocellular carcinoma, and several animal experiments have shown that such substances have direct inhibitory effects on hepatic carcinogenesis (46). Adjusting for all potential confounding factors including hepatitis virus infection rendered the effects of coffee drinking on hepatocellular carcinoma risk marginally significant, whereas adjusting for all factors, except hepatitis virus infection, revealed significant effects of coffee drinking (data not shown). Furthermore, adjusting for all factors including severity of liver fibrosis erased the effects of coffee drinking on hepatocellular carcinoma risk. These findings suggest that coffee drinking may somehow suppress liver fibrosis and thereby indirectly reduce hepatocellular carcinoma risk.

The main strengths of our study are its prospective cohort-based, nested case-control design, which minimized selection bias and provided for the use of stored sera and a wealth of epidemiologic information obtained before hepatocellular carcinoma diagnosis. Indeed, the distributions of HBV and HCV infection status among hepatocellular carcinoma cases and controls and mean age at diagnosis among hepatocellular carcinoma cases were similar to those in previous reports on Japanese populations (2, 4). Another major strength of our study is that it incorporated, in a strict and in-depth manner, HBV and HCV infection status and showed the interrelationship between these and numerous other epidemiologic factors. It is difficult and expensive to perform full cohort serum analyses, whereas the nested case-control design used here can provide substantial reductions in cost and effort with little loss of statistical efficiency.

The main limitation of our study is that the severity of liver fibrosis could not be classified into fibrosis stage of F0 to F4 based on liver specimens. We used platelet counts and type IV collagen concentrations as surrogate, but independent, markers of liver fibrosis. Previous reports showed a strong correlation between platelet count and fibrosis stage in the presence of chronic hepatitis C (47) and a close association between levels of type IV collagen, a basic component of the hepatic basal membrane, and severity of liver fibrosis. Another limitation of our study is the usage of sera that had been stored for long periods of time. Proteins and HCV RNA trend to degrade during prolonged storage of either frozen or freeze-dried sera. However, we minimized this degradative effect by the selection of matched controls relative to time and method of serum storage. Furthermore, we have previously shown that the freeze-dried sera are interchangeable with frozen sera in serologic and molecular biological detection of HBV and HCV (22, 25). Finally, some hepatocellular carcinoma cases had to be excluded because of nonavailability of stored sera. We did not detect any differences between included and excluded cases in terms of demographic variables or BMI.

In conclusion, HBV and HCV infection and obesity were independent risk factors for hepatocellular carcinoma, even after taking into account the severity of liver fibrosis. Moreover, the combination of HCV infection and increased BMI exerted a synergistic effect on the risk for hepatocellular carcinoma. Alcohol consumption of ≥40 g of ethanol per day was also an independent risk factor for hepatocellular carcinoma, likely contributing to the development of hepatocellular carcinoma through liver fibrosis. The radiation effect on hepatocellular carcinoma risk was shown to be marginally significant in univariate analysis; whether the radiation effect is confounded with other factors will be closely examined in a separate report. A precise understanding of the mechanism by which obesity contributes to development of hepatocellular carcinoma should lead to better therapeutic strategies, public health policies, and cost-effectiveness.

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HEPATOLOGY

Prospective study of short-term peginterferon-α-2a monotherapy in patients who had a virological response at 2 weeks after initiation of interferon therapy

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adverse effects, clinical trial, efficacy, hepatitis C virus, peginterferon-α-2a.

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Abstract

Background and Aims: Long-term interferon (IFN) therapy is effective in eliminating hepatitis C virus (HCV). However, it carries the risk of adverse effects and reduced quality of life. To assess whether short-term IFN therapy effectively eliminates HCV, we performed a prospective pilot study of pegylated (peg)IFN-α-2a therapy for 8 or 24 weeks.

Methods: After excluding patients with high titers of genotype-1, 55 HCV patients received pegIFN-α-2a. Patients who became negative for HCV-RNA at week 2 were allocated to either an 8-week ($n = 19$) or 24-week ($n = 15$) course of IFN. We evaluated the efficacy of and tolerance to IFN therapy.

Results: The sustained virological response rate was excellent in the two groups (8 weeks, 89.5% [17/19]; 24 weeks, 100% [15/15], respectively.). IFN dose reduction was required in one patient of the 8-week group, but in six patients of the 24-week group ($P = 0.028$). Treatment was completed by all patients of the 8-week group, but discontinued in five patients of the 24-week group ($P = 0.011$).

Conclusions: The 8-week IFN therapy is more tolerable than the 24-week therapy and had similar outcomes. Excluding the patients with high titers of genotype-1, we recommend switching to an 8-week course of pegIFN-α monotherapy once patients show an ultra rapid virological response at week 2 from the start of IFN therapy.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease with an estimated 170 million chronic carriers worldwide.[1] Chronic HCV infection is usually associated with liver cirrhosis (LC) and hepatocellular carcinoma (HCC).[2–6] In Japan, 60–70% of patients with HCC or LC are HCV carriers.[7] Antiviral therapy of interferon (IFN) is widely used for the treatment of chronic HCV infection and is assumed to prevent progression to LC and HCC, especially in patients who show a sustained virological response (SVR).

The reported total HCV-RNA elimination rate is approximately 30–40% in patients treated with conventional IFN monotherapy.[8–10] However, better results have been reported when pegylated (peg)IFN-α is used in both naive patients and in those who fail to respond to or relapse after conventional IFN-α monotherapy. In Japan, two kinds of pegIFN are available: pegIFN-α-2a and pegIFN-α-2b. PegIFN-α-2b can be used with ribavirin, a purine nucleoside analog, in naive patients with genotypes 1 and 2 with a high viral load (>100 KIU/mL of HCV-RNA) or patients with any viral load in whom previous IFN treatment did not eliminate HCV-RNA. PegIFN-α-2a has been used in Japan without ribavirin only since December 2003 because of health insurance restrictions. However, ribavirin combination therapy has been covered by public health insurance since March 2007 in Japan. The HCV elimination rate with pegIFN-α-2b plus ribavirin combination therapy is up to 54% in patients with genotype 1.[11] Several investigators have reported that pegIFN and ribavirin combination therapy for a period of 24 or 48 weeks ensures a viral clearance in most patients with HCV genotypes 2 or 3 infection.[12,13] However, ribavirin combination therapy frequently causes anemia and should be carefully used in the elderly, anemic, or pregnant young patients, and in those who require long-term treatment.[14] Apart from patients with a high viral load of genotype 1, IFN monotherapy is also effective in HCV elimination even when used without ribavirin. Previous studies suggest that the SVR achieved with pegIFN-α-2a is similar to that observed with pegIFN-α-2a combined with ribavirin in patients with hepatitis C.[15,16]

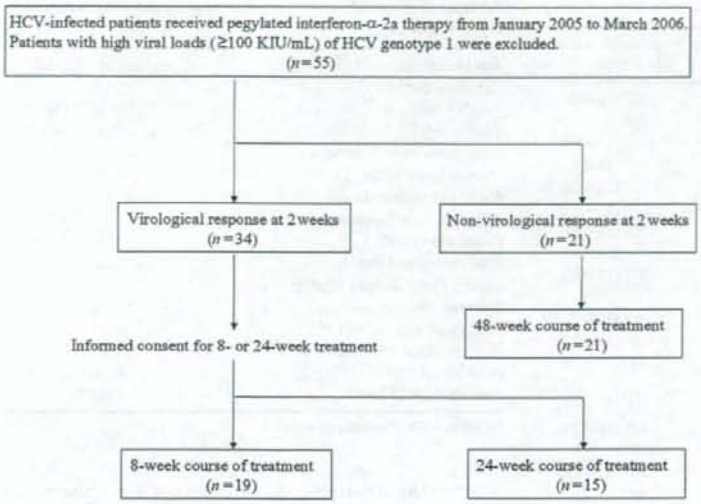

Figure 1 Flow diagram of the clinical trial. HCV, hepatitis C virus.

Although the tolerability of pegIFN is similar to that of the conventional IFN,[15] the 180 μg dose of pegIFN-α-2a therapy for 48 weeks is sometimes not tolerated by some patients. With the exception of those with high viral loads of genotype 1, the above regimen is expected to produce a high viral clearance rate, especially in patients with an early virological response. Several studies report the effectiveness of short-course IFN therapy (<24 weeks) for patients with an early virological response.[17–20] Therefore, a treatment duration of 48 weeks may be too long or more than sufficient for some patients, especially when one considers the undesirable adverse effects or the cost of treatment.

In the present study, we conducted a prospective controlled trial to compare the efficacy of an 8-week versus a 24-week course of pegIFN-α-2a (180 μg/time/week) for patients negative for HCV-RNA at 2 weeks after the initiation of therapy.

Methods

Patients

Between January 2005 and March 2006, a total of 55 HCV-infected patients received pegIFN-α-2a therapy at Hiroshima University Hospital (Hiroshima, Japan) and its associated hospitals in Japan. Patients with high viral loads (≥100 KIU/mL) of HCV genotype 1 were excluded from this study because of their low SVR rate. Among the 55 patients, 34 consecutive patients who showed a rapid virological response at 2 weeks were enrolled in this study (Fig. 1). Eligible patients had antibodies to HCV, were positive for HCV-RNA at study entry, and had not received previous IFN therapy. They included 21 men and 13 women, with a mean age of 53 years (range, 21–71 years). Their HCV genotypes were 1b, 2a, and 2b with variant HCV-RNA (5.1–400 KIU/mL by a reverse transcriptase-polymerase chain reaction [RT-PCR]). All patients underwent liver biopsies within 12 weeks before the start of IFN therapy and were confirmed to have chronic hepatitis by histopathological examination. Patients with any other cause of liver disease including coinfection with hepatitis-B virus or HIV, alcoholic hepatitis, fatty liver, autoimmune hepatitis, or previous organ transplantation were excluded from this study.

Study design

This multicenter prospective controlled study compared the efficacy and safety of 8 weeks versus 24 weeks of pegIFN-α-2a monotherapy in previously untreated patients with chronic hepatitis C who had a virological response at 2 weeks after the start of IFN. Patients with a virological response at 2 weeks were invited to sign a consent form accepting treatment with IFN for 8 weeks only. Those patients who refused consent received a 24-week course of treatment. The primary measure of efficacy was SVR, which was defined as undetectable HCV-RNA in the serum at 24 weeks after the cessation of treatment. All patients agreed to participate in the research protocol, which was approved by the hospital research ethics board, and gave written informed consent. The eligible patients received pegIFN-α-2a (Pegasys, F. Hoffmann-LaRoche, Basel, Switzerland) at 180 μg once per week subcutaneously, either for 8 weeks or 24 weeks, without ribavirin. Other patients who showed no rapid virological response at 2 weeks after the start of pegIFN-α-2a were treated for 24–48 weeks.

All patients were evaluated in an outpatient setting for safety, tolerance, and efficacy every week during the IFN treatment. Blood count was checked just before the IFN injection every week. The qualitative detection of HCV-RNA was performed by a standardized qualitative RT–PCR assay (Amplicor HCV monitor v2.0; Roche diagnostics Co., Tokyo, Japan) at the first 2 weeks and every 4 weeks during and after IFN treatment. The primary efficacy end point for this study was defined as a disappearance of detectable serum HCV-RNA at week 24 after the completion of the IFN treatment.

Table 1 Patients' characteristics

	8-week group ($n = 19$)	24-week group ($n = 15$)
Age (years)	51† (21–71)	47† (25–68)
Sex (male/female)	14/5	7/8
Height (cm)	169† (147–178)	161† (139–178)
Weight (kg)	64.6† (40.6–85)	59† (47–92.4)
Body mass index (kg/m²)	23.4† (18.0–27.8)	21.5† (18.6–30.5)
Platelet count (×10⁴/μL)	19.5† (9.6–30.7)	18.1† (8.9–31.7)
Alanine aminotransferase (IU/L)	55† (22–152)	60† (21–184)
γ-Glutamyl transpeptidase (IU/L)	25† (9–155)	47† (14–137)
Creatinine (mg/dL)	0.76† (0.6–0.9)	0.68† (0.38–0.85)
Total cholesterol (mg/dL)	160† (116–219)	154† (125–201)
Fasting blood glucose (mg/dL)	90† (72–104)	96† (84–115)
Diabetes mellitus	0	1
Hyaluronic acid (ng/mL)	24† (13–72)	78† (16–191)
HCV genotype (1b/2a/2b)	2/15/2	2/10/3
HCV-RNA (KIU/mL)	45† (5.1–370)	43† (5.3–400)
Fibrosis (F1/F2/F3/F4)	6/8/5/0	5/6/4/0

†Median. HCV, hepatitis C virus.

Statistical analysis

We compared the response to an 8-week course of pegIFN-α-2a with that to a 24-week course of pegIFN-α-2a. The χ^2-test and Fisher's exact test were used for comparisons of categorical variables between groups, while Student's *t*-test and the Wilcoxon test were used for continuous and ordinal variables as appropriate. *P*-values less than 0.05 were considered to indicate statistical significance. The JMP version 5.1 statistical software package (SAS Institute, Cary, NC, USA) was used for the statistical analysis of data.

Results

Baseline characteristics

Thirty-four patients who became HCV-RNA-negative at week 2 subsequently received either an 8-week course ($n = 19$) or 24-week ($n = 15$) course of 180 μg pegIFN-α-2a. The baseline characteristics of the two groups at the start of the IFN therapy are summarized in Table 1. None of the patients had LC, based on clinical, laboratory, and histopathological findings. Table 2 also shows the data of 21 patients with a non-rapid virological response at 2 weeks after the start of pegIFN-α-2a. The pretreatment viral loads of non-rapid virological responders were significantly higher than those of rapid virological responders ($P < 0.0001$).

Tolerance of IFN therapy and adverse events

Among the 19 patients of the 8-week group, the dose was reduced by 50% (to 90 μg of pegIFN-α-2a) in one patient with SVR at 3 weeks due to a fall in platelet count. However, all other patients were able to complete the full 8-week course without discontinuation. In 15 patients of the 24-week course, the dose was reduced to half (90 μg of pegIFN-α-2a) in six patients due to neutropenia ($n = 2$; one patient at 8 weeks and one patient at 10 weeks), thrombocytopenia ($n = 3$; two patients at 9 weeks and one patient at 10 weeks) and epigastralgia ($n = 1$; at 14 weeks). Furthermore, IFN therapy was withdrawn in another five patients, including two patients at 8 weeks due to thrombocytopenia, two patients at 12 weeks due to generalized fatigue, and one patient at 18 weeks due to various neurological symptoms, such as hand numbness. Thus the proportion of patients who required a dose reduction was lower in the 8-week group than in the 24-week group ($P = 0.028$). Furthermore, the proportion of patients who completed the treatment was significantly higher in the 8-week group than the 24-week group ($P = 0.011$). We concluded that our patients with HCV could tolerate 8 weeks of IFN therapy better than 24 weeks.

Table 2 Characteristics of 21 patients who did not show a rapid virological response

Age (years)	51† (22–76)
Sex (male/female)	11/8
Height (cm)	164.5† (148–175.5)
Weight (kg)	58.5† (42.5–75)
Body mass index (kg/m2)	22.5† (16.9–27.3)
Platelet count (×104/_L)	20.5† (12–28.6)
Alanine aminotransferase (IU/L)	93† (17–157)
γ-Glutamyl transpeptidase (IU/L)	39† (10–145)
Creatinine (mg/dL)	0.58† (0.5–0.96)
Total cholesterol (mg/dL)	158† (111–214)
Fasting blood glucose (mg/dL)	87† (68–119)
Diabetes mellitus	0
Hyaluronic acid (ng/mL)	45.6† (10–100)
HCV genotype (1b/2a/2b)	0/15/6
HCV-RNA (KIU/mL)	660† (40–830)
Fibrosis (F1/F2/F3/F4)	12/6/3/0

†Median. HCV, hepatitis C virus.

Biochemical and virological responses to therapy

With regard to the alanine aminotransferase (ALT) response to IFN therapy, all patients of both groups showed biochemical normalization at the end of treatment and at 6 months after the end of treatment. There was no difference in the sustained ALT response between the 8-week group and 24-week group. With regard to the virological response to IFN therapy, all patients of both groups exhibited a rapid decrease in HCV-RNA, reaching undetectable levels (HCV-RNA ≤ 100 copies/mL) by week 2. All patients had negative HCV-RNA levels at the end of treatment and none showed a null response. There was no significant difference in the rate of fall of the virological load between patients who had a sustained response and those who had a relapse, as discussed later. The proportions of patients who showed a SVR in the 8-week group and 24-week group were not significantly different (89.5% [17/19] and 100% [15/15], respectively [$P = 0.195$]). Two patients of the 8-week group had viral relapse after the end of treatment; one who had HCV genotype 2a with 50 KIU/mL pretreatment viral load relapsed at 12 weeks after the end of the treatment while the other had genotype 2b with 230 KIU/mL pretreatment viral load and relapsed at 8 weeks after the end of the treatment. The non-

rapid virological responders had a lower SVR rate. Eight (38%) patients showed SVR, 11 (52%) patients developed relapse after discontinuation of IFN, and two (10%) patients had no virological response.

Discussion

In Japan, pegIFN-α-2a monotherapy has been covered by public health insurance since December 2003. The standard duration of treatment with pegIFN-α-2a is 24 weeks for patients with low viral loads of genotype HCV-1 and any viral loads of genotype HCV-2 infection. Recent studies have reported that a treatment duration of more than 24 weeks in such cases does not increase the SVR rate.[11,13,21,22] Moreover, patients with early virological response seem to have a high rate of SVR.[23–25] In those patients, to reduce unnecessary exposure to treatment and its potential side-effects and to reduce costs, short-term IFN therapy has been used by several groups.[17–21] However, details of the IFN regimen differ from those of others and there are no studies that use short-term of pegIFN-α-2a treatment. We therefore conducted a prospective pilot study on the efficacies of an 8-week and 24-week pegIFN-α-2a regimen for patients with low viral titers of genotype HCV-1 and any viral titers of genotype HCV-2 who exhibited a virological response at 2 weeks after the initiation of IFN. In our study, patients with a relatively low viral load before the start of the IFN therapy tended to have a very early virological response.

Our results demonstrated that the virological response to the 8-week treatment (89.5% [17/19]) was excellent and was similar to the 24-week course (100% [15/15]). This high SVR rate of 8-week pegIFN-α-2a monotherapy seems as high as that reported in another short course study of 14-week pegIFN plus ribavirin combination treatment for patients with HCV genotype HCV-2 or HCV-3.[26] This high SVR rate of the 8-week course of pegIFN-α-2a may be associated with a rapid viral disappearance. Several studies have indicated that negative HCV-RNA at week 2 after the commencement of IFN is a predictor of SVR.[19,20,27,28] Therefore, for patients with a low HCV-1 viral load or those with HCV-2 infection with any viral load, we recommend switching to an 8-week course of pegIFN-α-2a monotherapy once they show an ultra rapid virological response, that is, negative HCV-RNA at week 2 from the start of IFN therapy. Furthermore, a longer course of IFN therapy with or without ribavirin can be prescribed when HCV-RNA becomes positive after discontinuation of the 8-week course of IFN therapy.

Although in our study all of the patients in the 24-week course showed SVR, it seems that 24 weeks is a long treatment period for those patients who become negative for HCV-RNA by week 2 of treatment to ascertain SVR. Our results showed that all patients in the 8-week course completed the course to the end of treatment. However, 33% of the patients of the 24-week course did not continue their treatment to the end of the course. Because patients tend to adhere to shorter regimens, which are also better tolerated than longer treatment regimens, a shorter exposure will probably translate into a better benefit–risk ratio in patients with early virological response.

Our study identified two relapsers among patients of the 8-week course after discontinuation of pegIFN-α-2a therapy. These two patients had a negative history of exposure to new HCV infection. One patient who had genotype HCV-2b and a high pretreatment viral load (HCV-RNA: 230KIU/mL) relapsed at 8 weeks after the discontinuation of IFN therapy, while the other who had genotype HCV-2a and a low pretreatment viral load (HCV-RNA: 50KIU/mL) relapsed at 12 weeks after the discontinuation of IFN therapy. These two patients could have SVR after additional IFN therapy for 24 weeks (one patient; pegIFN-α-2a monotherapy, one patient; pegIFN-α-2b and ribavirin combination therapy). We could not identify a definite factor associated with SVR or relapse. Although pretreatment factors, like genotype, viral load, and grade of fibrosis can be used to predict the mean treatment outcome for study cohorts, they are often of limited value in individual patients.[29,30]

As mentioned earlier, a short course of pegIFN-α-2a therapy for 8 weeks could be recommended in those patients who show an ultra rapid virological response at week 2 after the initiation of IFN therapy. Although the study by Shiffman *et al.*[31] demonstrates the inferiority of a shorter regimen in a large-scale, randomized, controlled study, the characteristics of their patients were largely different from those of our study, including racial difference (mostly Caucasian patients versus Japanese, a heavier body weight for the Caucasians versus Japanese patients) and differences in pretreatment viral load (variable and higher HCV-RNA level versus relatively low viral load in our patients). Patients' selection was also different between the two studies; our study was carried out only in cases negative for HCV-RNA at 2 weeks after the start of IFN compared to their randomized study, irrespective of a rapid virological response.

In conclusion, patients chronically infected with low titers of HCV-1 and those with HCV-2, regardless of their viral loads, who achieve an ultra rapid virological response, that is, HCV-RNA negativity at week 2, can receive only 8 weeks of pegIFN-α-2a monotherapy without compromising the chance of SVR. The results of our prospective study are encouraging, although the study population was small and was based on non-randomized methodology. The data of the present study are not conclusive for patients with very high pretreatment viremia who might achieve a rapid virological response or for those patients who do not achieve a rapid virological response. Further clinical trials are required to optimize the treatment duration in these patients.

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Eicosapentaenoic Acid Could Permit Maintenance of the Original Ribavirin Dose in Chronic Hepatitis C Virus Patients during the First 12 Weeks of Combination Therapy with Pegylated Interferon-α and Ribavirin

A Prospective Randomized Controlled Trial

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Key Words

Chronic hepatitis C · Ribavirin · PEG interferon · Combination therapy · Anemia · Eicosapentaenoic acid

Abstract

Objective: To evaluate the efficacy of eicosapentaenoic acid (EPA) against ribavirin (RBV)-associated hemolytic anemia during the first 12 weeks in chronic hepatitis C virus (HCV) combination therapy. ***Methods:*** This study was a prospective open-label, randomized controlled trial. 100 HCV patients were randomized to either the EPA group (n = 49) or non-EPA group (n = 51) who received combination therapy with or without EPA. We compared the changes in hemoglobin level and RBV plasma concentrations at week 12 in each group with RBV dose reduction rate and performed multivariate analysis to identify independent variables associated with RBV dose reduction. ***Results:*** 8 patients (17%) in the EPA group and 20 patients (29%) in the non-EPA group required RBV dose reduction, respectively. The cumulative RBV reduction rate was significantly lower in the EPA group than in the non-EPA group (p = 0.017), while the decrease of hemoglobin and RBV plasma concentrations from baseline was not significantly different. However, in the multivariate analysis, treatment with EPA showed significant variables for the reduction of RBV dose (odds ratio 3.235, p = 0.023). ***Conclusion:*** EPA could prevent the RBV dose reduction during the first 12 weeks in combination therapy, although further large-scale double-blind randomized controlled trials are required.

Introduction

Chronic hepatitis C virus (HCV) infection, the most common cause of chronic liver disease worldwide, can lead to cirrhosis and hepatocellular carcinoma. HCV infects an estimated 170 million individuals worldwide, and 2 million people in Japan are infected [1–4]. Pegylated in-

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terferon-α (PEG-IFN-α) plus ribavirin (RBV), which currently represent standard treatment for chronic HCV infection, can increase sustained virological response (SVR) [5–11]. Some studies have reported a decrease in virus level during the several weeks after starting treatment, which might correlate with the likelihood of SVR [12–16]. Davis et al. [16] reported that most patients who are able to complete the first 12 weeks of therapy achieve an early virological response and have a high probability of SVR.

Although high RBV doses offer the best chance of a SVR, they increase the risk of hemolytic anemia. RBV-associated hemolysis, which frequently results in anemia, may exacerbate symptoms such as fatigue and shortness of breath, resulting in reduced patient compliance [7–11]. This common side effect of RBV is dose-related and usually resolves when therapy is discontinued [7–19] but the mechanism causing this hemolytic anemia is unknown. Previous studies reported that none of the patients who discontinued therapy achieved SVR, and adherence to combination therapy enhanced SVR in genotype 1 HCV patients [20, 21].

Eicosapentaenoic acid (EPA), used widely to treat hyperlipidemia and atherosclerosis [23, 24], is readily assimilated into erythrocyte membrane phospholipids and correlates positively with erythrocyte deformability [25]. In an uncontrolled pilot study by Ide et al. [26], 6 patients given EPA after the development of anemia showed significant increases in mean hemoglobin (Hb) values. Although these prospective study data indicate that EPA may suppress RBV-induced anemia, the efficacy of EPA for combination therapy is uncertain. Furthermore, no systematic studies have been published.

In the present study, we assessed HCV-infected patients receiving PEG-IFN-α and RBV combination therapy to determine whether efficacy of EPA against RBV-associated hemolytic anemia was sufficient to avoid RBV dose reduction during the early period of combination treatment.

Method

For this prospective open-label randomized controlled trial, 102 patients chronically infected with HCV genotype 1b at a high viral load were enrolled between December 2004 and January 2005. This protocol was approved by the local ethics committees, and was conducted according to the principles of the Helsinki Declaration. Patients from 14 hospitals in the Hiroshima Liver Study Group were included after they gave informed written consent and were found to meet all of the following inclusion criteria: infection with HCV genotype 1b at a high viral load (>100 kIU/ml) according to seropositivity for anti-HCV antibodies, using a third-generation enzyme-linked immunosorbent assay, and serum HCV RNA determination using a quantitative polymerase chain reaction assay; Hb of at least 12.0 g/dl; compensated liver disease; age of at least 18 years; no hepatocellular carcinoma, and no bleeding tendency.

Exclusion criteria were decompensated liver disease; autoimmune disease; clinically significant cardiovascular, metabolic, renal, hematologic, neurologic, or psychiatric disease; systemic infections; neoplastic disease; systemic immunosuppressive treatment; active alcohol or drug abuse within the previous year, and pregnancy.

All patients received both PEG-IFN-α_{2b} (PegIntron; Schering Plough, Kenilworth, N.J., USA), 1.5 μg/kg/week, s.c., and oral RBV at 600 mg/day (<60 kg body weight (BW)), 800 mg/day (between 60 and 80 kg BW), or 1,000 mg/day (>80 kg BW), with the manufacturer's drug information for RBV. The lower doses of RBV rather than the higher doses were chosen because of the difference in weight between the Japanese and American population. All patients were assigned randomly to either the EPA group or non-EPA group. Randomization was carried out using sealed envelopes and a computer-generated randomization list. EPA patients received oral EPA (Epadel S900; Mochida Pharmaceutical, Tokyo, Japan) at 900 mg/day divided into two daily doses throughout treatment.

All patients were evaluated for treatment efficacy and safety by each attending a doctor based on the World Health Organization classification of adverse events. Hematologic, biochemical, and virological parameters were determined by the local laboratories at each study center.

For anemia, the daily RBV dose was reduced by 200 mg when Hb fell to <10 g/dl, acute decrease and remains of Hb concentrations >3 g/dl from baseline, or clinical symptoms of anemia associated with a decrease of Hb >2 g/dl from the start of treatment, for example, palpitation, dyspnea on efforts, and fatigue. Once lowered, the RBV dose was used throughout the rest of the study. When patients complained of symptom fatigue or pallor, RBV was discontinued when Hb fell to <8.5 g/dl or when patients manifested more severe anemia including orthostatic hypotension. These RBV dose reduction criteria were adhered to in each center.

The primary efficacy endpoint of this study was comparison of the RBV dose reduction rate in each group to determine whether EPA maintains RBV dosage. The second endpoint was changes in Hb level between treatment groups and change in RBV dosage and plasma concentrations at week 12; all patients who were randomized and received this therapy were included in this analysis. The RBV plasma concentrations were determined by a validated high-performance liquid chromatography tandem mass spectrometric assay using ^{13}C-RBV as an internal standard [27, 28]. However, for patients who discontinued before week 12, the last available data were used in this study. Since pharmacokinetic studies showed that serum RBV concentrations take 4–8 weeks to reach a plateau, we chose week 12 as the endpoint in order to evaluate the influence of RBV-induced anemia.

Statistical Analysis

To detect a difference in Hb levels of 2 g/dl, using a two-sided test with a significance level of 0.05 and a power of 90%, 35 patients were required for each group. To compensate for non-evaluable patients, we planned to enroll 50 patients per group.

The χ^2 test or the Mann-Whitney U test was used for statistical comparisons between groups, as appropriate. Treatment outcomes

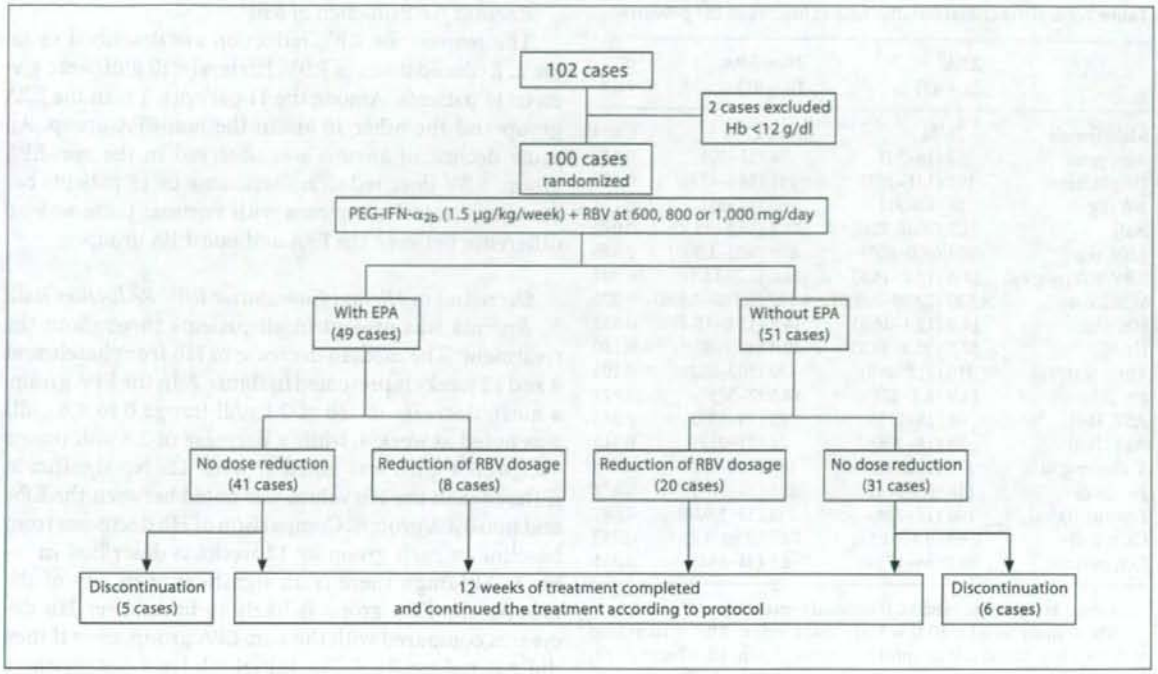

Fig. 1. Flow chart showing progression of participants throughout the protocol.

were analyzed on an intention-to-treat basis. Cumulative reduction rates of RBV dose were calculated using the Kaplan-Meier method and compared by the log-rank test. Multivariate analysis (multivariate logistic regression) was used to identify factors independently associated with RBV dose reduction. The influence of 19 variables on RBV dose reduction required by RBV-induced anemia was examined. Variables considered included: gender, age, height, pretreatment body weight (BW), body mass index (BMI), dose of RBV (absolute and per kg BW), pretreatment Hb, hematocrit (Ht), pretreatment red blood cell count (RBC), white blood cell count (WBC), platelet counts (PLT), pretreatment serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (T-cho), iron (Fe), ferritin, creatinine (Cr), creatinine clearance (Ccr), and treatment with EPA.

All p values <0.05 were considered to indicate statistical significance. Calculations were performed with the SPSS Statistical Package, Version 11.0 (SPSS, Chicago, Ill., USA).

Results

Among 102 patients with HCV genotype 1b at a viral load of at least 100 kIU/ml, 2 were excluded because of an Hb value <12 g/dl. The remaining 100 patients were randomized to receive or not receive EPA. The baseline characteristics are summarized in table 1, and the flow chart in figure 1 shows the progression of participants from screening to 12 weeks of treatment.

EPA Group

Of 49 patients randomized to receive combination therapy with EPA, 41 needed no RBV reduction, while 8 patients required RBV dose reduction within 12 weeks of beginning treatment. Among them, 5 withdrew from the study before completing 12 weeks because of neutropenia depression and were lost to follow-up, all of them stopped the treatment without RBV dose reduction. Therefore, 44 patients completed the initial 12 weeks and continued according to the protocol. Patient compliance was not affected by EPA. No untoward side effects were attributed to EPA in this trial.

Non-EPA Group

Of 51 patients randomized to receive combination therapy without EPA, 31 needed no RBV reduction, while 20 required RBV dose reduction within 12 weeks of be-

Table 1. Baseline characteristics and valuables of the patients

	EPA (n = 49)	Non-EPA (n = 51)	p value
Male/female	25/24	24/27	0.699
Age, years	57 (18–74)	58 (21–72)	0.833
Height, cm	157 (141–167)	161 (144–179)	0.672
BW, kg	58 (42–91)	62 (42–85)	0.194
BMI	24.3 (20.0–32.0)	23.2 (18.8–29.9)	0.072
RBV, mg	694 (600–800)	670 (600–1,000)	0.465
RBV/BW, mg/kg	11.6 (10.2–14.5)	11.6 (7.7–14.3)	0.302
WBC, μ/l	4,520 (2,690–7,400)	4,480 (2,410–7,400)	0.220
Hb, g/dl	14.2 (12.1–16.3)	14.3 (12.0–16.8)	0.532
Ht, %	37.5 (30.8–44.2)	39.0 (33.1–47.0)	0.180
RBC, $\times 10^4/\mu l$	410 (317–490)	430 (362–522)	0.103
Plt, μ/l	14.9 (8.5–27)	14.5 (7–57)	0.171
AST, IU/l	58 (18–213)	62 (24–187)	0.343
ALT, IU/l	65 (18–256)	78 (21–217)	0.315
T-cho, mg/dl	133 (102–249)	144 (121–235)	0.933
Fe, μg/dl	116 (65–294)	105 (51–301)	0.872
Ferittin, ng/ml	190 (16–724)	203 (13–1,024)	0.081
Cr, mg/dl	0.68 (0.40–1.1)	0.67 (0.50–1.1)	0.753
Ccr, ml/min	98.1 (56–179)	94.1 (51–191)	0.351

Values are given as median (minimum–maximum).

BW = Body weight; BMI = body mass index; RBV = ribavirin; WBC = white blood cell count; Hb = hemoglobin; Ht = hematocrit; RBC = red blood cell count; PLT = platelet counts; AST = aspartate aminotransferase; ALT = alanine aminotransferase; T-cho = total cholesterol; Fe = iron; Cr = creatinine; Ccr = creatinine clearance.

Table 2. Reason for RBV dose reduction during 12 weeks after starting combination treatment

	EPA (n = 8)	Non-EPA (n = 20)	Total (n = 28)
(1) Hb levels <10 g/dl	1	10	11
(2) Acute decrease of Hb levels >3 g/dl	0	2	2
(3) Clinical symptoms of anemia associated with a decrease of Hb levels >2 g/dl	7	8	15

ginning treatment. Among them, 6 withdrew from the study before 12 weeks because of adverse effects including depression, thrombopenia, pneumonia, and were lost to follow-up. 45 patients completed the initial 12 weeks and continued according to the protocol. No patient required a blood transfusion and no other adverse effects were noted in either of the groups.

Reasons for Reduction of RBV

The reasons for RBV reduction are described in table 2. Reduced doses of RBV, Hb level <10 g/dl, were given to 11 patients. Among the 11 patients, 1 is in the EPA group and the other 10 are in the non-EPA group. An acute decline of anemia was observed in the non-EPA group. RBV dose reduction was done in 15 patients because of clinical symptoms with anemia; there was no difference between the EPA and non-EPA group.

Decreases in Hb and Cumulative RBV Reduction Rate

Anemia was present in all patients throughout the treatment. The median decrease in Hb from baseline at 4 and 12 weeks is presented in figure 2. In the EPA group, a mean decrease in Hb of 2.1 g/dl (range 0 to 4.6 g/dl) was noted at week 4, while a decrease of 2.4 g/dl (range −0.1 to 6.8 g/dl) was noted at week 12. No significant difference in the Hb values was noted between the EPA and non-EPA groups. Comparison of Hb decreases from baseline in each group by 12 weeks is described in table 3. Although there is no significance in any of the groups, the EPA group is likely to have lower Hb decreases compared with the non-EPA group, even if they did not reduce RBV. The initial Hb level distributions are described in figure 3. No difference was observed between the EPA and non-EPA groups after distribution of initial Hb levels.

However, the cumulative RBV dose reduction rate during 12 weeks of treatment (EPA 17%, non-EPA 29%) was significantly lower in the EPA group than the non-EPA group (fig. 4). In the non-EPA group, respective mean RBV plasma concentrations at weeks 4 and 12 were 2,349 ± 897 and 2,249 ± 874 mg/ml. In the EPA group, respective mean RBV plasma concentrations at weeks 4 and 12 were 2,201 ± 887 and 2,508 ± 705 mg/ml. Although the EPA group had a higher RBV concentration at 12 weeks, differences in RBV concentrations between the EPA group and non-EPA group were not significant (table 4).

Uni- and Multivariate Analysis

Variables were analyzed for the relationship to RBV dose reduction by both uni- and multivariate methods. Gender, age, height, BW, BMI, dose of RBV (both absolute and by patient BW), pretreatment Ht concentration, RBC, WBC, PLT, AST, ALT, T-cho, Fe, ferritin, Cr, and Ccr were not associated with development of anemia by univariate analysis, while higher pretreatment Hb and treatment with EPA were related to maintenance RBV dose. By multivariate analysis, only treatment with EPA

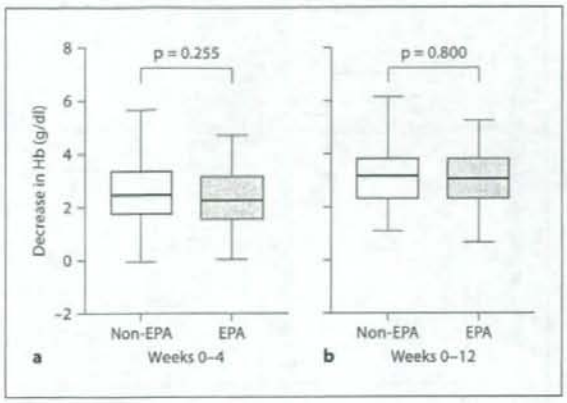

Fig. 2. Box-and-whisker plot comparing the decrease of Hb from baseline at week 4 (**a**) and at week 12 (**b**). No significant difference was seen between the EPA and non-EPA groups (week 4, p = 0.255, week 12, p = 0.800).

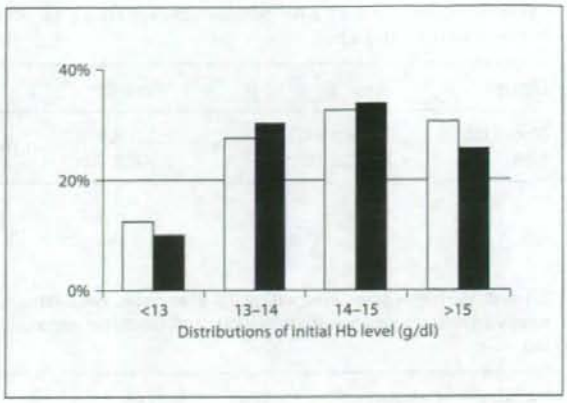

Fig. 3. No difference was observed between the EPA and non-EPA groups after distributing initial Hb levels. □ = EPA; ■ = non-EPA.

Table 3. Comparison of Hb decrease from baseline in each group by 12 weeks

	EPA (n = 44)			Non-EPA (n = 45)		
	RBV reduction (n = 8)	no reduction (n = 36)	p	RBV reduction (n = 20)	no reduction (n = 25)	p
4 weeks	2.4 (1.1)	1.7 (1.4)	0.08	2.8 (0.9)	2.3 (1.7)	0.31
8 weeks	2.5 (1.2)	2.1 (1.6)	0.46	3.3 (1.2)	2.5 (1.1)	0.09
12 weeks	2.9 (1.5)	2.4 (1.2)	0.30	3.1 (0.9)	2.8 (1.2)	0.35

Values are given as mean (SD).

significantly and independently reduced the likely need for reduction of RBV dose (odds ratio 3.235, p = 0.023) (table 5).

Virological Response

In the EPA group, RVR (rapid virological response), EVR (early virological response) and ETR (end of treatment response) were achieved in 8 of 49 (16%), 27 of 49 (55%), and 30 of 49 patients (61%), whereas in the non-EPA group, RVR, EVR and ETR were achieved in 16 of 51 (31%), 33 of 51 (65%), and 35 of 51 patients (68%), respectively. Since after the end of the treatment some patients in each group continued the combination therapy, the number of patients with SVR decreased. SVR was achieved by 15 of 36 (42%) in the EPA group and by 19 of 40 (45%) in the non-EPA group, respectively. In each viral response, no significant differences were achieved in either of the groups.

Discussion

The results of this study demonstrated that with EPA treatment anemic HCV-infected patients undergoing combination therapy more often could avoid RBV dose reduction in the first 12 weeks of therapy, although a lower pretreatment increased the likelihood of RBV dose reduction. To our knowledge this is the first reported randomized clinical trial of EPA in combination therapy for chronic hepatitis C.

Table 4. Comparison of RBV plasma concentrations (mg/ml) between weeks 4 and 12

Group	Week 4	p	Week 12	p
Non-EPA	2,349 ± 897	0.899	2,249 ± 874	0.101
EPA	2,201 ± 887		2,508 ± 705	

Table 5. Variables associated with RBV dose reduction during 12 weeks after starting combination treatment (multivariate analysis)

Variable	Category	OR	95% CI	p
Hb	(1) >13 g/dl	1	0.620–9.919	0.199
	(2) <13 g/dl	2.480		
EPA	(1) With EPA	1	1.173–8.920	0.023
	(2) Without EPA	3.235		

OR = Odds ratio; 95% CI = 95% confidence interval.

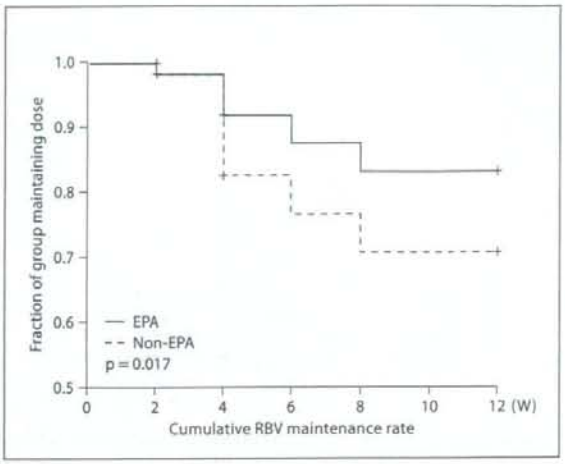

Fig. 4. Cumulative RBV dose maintenance rates during 12 weeks of treatment were 83% for the EPA and 71% for the non-EPA (RBV dose reduction rate: 17% for the EPA and 29% for the non-EPA), respectively. The original dose could be maintained in significantly more EPA than non-EPA patients (p = 0.017).

Many previous studies reported combination therapy with PEG-IFN and RBV for 24–48 weeks to be effective in treating chronic hepatitis C [1–5]. The most frequent toxic side effect of RBV is a reversible hemolytic anemia, which requires some patients to reduce or discontinue combination therapy. The mechanism underlying the hemolytic anemia is unclear, but is thought to involve accumulation of RBV triphosphate in erythrocytes [18, 19]. The effect of RBV accumulation on cellular respiration reduces the half-life of erythrocytes through extravascular hemolysis. EPA, the principal fatty acid in fish oils, has a wide variety of pharmacologic actions including an increase in deformability of erythrocytes [22]. EPA has been shown to improve erythrocyte deformability by incorporation into erythrocyte membrane phospholipid [23]. While Hino et al. [29] show a decrease of EPA content in erythrocyte membrane phospholipids in HCV patients with IFN and RBV therapy, it is thought that EPA supplementation would be useful for preventing RBV-induced anemia. Ide et al. [26] reported that EPA increased Hb in patients with RBV-related anemia. However, clinical trials have been limited to a pilot study, and a randomized trial was needed. In performing such a trial we focused on whether EPA administration diminished RBV-associated hemolysis and improved patient tolerance sufficiently to avoid RBV dose reductions in patients with chronic hepatitis C treated with a combination therapy. However, in our results there was no difference of decrease in Hb from baseline at 4 and 12 weeks. It is considered that the anemia symptom led to reduce the RBV dose before 10 g/dl for all anemia patients, therefore in the present studies no significant difference in Hb values was noted between the EPA and non-EPA groups. It is thought that EPA could be useful to decrease the symptom of RBV-induced anemia.

Previous studies have found full-dose RBV to be important in the early phase after initiating treatment. McHutchinson et al. [30] reported that patients who were unable to adhere to full-dose therapy during the first 12 weeks after initiating treatment had a lower rate of SVR than patients who did not require dose reduction until after week 12. Shiffman et al. [31] reported that reducing the RBV dose to <80 or 60% of the starting dose during the first 20 weeks of treatment was associated with a decline in SVR rate from 21 to 11% at 72 weeks. Furthermore, previous studies suggested that high RBV concentrations could contribute to the improvement in viral response in this group of patients [31–34].

Since the RBV concentrations remained stable after 4–8 weeks of treatment [5, 6], it is thought that RBV does not contribute to the antiviral effect during 4 weeks after the beginning of combination treatment. However, Arase

et al. [34] showed that a higher RBV concentration contributes to SVR and also showed that in order to raise the RBV concentration it is important to maintain a RBV dose in the early phase after the beginning of combination treatment. In our study, although RVR was lower in the EPA group, EVR, ETR, and SVR showed almost equal results between the EPA group and non-EPA group. We consider it is one of the reasons why RBV dose maintenance during the early phase would be more effective in the EPA group than in the non-EPA group in spite of other factors (e.g. IFN sensitivity, LDL-C, G-GTP, ICG-R15, and amino acid substitutions in the core region [35–38]). However, in our study, patients had combination treatment during the initial 12 weeks, but the effect of combination therapy with EPA on HCV was unknown. It is thought that further investigation is needed.

In summary, giving EPA could permit maintenance of the original RBV dose in HCV patients during the first 12 weeks of PEG-IFN-α and RBV combination therapy. However, since this study was of only intermediate size and an open-label study, which might introduce a bias to reduce the dose of RBV, further large-scale double-blind randomized controlled trials are required to investigate whether EPA could be useful for RBV hemolytic anemia.

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