A previous study indicated that a late evening meal, including carbohydrate-rich snacks, had the same effect as BCAA administration,[8] although a recent randomized, controlled trial suggested that the impact of BCAA administration on the improvement of nutritional parameters was superior to that of ordinary food containing matched daily energy and protein intake.[20]

However, few studies have investigated the long-term effect of nutritional support on hepatocellular carcinoma (HCC) development. To the best of our knowledge, only two studies have evaluated the effect of nutritional intervention on the risk of HCC recurrence among postoperative HCC patients.[21,22] In these studies, the intervention group had a significantly lower recurrence rate when compared to the control group. These reports suggested that nutritional support might act to prevent HCC occurrence. Thus we conducted a case-control study to examine the hypothesis that nutritional support might reduce the risk of HCC incidence. The present study took special notice of a late evening meal as a nutritional factor since this has been considered to be one of the most effective approaches for improvement of nocturnal starvation. In Japan, 80% of HCC cases are caused by hepatitis C virus (HCV) infection,[23] so the source population was restricted to patients with chronic type C liver disease.

METHODS

Selection of cases and controls

THE METHOD OF the present study has been described elsewhere.[24,25] We identified all consecutive patients with chronic hepatitis C who visited the Department of Hepatology of Osaka City University Hospital (OCUH; Osaka, Japan) for clinical follow up between 1 November 2001 and 31 January 2002 (the recruitment period). The following patients were excluded: patients with other types of liver disease (e.g. co-infection with hepatitis B virus, primary biliary cirrhosis, autoimmune hepatitis, and idiopathic portal hypertension), referred patients who had already been diagnosed with HCC at other hospitals, and patients in poor health (e.g. liver failure and terminal stage of HCC). This resulted in 1159 patients who were regarded as a source population.

From the source population, 86 patients were first diagnosed with HCC between 1 November 1998 and 31 March 2002. The diagnosis of HCC was based either on a histopathological examination or a positive result in at least one imaging study (computed tomography, magnetic resonance imaging, angiography) combined with an elevated serum á-fetoprotein level. For each HCC case, we selected 1–5 control patients, matching for age (±2 years), sex, and the date of the first OCUH visit (±2 years). Eventually, 86 cases and 333 controls were identified as candidates.

The study protocol was approved by the ethics committee at the Osaka City University Graduate School of Medicine.

Information collection

From 1 June 2002 to 31 December 2002 (the study period), the physician-in-charge explained this study to the candidate cases and controls each time they underwent regular medical examinations. After obtaining informed consent verbally, the physician-in-charge gave the patients a self-administered, mail-back questionnaire. We mailed reminders to non-respondents twice at 1-month intervals. The questionnaire included items on demographic factors; past medical history; age of first identification of liver disease (e.g. abnormality of liver enzyme level or positive results for HCV infection); family history of liver diseases; smoking; alcohol drinking; dietary habits, including a late evening meal; occupation; physical exercise; and reproductive history. A late evening meal was defined as a snack or meal within 2 h before bedtime. The habit of eating a late evening meal after first identification of liver disease was investigated retrospectively by reporting a dichotomous answer (yes or no). Patients who answered "yes" also reported the average weekly frequency of eating a late evening meal and the major food items they consumed.

We also collected the findings of abdominal ultrasonography and laboratory data at the first OCUH visit from medical records. At OCUH, the findings from the abdominal ultrasonography had been scored to show disease severity on a semiquantitative scale called the "US score." This score is the sum of the five leveled scores (0, 0.5, 1.0, 1.5, and 2.0) for five variables (liver deformity, nature of the liver edge, nature of the liver surface, coarsening of intrahepatic echo signals, and size of the spleen). This was evaluated in patients with chronic type C liver disease and proved to be highly correlated with the degree of liver fibrosis according to the new European classification or Child–Turcotte criteria.[26] While US scores ≥3.5 indicated chronic liver disorders and US scores >5.0 indicated liver cirrhosis, the sensitivity and specificity of this approach to classifying the presence or absence of liver cirrhosis were estimated to be 83–97% and 91–96%, respectively.[27,28] Laboratory data included white blood cell, red blood cell, platelet

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count, total bilirubin, aspartate aminotransferase, alanine aminotransferase, total protein, albumin, α-fetoprotein, virus titer of HCV-RNA, and fasting blood sugar. Information about interferon therapy was obtained from medical records.

Data analyses

The frequency of intake of a late evening meal was recategorized into three levels according to the distribution of controls, with category boundaries that were drawn to make the size of groups as equal as possible. The χ^2-test and Wilcoxon rank sum test were used to compare selected characteristics between cases and controls. To consider the presence of confounding, the distribution of potential confounders was compared between patients who consumed a late evening meal and those who did not only among the control patients using χ^2-test or Wilcoxon rank sum test. The conditional logistic regression model was used to calculate the odds ratios (OR) and 95% confidence intervals (CI) for HCC risk. Variables that showed *P*-values less than 0.1 or seemed to correlate with the late evening meal were considered to be potential confounders for adjustment.

We performed an additional analysis to consider the effect of possible confounding variables, such as markers of progression of liver disease, potential malnutrition, obesity, and treatment with interferon. In the additional stratified analyses, patients were divided into two groups according to the following cut-off point: median level of US score, presence or absence of suspected liver cirrhosis (ratio of aspartate to alanine aminotransferase >1.0,[29–31] platelet count $<10 \times 10^4/\mu l$[29]), normal level of α-fetoprotein, median level of serum albumin, and presence or absence of obesity.[32] In the stratified analyses, the unconditional logistic regression model was used to calculate OR and 95% CI of a late evening meal for HCC. Each model included three matching factors (i.e. age, sex, and duration from first OCUH visit) and the potential confounders other than stratified factors. The homogeneity of OR across stratified categories was tested as the *P*-value of the interaction term between a late evening meal and each stratified variable.

All statistical analyses were performed using SAS version 8.2 (SAS Institute, Cary, NY, USA).

RESULTS

AMONG THE 419 enrolled patients, 51 were excluded. Ten patients were subsequently found to be ineligible (e.g. co-infection with HBV and complete recovery from HCV infection) and 41 patients did not visit OCUH during the study period. There were 23 non-respondents (6%) for the following reasons: death (4 patients: 3 cases and 1 control), poor health (6 patients: 3 cases and 3 controls), and refusal to participate (13 patients: 1 case and 12 controls). Eventually, 326 patients (73 cases and 253 controls, 73 matched sets) maintained the initial matched combination and comprised the patients for the analysis.

Table 1 shows the selected characteristics of the cases and controls. Cases and controls were well matched for age, sex, and duration from the first OCUH visit until the beginning of the study. A significant difference between cases and controls was observed in the duration from first identification of liver disease until the beginning of the study period (17 *vs* 13 years). Cases had more family history of liver diseases and received less interferon therapy with marginal significance. Laboratory data and US scores at the first OCUH visit indicated that cases had more severe disease condition than controls during the 7 years before the beginning of the study period.

Table 2 provides the distribution of selected potential confounders between patients who had consumed a late evening meal and those who did not among the control patients. No measurable differences were found in the distribution of potential confounders, including liver disease progression, body mass index, and interferon therapy, across the groups who did or did not consume a late evening meal.

Table 3 shows the OR for HCC according to late evening meal, adjusted for duration from first identification of liver disease, disease severity at the first OCUH visit (US score, platelet count, aspartate aminotransferase, α-fetoprotein, and fasting blood sugar), and interferon therapy. The group who consumed a late evening meal had a reduced risk of HCC as compared to those who did not consume one (OR, 0.08; 95% CI, 0.01–0.48). In addition, higher frequency intake of a late evening meal was associated with lower OR with a significant dose–response relationship (trend $P = 0.009$). Thus a late evening meal was associated with a lower risk of HCC.

The inverse associations of a late evening meal with the development of HCC did not differ between groups with or without possible liver cirrhosis (Table 4). When the study patients were divided according to the absence or presence of possible liver cirrhosis (e.g. a platelet count of less than $10 \times 10^4/\mu l$ or ratio of aspartate to alanine aminotransferase of more than 1.0), no measurable difference was observed in the inverse association

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Table 1 Comparison of selected characteristics between cases and controls†

Characteristics	Case ($n = 73$)	Control ($n = 253$)	*P*-value‡
Age (years)	69 (65–73)	69 (65–72)	0.389
Sex (%)			
Male	47	52	0.434
Duration until beginning of the study (years)			
From first identification of liver disease	17 (12–26)	13 (10–21)	0.011
From first OCUH visit	7 (4–9)	7 (4–9)	0.289
Family history of liver diseases (%)			
Present	38	27	0.069
Interferon therapy (%)			
Present	25	36	0.072
Body mass index (kg/m^2)	22 (21–26)	23 (21–25)	0.986
Platelet count (×10^4/μL)	11 (8–15)	16 (12–20)	0.000
Aspartate aminotransferase (IU/L)	86 (59–112)	67 (43–101)	0.003
Albumin (g/dL)	3.8 (3.6–4.1)	4.1 (3.9–4.3)	0.000
Alpha-fetoprotein (ng/mL)	15 (7–36)	5 (4–11)	0.000
Fasting blood sugar (mg/dL)	100 (94–118)	98 (92–108)	0.066
US score	4.0 (3.0–5.5)	3.0 (2.0–3.5)	0.000

†Data are expressed as median (inter-quartile range) unless otherwise indicated. ‡χ^2-test and Wilcoxon rank sum test were used where appropriate. OCUH, Osaka City University Hospital; US score, ultrasonography score.

Table 2 Comparison of selected characteristics between patients who consumed a late evening meal and those who did not among control patients†

Characteristics	Patients who consumed a late evening meal ($n = 46$)	Patients who did not consume a late evening meal ($n = 207$)	*P*-value‡
Age (years)	68 (64–74)	69 (65–72)	0.960
Sex (%)			
Male	52	52	0.953
Duration until beginning of the study (years)			
From first identification of liver disease	16 (11–26)	13 (10–21)	0.152
From first OCUH visit	7 (4–9)	7 (4–9)	0.974
Family history of liver diseases (%)			
Present	28	27	0.868
Interferon therapy (%)			
Present	37	36	0.877
Body mass index (kg/m^2)	23 (21–25)	23 (21–25)	0.842
Platelet count (×10^4/μL)	17 (11–21)	16 (12–19)	0.677
Aspartate aminotransferase (IU/L)	66 (41–96)	67 (47–101)	0.482
Albumin (g/dL)	4.1 (3.8–4.3)	4.1 (3.9–4.3)	0.352
α-Fetoprotein (ng/mL)	5 (4–10)	6 (4–11)	0.452
Fasting blood sugar (mg/dL)	99 (93–110)	98 (92–107)	0.330
US score	3.0 (2.0–4.0)	3.0 (2.5–3.5)	0.471

†Data are expressed as median (interquartile range) unless otherwise indicated. ‡χ^2-test and Wilcoxon rank sum test were used where appropriate. OCUH, Osaka City University Hospital; US score, ultrasonography score.

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Table 3 Odds ratio (OR)† for hepatocellular carcinoma according to frequency of intake of a late evening meal

Characteristics	Level	Case ($n = 73$) n (%)	Control ($n = 253$) n (%)	Univariate			Multivariate‡		
				OR	(95% CI)	P-value	OR	(95% CI)	P-value
Late evening meal	Never	66 (90)	207 (82)	1			1		
	Intake	7 (10)	46 (18)	0.47	(0.20–1.07)	0.071	0.08	(0.01–0.48)	0.005
Frequency	Never	66 (90)	207 (82)	1			1		
	<4 times/week	6 (8)	26 (10)	0.70	(0.28–1.75)	0.440	0.12	(0.02–1.02)	0.052
	≥4 times/week	1 (1)	20 (8)	0.16	(0.02–1.19)	0.073	0.06	(0.01–0.57)	0.015
				(Trend $P = 0.041$)				(Trend $P = 0.009$)	

†Calculated by the conditional logistic regression model. ‡Model includes: duration from first identification of liver disease, body mass index at first identification of liver disease, severity of liver disease at first Osaka City University Hospital visit (ultrasonography score, platelet count, aspartate aminotransferase, albumin, α-fetoprotein, fasting blood sugar), family history of liver disease, and interferon therapy. CI, confidence intervals.

of a late evening meal with HCC across the groups, although valid estimates could not be calculated in the assessment of the ratio of aspartate to alanine aminotransferase of more than 1.0. As for the α-fetoprotein level, the relationship between a late evening meal and HCC was demonstrated with smaller OR among patients with a normal α-fetoprotein level. Regarding albumin level or obesity, inverse associations of a late evening meal with HCC were more pronounced in patients with an albumin level less than 4 g/dL and those with a body mass index less than 25 kg/m^2. Furthermore, the interaction between body mass index and a late evening meal for HCC was statistically significant ($P = 0.022$ for the homogeneity of OR). A late evening meal indicated a smaller OR for HCC risk irrespective of the absence or presence of a history of interferon therapy, although the relationship reached statistical significance only in patients without a history of interferon therapy.

The results from stratified analyses suggested that the interaction between a late evening meal and α-fetoprotein level, albumin level, or body mass index existed. In that case, it may be more appropriate that the interaction terms were included in the overall multivariate analyzes. Thus we conducted additional multivariate analyses in which each interaction term was added as an adjustment. When the interaction term between a late evening meal and α-fetoprotein was included in the multivariate analysis, OR of a late evening meal was almost similar with the results in Table 3 (OR, 0.02; 95% CI = 0.00–1.21; $P = 0.062$). Considering the interaction with albumin level, OR were nearly the same as the results in Table 3 (OR, 0.09; 95% CI = 0.01–0.62; $P = 0.014$). When we included the interaction term between a late evening meal and body mass index, the model did not converge because there was only one case who consumed a late evening meal and had a body mass index of less than 25 kg/m^2. Thus we could not simultaneously consider these three interactions. In order to consider these interactions, further large-scale studies are needed.

DISCUSSION

THE PRESENT RESULTS support the hypothesis that a late evening meal may decrease the risk of HCC. This finding is consistent with those of previous studies in which nutritional intervention was associated with a lowered recurrence rate of HCC among postoperative HCC patients.[18,19] In addition, a past experimental study indicated that higher administration of a nutritional

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Table 4 Adjusted odds ratio (OR) of late evening meal intake for hepatocellular carcinoma stratified according to selected potential confounders

Stratified category	Proportion of late evening meal intake		OR†‡ (95% CI)	*P*-value	Homogeneity of OR across stratified categories§
	Case n/N (%)	Control n/N (%)			
US score (Severity of liver disease)					
<3.5¶	0/20 (0)	27/162 (17)	- -	-	0.951
3.5+	6/51 (12)	19/90 (21)	0.39 (0.11–1.37)	0.142	
Ratio of aspartate to alanine aminotransferase					
<1.0	6/57 (11)	33/194 (17)	0.50 (0.17–1.47)	0.209	0.947
1.0+¶	1/16 (6)	13/59 (22)	- -	-	
Platelet count ($\times 10^4/\mu L$)					
10	4/42 (10)	36/225 (16)	0.36 (0.08–1.66)	0.191	0.563
<10	3/29 (10)	10/28 (36)	0.26 (0.04–1.53)	0.136	
α-Fetoprotein (ng/mL)					
20.0+	5/25 (20)	6/37 (16)	0.75 (0.10–5.62)	0.779	0.067
<20.0	2/44 (5)	37/194 (19)	0.02 (0.001–0.36)	0.007	
Albumin level (g/dL)					
4.0+	3/30 (10)	25/167 (15)	0.72 (0.16–3.23)	0.665	0.148
<4.0	4/43 (9)	21/86 (24)	0.13 (0.02–0.68)	0.016	
Body mass index (kg/m^2)					
25.0+	5/18 (28)	10/54 (19)	1.59 (0.22–11.2)	0.644	0.022
<25.0	1/53 (2)	36/198 (18)	0.05 (0.01–0.44)	0.008	
History of interferon therapy					
Absent	6/55 (11)	29/162 (18)	0.27 (0.08–0.99)	0.048	0.672
Present	1/18 (6)	17/91 (19)	0.15 (0.01–2.76)	0.203	

†Calculated by unconditional logistic regression model. ‡Model includes three matching factors (age, sex, and duration from first OCUH visit) and the following potential confounders other than stratified factor: body mass index at first identification of liver disease, severity of liver disease at first OCUH visit (US score, platelet count, aspartate aminotransferase, albumin, α-fetoprotein, fasting blood sugar), family history of liver disease, duration from first identification of liver disease, and interferon therapy. §Homogeneity of OR across stratified categories was tested as the *P*-value of the interaction term between a late evening meal and each stratified variable. ¶Model did not converge because there were no cases or too limited cases who consumed a late evening meal. CI, confidence intervals; OCUH, Osaka City University Hospital visit US score; ultrasonography score.

factor prevented human HCC cells from increasing.[33] Thus it seems reasonable to infer that a late evening meal has a protective effect against HCC.

It is important to clarify the optimal timing of nutritional support. Some studies have indicated that starting nutritional support in the early stage of cirrhosis may be useful in improving nutritional parameters.[34,35] In the present stratified analyses, the protective impact of a late evening meal was observed irrespective of the presence or absence of possible liver cirrhosis. The inverse effect of a late evening meal for HCC development was more pronounced among patients with an α-fetoprotein level of less than 20 ng/mL. About this association, we considered the following: (i) patients with a higher α-fetoprotein level might have a higher risk for HCC development. This background caused difficulty in the detection of the negative association with a late evening meal among these patients, but ease in determining the relationship among those with a normal α-fetoprotein level; (ii) potentially undetectable HCC cells might be developed among patients with a higher α-fetoprotein level. A late evening meal might no longer operate on the prevention of HCC among these patients. Contrary to this, the impact of late evening meal might be more easily demonstrated among those with a normal α-fetoprotein level. Further studies with larger study sizes are needed to corroborate these findings in order to consider the underlying mechanisms.

As for the interaction between a late evening meal and body mass index, the inverse associations of a late evening meal with HCC were further pronounced in

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patients with a body mass index less than 25 kg/m^2. It was recently indicated that obesity might be a risk factor of HCC development. Thus it brought about difficulties in the detection of the negative association with a late evening meal among the obesity group, but ease in demonstrating the decreasing OR of a late evening meal for HCC among patients with a body mass index less than 25 kg/m^2. A recent randomized, controlled trial among patients with decompensated liver cirrhosis demonstrated that the impact of BCAA in reducing the risk of liver cancer is superior to that of the ordinary food group among patients with a body mass index of more than 25 kg/m^2, although there was no difference in the risk of HCC between BCAA and ordinary food among those with a body mass index below 25 kg/m^2.[36] Taken together, these findings seem to indicate that a late evening meal has a preventive effect against HCC to the same extent as BCAA administration among patients who are not obese, while the effect of a late evening meal for HCC prevention is less than that of BCAA among those who are obese. It is therefore likely that BCAA and a late evening meal exert their effects by different mechanisms among patients who are obese.

The effect of a late evening meal was found to be statistically significant only in patients without a history of interferon therapy. However, point estimates of the effect of a late evening meal were similar in the absence or presence of a history of interferon. Thus decreased statistical power in the category of the presence of interferon therapy (i.e. only a small number of patients had experienced interferon therapy) might be responsible for the lack of statistical significance.

Regarding the mechanism of a late evening meal in HCC prevention, several previous studies indicated that malnutrition, including nocturnal starvation, is related to a poorer prognosis of liver cirrhosis[4–9] and that a late evening meal or BCAA supplement before bedtime improves protein–energy nutrition, imbalance of amino acids, or glucose tolerance.[13–15,17–19] In addition, some reports have indicated that a nibbling pattern of food intake, including a good breakfast and a late evening meal, would be preferable in order to have shorter episodes of catabolism during the day.[37–39] Some intervention studies have suggested that nutritional supplementation with oral BCAA is useful in preventing progressive hepatic failure and improving surrogate markers and perceived health status.[40–42] Thus it seems quite probable that a late evening meal acts to counteract malnutrition or nocturnal starvation, suppress the aggravation of liver disease, and as a result, prevent the development of HCC.

The strength of the present study is that the source population was restricted to patients with chronic type C liver disease, which enabled us to make a straightforward interpretation regarding any risk factors for HCV-associated HCC. In addition, we could analyze the data allowing for differences of background factors between the compared groups (e.g. severity of liver disease and the duration from first identification of liver disease).

However, due to the case-control study design within a very special population, that is, patients with chronic hepatitis C, the following three limitations may be present. First, selection bias might be introduced since the source population consisted of patients who had survived to the recruitment period. Patients who developed HCC but died before the recruitment period were not included in the case series, although cases were defined as those patients who had been first diagnosed with HCC in the recent past, that is, within 3 years. However, previous studies have reported that the mortality rate was significantly lower among a nutritional intervention group than among a placebo group.[40,43] It is therefore likely that patients without nutritional support have a higher risk of death. If, hypothetically speaking, cases excluded because of death had been included in this study, the prevalence of never consuming a late evening meal would increase in the hypothetical case series and the OR would decrease. Thus this selection bias may operate to bias the association toward the null, but not lead to exaggerated results.

A second limitation is an information bias resulting from imperfect memory of distant past history of consuming late evening meals. However, the hypothesis that a late evening meal is related to HCC or chronic liver disease was not generally recognized. Thus all patients would receive similar recall stimuli about past late evening meals. The misclassification due to such information bias, if any, is probably non-differential and would not affect the plausibility of the results.

Reverse causation is a third limitation for the observed association, although most retrospective studies suffer from this limitation. The habit of a late evening meal may change over time. However, this results of this study were interpreted from the information of a late evening meal at only one point without considering the potential changes in a late evening meal associated with liver dysfunction. Since more than 30 years may elapse between HCV infection and developing HCC, a late evening meal in the recent past may be affected by already manifested liver dysfunction. A long induction period in HCC can bring about the apparent causative associations, and exposure might be

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of importance only during an age-specific window or a specific time interval before diagnosis.

It is possible that other lifestyle characteristics can account for the protective effect of a late evening meal. However, we estimated the effect of late evening meal after correcting for the known HCC risk factors (liver disease severity, diabetes mellitus, family history, and interferon therapy) and for other putative confounders (duration of liver disease, and body mass index). In addition, similar results were obtained even when alcohol drinking and smoking were included in the analysis as additional potential confounders (data not shown). However, other uncontrolled factors might have affected the validity of our results. Previous studies indicated that riboflavin or vitamin B12 might reduce the risk of HCC.[44,45] One report indicated that some nutrients were positively associated with liver cirrhosis.[46] In addition, current guidelines define a late evening meal as a type of divided meal, and thus recommend fixing the total energy intake.[1,10] Due to the retrospective epidemiological analysis, a late evening meal in the present study could not be well characterized in terms of total energy intake as well as specific nutrients. Thus a late evening meal could be correlated with energy intake or specific nutrients.

In summary, this study showed a negative association between a late evening meal and HCC occurrence among patients with chronic hepatitis C. Further studies with larger study sizes are needed to corroborate these findings.

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REFERENCES

1 Plauth M, Merli M, Kondrop J, Weimann A, Ferenci P, Muller MJ. ESPEN guidelines for nutrition in liver disease and transplantation. *Clin Nutr* 1997; 16: 43–55.
2 Kondrup J, Muller MJ. Energy and protein requirements of patients with chronic liver disease. *J Hepatol* 1997; 27: 239–47.
3 Muller MJ, Lautz HU, Plogmann B, Burger M, Korber J, Schmidt FW. Energy expenditure and substrate oxidation in patients with cirrhosis: the impact of cause, clinical staging and nutritional state. *Hepatology* 1992; 15: 782–94.
4 Alberino F, Gatta A, Amodio P *et al.* Nutrition and survival in patients with liver cirrhosis. *Nutrition* 2001; 17: 445–50.
5 Tajika M, Kato M, Mohri H *et al.* Prognostic value of energy metabolism in patients with viral liver cirrhosis. *Nutrition* 2002; 18: 229–34.
6 Merli M, Riggio O, Dally L, PINC (Policentrica Italiana Nutrizione Cirrosi). Does malnutrition affect survival in cirrhosis? *Hepatology* 1996; 23: 1041–6.
7 O'Keefe SJ, El-Zayadi AR, Carraher TE, Davis M, Williams P. Malnutrition and immuno-incompetence in patients with liver disease. *Lancet* 1980; 20: 615–17.
8 Nakaya Y, Harada N, Kakui S *et al.* Severe catabolic state after prolonged fasting in cirrhotic patients: effect of oral branched-chain amino-acid-enriched nutrient mixture. *J Gastroenterol* 2002; 37: 531–6.
9 Moriwaki H, Tajika M, Miwa Y *et al.* Nutritional pharmacotherapy of chronic liver disease: from support of liver failure to prevention of liver cancer. *J Gastroenterol* 2000; 35 (Suppl. 12): 13–17.
10 ASPEN Board of Directors and the Clinical Guidelines Task Force. Guidelines for the use of parenteral and enteral nutrition in adult and pediatric patients. *JPEN* 2002; 26: 65SA–8.
11 Okamoto M, Sakaida I, Tsuchiya M, Suzuki C, Okita K. Effect of a late evening snack on the blood glucose level and energy metabolism in patients with liver cirrhosis. *Hepatol Res* 2003; 27: 45–50.
12 Donaghy A. Issues of malnutrition and bone disease in patients with cirrhosis. *J Gastroenterol Hepatol* 2002; 17: 462–6.
13 Fukushima H, Miwa Y, Ida E *et al.* Nocturnal branched-chain amino acid administration improves protein metabolism in patients with liver cirrhosis: comparison with daytime administration. *J Parenter Enteral Nutr* 2003; 27: 315–22.
14 Tsuchiya M, Sakaida I, Okamoto M, Okita K. The effect of a late evening snack in patients with liver cirrhosis. *Hepatol Res* 2005; 31: 95–103.
15 Yamauchi M, Takeda K, Sakamoto K, Ohata M, Toda G. Effect of oral branched chain amino acid supplementation in the late evening on the nutritional state of patients with liver cirrhosis. *Hepatol Res* 2001; 21: 199–204.
16 Muto Y, Sato S, Watanabe A *et al.* Effects of oral branched-chain amino acid granules on event-free survival in patients with liver cirrhosis. *Clin Gastroenterol Hepatol* 2005; 3: 705–13.
17 Chang WK, Chao YC, Tang HS, Lang HF, Hsu CT. Effect of extra-carbohydrate supplementation in the late evening on energy expenditure and substrate oxidation in patients with liver cirrhosis. *J Parenter Enteral Nutr* 1997; 21: 96–9.
18 Zillikens MC, Berg JWO, Wattimena JTD, Rietveld T, Swart GR. Nocturnal oral glucose supplementation. *J Hepatol* 1993; 17: 377–83.

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19 Miwa Y, Shiraki M, Kato M *et al.* Improvement of fuel metabolism by nocturnal energy supplementation in patients with liver cirrhosis. *Hepatol Res* 2000; 18: 184–9.
20 Nakaya Y, Okita K, Suzuki K *et al.* BCAA-enriched snack improves nutritional state of cirrhosis. *Nutrition* 2007; 23: 113–20.
21 Matsui Y, Uhara J, Satoi S *et al.* Improved prognosis of postoperative hepatocellular carcinoma patients when treated with functional foods: a prospective cohort study. *J Hepatol* 2002; 37: 78–86.
22 Muto Y, Moriwaki H, Ninomiya M *et al.*, for the Hepatoma Prevention Study Group. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. *N Eng J Med* 1996; 334: 1561–7.
23 Wada I, Hara T, Kajihara S *et al.* Population-based study of hepatitis C virus infection and hepatocellular carcinoma in western Japan. *Hepatol Res* 2002; 23: 18–24.
24 Ohfuji S, Fukushima W, Tanaka T *et al.* Coffee and reduced risk of hepatocellular carcinoma among chronic hepatitis C patients: a case-control study. *Hepatol Res* 2006; 36: 201–8.
25 Fukushima W, Tanaka T, Ohfuji S *et al.* Does alcohol increase the risk of hepatocellular carcinoma among patients with hepatitis C virus infection? *Hepatol Res* 2006; 34: 141–9.
26 Habu D, Nishiguchi S, Enomoto M *et al.* Ultrasonographic diagnosis of degree of chronic type C liver disease. *Hepatogastroenterology* 2005; 52: 1820–4.
27 Kurioka N, Asai H, Harihara S, Yamamoto S. Liver cirrhosis. *Kan Tan Sui* 1985; 10: 383–9.
28 Ohtake K. Evaluation of criteria on liver cirrhosis used for ultrasonic mass survey. *Osaka City Med J* 1991; 40: 173–94.
29 Ikeda K, Saitoh S, Kobayashi M *et al.* Distinction between chronic hepatitis and liver cirrhosis in patients with hepatitis C virus infection. Practical discriminant function using common laboratory data. *Hepatol Res* 2000; 18: 252–66.
30 Sheth SG, Flamm SL, Gordon FD, Chopra S. AST/ALT ratio predicts cirrhosis in patients with chronic hepatitis C virus infection. *Am J Gastroenterol* 1998; 93: 44–8.
31 Williams ALB, Hoofnagle JH. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. *Gastroenterol* 1988; 95: 734–9.
32 The Examination Committee of Criteria for 'Obesity Disease' in Japan, Japan Society for the Study of Obesity. New criteria for 'obesity disease' in Japan. *Circ J* 2002; 66: 987–92.
33 Sugiyama K, Yu L, Nagasue N. Direct effect of branched-chain amino acids on the growth and metabolism of cultured human hepatocellular carcinoma cells. *Nutr Cancer* 1998; 31: 62–8.
34 Nishiguchi S, Habu D. Effect of oral supplementation with branched-chain amino acid granules in the early stage of cirrhosis. *Hepatol Res* 2004; 30 (Suppl.): 36–41.
35 Habu D, Nishiguchi S, Nakatani S *et al.* Effect of oral supplementation with branched-chain amino acid granules on serum albumin level in the early stage of cirrhosis: a randomized pilot trial. *Hepatol Res* 2003; 25: 312–18.
36 Muto Y, Sato S, Watanabe A *et al.* Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. *Hepatol Res* 2006; 35: 204–14.
37 Marchesini G, Bianchi G, Rossi B, Brizi M, Melchionda N. Nutritional treatment with branched-chain amino acids in advanced liver cirrhosis. *J Gastroenterol* 2000; 35 (Suppl. 12): 7–12.
38 Swart GR, Zillikens MC, Vuure JK, Berg JWO. Effect of a late evening meal on nitrogen balance in patients with cirrhosis of the liver. *BMJ* 1989; 299: 1202–3.
39 WPHG Venne V, Westerterp KR, Hoek B, Swart GR. Energy expenditure and substrate metabolism in patients with cirrhosis of the liver: effects of the pattern of food intake. *Gut* 1995; 36: 110–16.
40 Marchesini G, Bianchi G, Merli M *et al.* for the Italian BCAA Study Group. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. *Gastroenterology* 2003; 124: 1792–801.
41 Marchesisni G, Dioguardi FS, Bianchi GP *et al.* and the Italian Multicenter Study Group. Long-term oral branched-chain amino acid treatment in chronic hepatic encephalopathy: a randomized double-blind casein-controlled trial. *J Hepatol* 1990; 11: 92–101.
42 The San-In Group of Liver Surgery. Long-term oral administration of branched chain amino acids after curative resection of hepatocellular carcinoma: a prospective randomized trial. *Br J Surg* 1997; 84: 1525–31.
43 Yoshida T, Muto Y, Moriwaki H, Yamato M. Effect of long-term oral supplementation with branched-chain amino acid granules on the prognosis of liver cirrhosis. *Gastroenterol Jpn* 1989; 24: 692–8.
44 Corrao G, Torchio P, Zambon A, D'Amicis A, Lepore AR, Orio F, The Provincial Group for The Study of Chronic Liver Disease. Alcohol consumption and micronutrient intake as risk factors for liver cirrhosis: a case-control study. *Ann Epidemiol* 1998; 8: 154–9.
45 Habu D, Shiomi S, Tamori A *et al.* Role of vitamin K2 in the development of hepatocellular carcinoma in women with viral cirrhosis of the liver. *JAMA* 2004; 292: 358–61.
46 Corrao G, Zambon A, Bagnardi V *et al.* Nutrient intakes, nutritional patterns and the risk of liver cirrhosis: an explorative case-control study. *Eur J Epidemiol* 2004; 19: 861–9.

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Short Communication

Optimal duration of additional therapy after biochemical and virological responses to lamivudine in patients with HBeAg-negative chronic hepatitis B: a randomized trial

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Aim: The endpoint of treatment with nucleoside analogs remains unclear for patients with hepatitis B e antigen (HBeAg)-negative chronic hepatitis B. We report the results of a randomized trial to determine the optimal duration of additional therapy after response to lamivudine in HBeAg-negative patients.

Methods: Twenty-two patients with HBeAg-negative chronic hepatitis B who exhibited biochemical and virological responses to lamivudine were enrolled. When patients responded to treatment, they were randomly assigned to receive 12 more months of therapy (Group A, 11 patients) or 24 more months of therapy (Group B, 11 patients).

Results: The baseline characteristics of the patients were similar in the two groups. Biochemical and virological responses were obtained in all patients within 6 months. Drug resistance developed in one patient in Group A during month 7 of additional therapy, and in five patients in Group B from months 13–23 of additional therapy. Ten patients in Group A and six in Group B completed the protocol and were included in analysis. Eight of the 10 patients in Group A experienced relapse between months 2 and 14 after the discontinuation of therapy, while three of the six patients in Group B experienced relapse between months 2 and 24. There was no difference in cumulative relapse rate between the groups ($P = 0.275$).

Conclusion: Additional therapy with lamivudine for longer than 12 months after biochemical and virological responses in patients with HBeAg-negative chronic hepatitis B could increase the risk of drug resistance, but did not reduce the rate of relapse.

Key words: HBeAg-negative chronic hepatitis B, hepatitis B virus, lamivudine, YMDD variant

INTRODUCTION

INFECTION WITH HEPATITIS B virus (HBV) affects more than 350 million people worldwide.[1,2] Seroconversion from hepatitis B e antigen (HBeAg) to anti-HBe usually represents a transition from chronic hepatitis to an inactive carrier state with normal alanine aminotransferase (ALT) and decreased HBV-DNA levels. However, in a portion of patients with HBV variants with mutations in the precore or core promoter regions that abolish or downregulate HBeAg synthesis,[3,4] serum ALT and HBV-DNA levels sometimes remain persistently elevated even after HBeAg seroconversion. Sustained spontaneous remission is uncommon in such patients with HBeAg-negative chronic hepatitis B.

Currently available antiviral therapy for chronic hepatitis B includes interferon[5,6] and nucleos(t)ide analogs, such as lamivudine,[7–10] adefovir dipivoxil[11,12] and entecavir.[13,14] Although interferon-induced remission of chronic hepatitis B is durable, it is achieved in only a minority of patients. In contrast, treatment with nucleos(t)ide analogs induces biochemical and virological responses in a majority of patients, but viral relapse and

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exacerbations of hepatitis are common after discontinuation of treatment. One possible approach to reducing the risk of relapse is extending the duration of treatment.

However, a major concern with the long-term use of nucleos(t)ide analogs is the selection of antiviral-resistant mutations in the reverse transcriptase (rt) domain of the polymerase gene. For instance, mutations at amino acid rt204 in the tyrosine–methionine–aspartate–aspartate (YMDD) motif are associated with resistance to some nucleoside analogs.[15–17] The decision of how long to continue treatment after response to therapy should be based on a careful assessment of the balance between the likelihood of sustained remission and the risk of development of drug resistance.

Lamivudine was the first approved oral nucleoside analog for the treatment of chronic hepatitis B. The recently updated guidelines recommend that, in HBeAg-positive patients, lamivudine treatment be continued for an additional 6–12 months after seroconversion to anti-HBe.[18,19] However, the ideal endpoint of treatment remains unclear for HBeAg-negative patients. One reason for this is that the endpoint is difficult to assess, since normal ALT and undetectable HBV–DNA levels are the only practical criteria for determination of it, rather than HBeAg seroconversion. Another reason is that longer term treatment is needed for HBeAg-negative patients because of the high rates of post-treatment relapse.[20]

We report here the results of a randomized trial to determine the optimal duration of additional therapy after response to lamivudine in patients with HBeAg-negative chronic hepatitis B. Patients with HBeAg-negative chronic hepatitis B who had biochemical and virological responses to lamivudine were randomly assigned to receive 12 or 24 more months of therapy.

METHODS

Patients

BETWEEN SEPTEMBER 2000 and May 2004, 22 patients (16 men and 6 women; mean age, 49 ± 11 years) with HBeAg-negative chronic hepatitis B who exhibited biochemical and virological responses to lamivudine therapy were enrolled in this study. The inclusion criteria were as follows: (1) persistent elevation of serum ALT levels for at least 6 months before the start of therapy; (2) presence of hepatitis B surface antigen; (3) absence of HBeAg and presence of anti-HBe; (4) presence of HBV–DNA > 10^5 copies/ml; (5) no use of immunomodulatory drugs, including interferon, within one year before the start of therapy; (6) no previous use of nucleos(t)ide analogs; (7) absence of other likely causes of chronic liver disease; and (8) no clinical signs of decompensated cirrhosis or hepatocellular carcinoma. The procedures of the study accorded with the Helsinki Declaration of 1975 (2000 revision) and were approved by the Ethics Committee of the Osaka City University Medical School.

Study protocol

Lamivudine was given orally in a dose of 100 mg once daily. After lamivudine therapy was started, serum ALT and HBV–DNA levels were tested every 1–2 months. When patients exhibited biochemical and virological responses, they were randomly assigned to one of two groups: (1) 11 were assigned to receive 12 more months of therapy (Group A); and (2) 11 were assigned to receive 24 more months of therapy (Group B). Biochemical response was defined as a decrease in serum ALT levels to within the reference range. Virological response was defined as an undetectable HBV–DNA level on polymerase chain reaction (PCR) testing. The endpoint for analysis was relapse, defined as increase in serum ALT to >2× the upper limit of normal and increase in HBV–DNA to >10^5 copies/ml.

Assays

Hepatitis B surface antigen, HBeAg and anti-HBe were detected by chemiluminescence enzyme immunoassay. Genotypes of HBV were identified by enzyme-linked immunosorbent assay (Institute of Immunology, Tokyo, Japan).[21] The mutations at nucleotide (nt) 1896 in the precore region, and at nt 1762 and nt 1764 in the basal core promoter region of HBV–DNA were detected by enzyme-linked minisequence assay (Genome Science Laboratory, Tokyo, Japan). HBV–DNA was measured by transcription-mediated amplification assay (Chugai Diagnostics, Tokyo, Japan).[22] The range of detection of the assay was between 3.7 and 8.7 $\log_{10}$ copies/ml of HBV–DNA. If HBV–DNA was not detected by this method, a PCR-based Amplicor Monitor test (Roche Molecular Systems, Pleasanton, CA, USA)[23] was utilized. The range of detection of the assay was between 2.6 and 7.6 $\log_{10}$ copies/ml. When virological breakthrough – defined as one $\log_{10}$ increase in HBV–DNA during continuous treatment – was observed, the mutations in the YMDD motif of the polymerase gene were examined by a line probe assay (INNO-LiPA HBV DR; Innogenetics NV, Ghent, Belgium).[24]

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Table 1 Baseline characteristics of enrolled patients with HBeAg-negative chronic hepatitis B

	Group A ($n = 11$)	Group B ($n = 11$)	*P*-value
Age (year)	52 ± 9	47 ± 12	0.412
Sex (male/female)	9/2	6/5	0.311
ALT (IU/l)	85 (40–545)	203 (53–612)	0.412
HBV genotype (A/B/C)	0/1/10	1/1/9	0.591
Precore (wild/mixed/mutant)	2/1/8	1/4/6	0.298
Core promoter (wild/mixed/mutant)	3/1/7	2/0/9	0.484
HBV-DNA (log_{10} copies/ml)	6.1 (4.9–7.6)	7.1 (3.7–8.1)	0.431
Grade of inflammation (mild/moderate/severe)	4/3/1	4/1/0	0.385
Stage of fibrosis (mild/moderate/severe/cirrhosis)	1/1/4/2	1/0/3/1	0.565

Values are means ± SD for normally distributed variables, and medians (range) for non-normally distributed variables.
ALT, alanine aminotransferase; HBV, hepatitis B virus.

Histopathology

After informed consent had been obtained, liver biopsy was undertaken within 6 months before the start of therapy. Histopathologic findings were assessed by grading of inflammatory activity and staging of fibrosis using the classification of Desmet *et al.*[25]

Statistical analysis

Statistical analysis was performed with the Statview SE+Graphics program, version 5.0 (SAS Institute, Cary, NC, USA). Distributions of continuous variables were analyzed by the Mann-Whitney *U*-test. Differences in proportions were tested by Fisher's exact test. Cumulative incidences were plotted using the Kaplan–Meier method, and the significance of differences was determined using the log–rank test. A two-tailed *P*-value less than 0.05 was considered significant.

RESULTS

Baseline characteristics of patients

THE BASELINE CHARACTERISTICS of the 22 patients with HBeAg-negative chronic hepatitis B included in this study are shown in Table 1. Liver biopsy was not performed in eight of the 22 patients, because informed consent could not be obtained. There were no significant differences between groups A and B with respect to mean age, sex ratio, serum ALT activity, proportions of HBV genotypes, serum HBV-DNA level, or histological findings for the liver.

Flow of participants though the trial

The flow of participants though the trial is shown in Figure 1. Biochemical and virological responses were obtained from all patients within 6 months. Drug-resistant YMDD variants emerged in one patient in Group A during month 7 of additional therapy and in five patients in Group B from months 13–23 of additional therapy. These patients were treated with adefovir dipivoxil in addition to lamivudine, and excluded from analysis. Ten patients in Group A and six in Group B

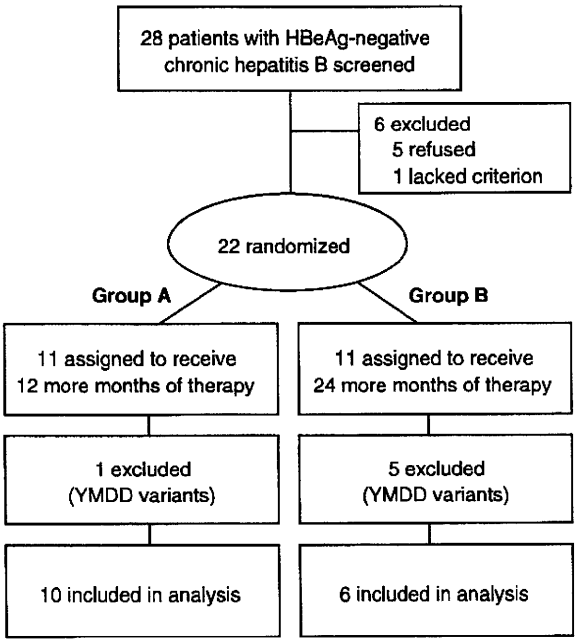

Figure 1 Flow of participants though the trial. Ten patients in Group A and six in Group B completed the study according to the protocol, and were included in analysis.
YMDD, tyrosine-methionine-aspartate-aspartate.

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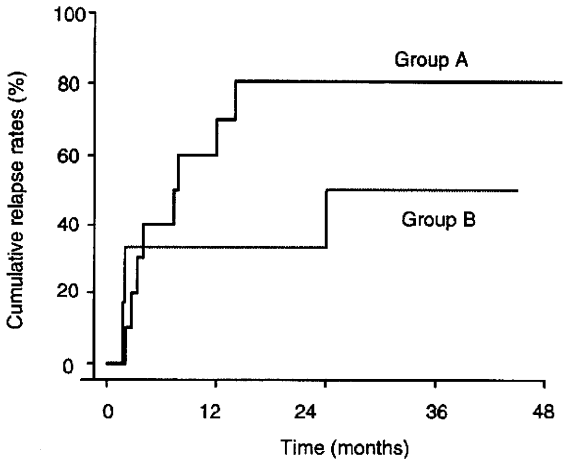

Figure 2 Cumulative rates of post-treatment relapse by duration of additional therapy after biochemical and virological responses to lamivudine. There was no significant difference in cumulative post-treatment relapse rates between the groups according to the duration of therapy ($P = 0.275$). Log-rank test.

completed the study according to the protocol, and were included in analysis.

Cumulative rates of post-treatment relapse

The cumulative rates of post-treatment relapse are shown by duration of additional therapy in Figure 2. Eight of the 10 patients in Group A experienced relapse between months 2 and 14 after discontinuation of therapy, while three of the six patients in Group B experienced relapse between months 2 and 24. There was no difference in cumulative relapse rate between the groups ($P = 0.275$). Reinstitution of lamivudine resulted in prompt responses in the patients with relapse, since drug resistance had not developed.

DISCUSSION

IN PREVIOUS LARGE multicenter trials, lamivudine treatment resulted in biochemical, virological and histological improvement,[7–9] as well as reducing the incidence of hepatic decompensation and hepatocellular carcinoma.[26] Currently, limited findings are available on the long-term efficacy of newer analogs in preventing the progression of liver disease to cirrhosis and hepatocellular carcinoma.

In HBeAg-positive chronic hepatitis B, a study in non-Asian countries found that 30 of 39 (77%) patients who experienced HBeAg seroconversion during 12-month lamivudine treatment had a durable response during a median follow-up of 37 months.[27] However, studies from Korea reported lower rates of durability (50–60%).[28,29] This discrepancy may be due, at least in part, to differences in viral genotypes among study populations.[30,31] In Asian countries, the most prevalent type is genotype C, which is associated with more progressive liver disease and a low likelihood of a sustained response to antivirals. In HBeAg-negative chronic hepatitis B, durable response during 12-month follow-up after the end of 12-month lamivudine treatment was found in only 2 of 15 (13%) patients.[20]

How long treatment with nucleos(t)ide analogs should be continued after response to treatment has yet to be tested in a randomized trial. Previous open trials suggested that long-term therapy with lamivudine increases rates of durable response. Fung *et al.*[32] reported that rates of durable virological response were increased to 50%, 70% and 50% at 6, 12, and 18 months after discontinuation of treatment, respectively, in HBe-negative patients who had completed 2 years of treatment and had persistently undetectable HBV-DNA on PCR testing during the second year. Ryu *et al.*[33] reported similar results for HBe-positive patients. However, virological breakthrough was found in 12–25% of the patients included in these studies during the 12–24 months of additional therapy.

The incidence of resistance to lamivudine increases with the duration of treatment: 24% at 1 year, increasing to 70% at 4 years.[8] Patients with detectable HBeAg and high HBV-DNA levels at baseline are at high risk for the emergence of drug-resistant variants.[34,35] In addition, patients who had a slow decrease in viral loads after the initiation of treatment are more likely to develop drug resistance. However, Kurashige *et al.*[36] reported that the cumulative rates of resistance to lamivudine were 4% at 1 year and 25% at 2 years of therapy, even when the initial viral response was achieved at 6 months. The benefits of extended therapy must thus be balanced against the risk of drug-resistant mutation.

Our randomized trial showed that additional therapy with lamivudine for longer than 12 months after biochemical and virological responses in patients with HBeAg-negative chronic hepatitis B could increase the risk of drug resistance, but did not reduce the rate of relapse. One limitation of this study was the small number of patients included. The cumulative rate of post-treatment relapse was lower in patients assigned to

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Group B than in patients assigned to Group A (Fig. 2). However, this difference was not statistically significant, and our analysis excluded patients in whom drug resistance developed during additional therapy. Overall, sustained remission after discontinuation of lamivudine therapy was observed at similar rates in the two groups (2 of 11 in Group A and 3 of 11 in Group B). Even larger studies would be unable to demonstrate the benefit of additional therapy for longer than 12 months.

Among the nucleos(t)ide analogs approved for use in treating hepatitis B, lamivudine is associated with the highest and entecavir with the lowest rate of drug resistance (<1% at year 4) in nucleos(t)ide-naïve patients.[37] Entecavir is the nucleoside analog most potent against HBV, and has exhibited superiority to lamivudine in randomized controlled trials.[13,14] The use of new analogs with potent antiviral effects and low rates of resistance could make it possible to extend the duration of therapy, which might reduce the risk of post-treatment relapse. Further studies are needed to determine the optimal duration of additional therapy after response to new analogs.

In lamivudine-refractory patients with YMDD variants, resistance to entecavir occurred more frequently (15% at year 4) due to an additional substitution (at rt169, rt184, rt202, or rt250) in the polymerase gene.[37] Drug-resistant mutations limit subsequent treatment options because of cross-resistance. Based on the results of this study, we recommend that lamivudine should be switched to entecavir before the emergence of YMDD variants, or be discontinued 12 months after response to therapy. Entecavir is the treatment of choice when relapse occurs.

In conclusion, this randomized trial showed that additional therapy with lamivudine for longer than 12 months after biochemical and virological response in patients with HBeAg-negative chronic hepatitis B can increase the risk of drug resistance, but did not reduce the rate of relapse. The optimal duration of therapy, with which both the risk of development of drug resistance during additional therapy and the risk of relapse after discontinuation of therapy are reduced, was not established, because of the high incidence of drug resistance and insufficient antiviral potency when lamivudine was used.

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REFERENCES

1 Lai CL, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. *Lancet* 2003; **362**: 2089–94.
2 Ganem D, Prince AM. Hepatitis B virus infection – natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118–29.
3 Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N Engl J Med* 1991; **324**: 1699–704.
4 Okamoto H, Tsuda F, Akahane Y *et al.* Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol* 1994; **68**: 8102–10.
5 Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Intern Med* 1993; **119**: 312–23.
6 Niederau C, Heintges T, Lange S *et al.* Long-term follow-up of HBeAg-positive patients treated with interferon alpha for chronic hepatitis B. *N Engl J Med* 1996; **334**: 1422–7.
7 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61–8.
8 Liaw YF, Leung NW, Chang TT *et al.* Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *Gastroenterology* 2000; **119**: 172–80.
9 Lau DT, Khokhar MF, Doo E *et al.* Long-term therapy of chronic hepatitis B with lamivudine. *Hepatology* 2000; **32**: 828–34.
10 Kohmoto M, Enomoto M, Yano Y *et al.* Detection of serum hepatitis B virus DNA by real-time quantitative polymerase chain reaction (TaqMan PCR) during lamivudine treatment: comparison with three other assays. *Hepatol Res* 2003; **26**: 125–33.
11 Hadziyannis SJ, Tassopoulos NC, Heathcote EJ *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. *N Engl J Med* 2003; **348**: 800–7.
12 Enomoto M, Nishiguchi S, Seki S, Yamane T, Hino M. Adefovir dipivoxil to prevent exacerbation of lamivudine-resistant hepatitis B infection during chemotherapy for non-Hodgkin's lymphoma. *Am J Gastroenterol* 2004; **99**: 1619–20.
13 Chang TT, Gish RG, de Man R *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; **354**: 1001–10.

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14 Lai CL, Shouval D, Lok AS *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; **354**: 1011–20.
15 Ling R, Mutimer D, Ahmed M *et al.* Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* 1996; **24**: 711–13.
16 Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. *Hepatology* 1996; **24**: 714–17.
17 Enomoto M, Tamori A, Kohmoto MT *et al.* Mutational patterns of hepatitis B virus genome and clinical outcomes after emergence of drug-resistant variants during lamivudine therapy: analyses of the polymerase gene and full-length sequences. *J Med Virol* 2007; **79**: 1664–70.
18 Keeffe EB, Dieterich DT, Han SH *et al.* A treatment algorithm for the management of chronic hepatitis B virus infection in the United States: an update. *Clin Gastroenterol Hepatol* 2006; **4**: 936–62.
19 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
20 Santantonio T, Mazzola M, Iacovazzi T, Miglietta A, Guastadisegni A, Pastore G. Long term follow-up of patients with anti-HBe/HBV-DNA-positive chronic hepatitis B treated for 12 months with lamivudine. *J Hepatol* 2000; **32**: 300–6.
21 Usuda S, Okamoto H, Iwanari H *et al.* Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. *J Virol Methods* 1999; **80**: 97–112.
22 Ide T, Kumashiro R, Hino T *et al.* Transcription-mediated amplification is more useful in the follow-up of patients with chronic hepatitis B treated with lamivudine. *Hepatol Res* 2001; **21**: 76–84.
23 Gerken G, Gomes J, Lampertico P *et al.* Clinical evaluation and applications of the Amplicor HBV Monitor test, a quantitative HBV-DNA PCR assay. *J Virol Methods* 1998; **74**: 155–65.
24 Stuyver L, Van Geyt C, De Gendt S *et al.* Line probe assay for monitoring drug resistance in hepatitis B virus-infected patients during antiviral therapy. *J Clin Microbiol* 2000; **38**: 702–7.
25 Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading, and staging. *Hepatology* 1994; 19: 1513–20.
26 Matsumoto A, Tanaka E, Rokuhara A *et al.* Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2795 patients. *Hepatol Res* 2005; **32**: 173–84.
27 Dienstag JL, Cianciara J, Karayalcin S *et al.* Durability of serologic response after lamivudine treatment of chronic hepatitis B. *Hepatology* 2003; **37**: 748–55.
28 Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. *Hepatology* 2000; **32**: 803–6.
29 Lee KM, Cho SW, Kim SW, Kim HJ, Hahm KB, Kim JH. Effect of virological response on post-treatment durability of lamivudine-induced HBeAg seroconversion. *J Viral Hepat* 2002; **9**: 208–12.
30 Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology* 2003; **46**: 329–38.
31 Enomoto M, Tamori A, Nishiguchi S. Hepatitis B virus genotypes and response to antiviral therapy. *Clin Lab* 2006; **52**: 43–7.
32 Fung SK, Wong F, Hussain M, Lok AS. Sustained response after a 2-year course of lamivudine treatment of hepatitis B e antigen-negative chronic hepatitis B. *J Viral Hepat* 2004; **11**: 432–8.
33 Ryu SH, Chung YH, Choi MH *et al.* Long-term additional lamivudine therapy enhances durability of lamivudine-induced HBeAg loss: a prospective study. *J Hepatol* 2003; **39**: 614–19.
34 Yuen MF, Sablon E, Hui CK, Yuan HJ, Decraemer H, Lai CL. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. *Hepatology* 2001; **34**: 785–91.
35 Suzuki F, Tsubota A, Arase Y *et al.* Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. *Intervirology* 2003; **46**: 182–9.
36 Kurashige N, Hiramatsu N, Ohkawa K *et al.* Initial viral response is the most powerful predictor of the emergence of YMDD mutant virus in chronic hepatitis B patients treated with lamivudine. *Hepatol Res* 2008; **38**: 450–6.
37 Colonno RJ, Rose RE, Pokornowski K *et al.* Four year assessment of entecavir resistance in nucleoside naïve and lamivudine refractory patients. *J Hepatol* 2007; **46** (Suppl 1): 294.

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CLINICAL STUDIES

Differences in molecular alterations of hepatocellular carcinoma between patients with a sustained virological response and those with hepatitis C virus infection

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Abstract

Background/Aims: The mechanism of hepatocarcinogenesis remains unclear in patients in whom hepatitis C virus (HCV) disappears after interferon (IFN) therapy. We compared molecular alterations in hepatocellular carcinoma (HCC) between patients with a sustained virological response (SVR) to IFN and patients with HCV. *Methods:* The study group comprised 44 patients with HCV and 13 patients with SVR. One patient in the SVR group had two tumour nodules, both of which were examined. Mitochondrial DNA (mtDNA) mutations in displacement-loop lesions were directly sequenced. Mutation of the TP53 gene was examined by direct sequencing. The methylation status of *p16, p15, p14, RB* and *PTEN* genes was evaluated by a methylation-specific polymerase chain reaction. *Results:* The average number of mtDNA mutations was 4.2 in 44 HCCs with HCV and 2.0 in 14 HCCs with SVR ($P = 0.0021$). mtDNA mutation was less frequently detected in HCCs from patients with SVR than in patients with HCV. TP53 mutations were detected in 12 (27%) of 44 HCCs with HCV and 2 (14%) of 14 SVR-HCCs. Hypermethylation of the *p16, p15, p14, RB* and *PTEN* promoters was, respectively, detected in 34, 13, 8, 12 and 11 of 44 HCCs from patients with HCV and 14, 0, 0, 2 and 2 of 14 HCCs from patients with SVR ($P = 0.049$, 0.021, 0.085, 0.322 and 0.402). Hypermethylation of *p16* was one of the most important alterations in SVR-HCC. *Conclusions:* Molecular alterations in hepatocarcinogenesis of patients with SVR-HCC were different from those of patients with continuous HCV infection.

Hepatitis C virus (HCV) is one of the most important risk factors for hepatocellular carcinoma (HCC). Clinical studies have suggested that HCV induces inflammation in the liver, followed by the accumulation of reactive oxygen species (ROS), which promote mutations in the human genome (1, 2). Persistent inflammation also results in repeated hepatocyte death and regeneration, leading to the gradual accumulation of DNA mutations in hepatocytes. Point mutations in tumour suppressor genes, including TP53, have been confirmed in hepatic cirrhosis in patients with HCV (3). Epigenetic alterations, such as methylation of the promoter of cell cycle gene inhibitors with the resulting loss of its expression, have been frequently detected in liver cirrhosis with viral infection (4, 5). Continuous inflammation induces genetic or epigenetic alterations, or both, in hepatocytes, culminating in a preneoplastic condition. HCV itself is an oncogenic virus. HCV core protein or HCV NS5A protein has oncogenic potential function in animal models without inflammation (6, 7). *In vitro* studies have suggested that HCV protein modifies host immunity to sustain infection (8). The suppression of immunological response is attributed to the failure to eliminate neoplastic cells from the liver. These findings suggest that cooperation between virus-induced chronic inflammation and HCV coding proteins accelerates carcinogenesis in the liver.

Interferon (IFN) has potent antiviral activity against HCV. Antiviral therapy with pegylated IFN in combination with ribavirin produces a sustained virological response (SVR) in approximately 60% of patients with chronic hepatitis C (9, 10). Complete eradication of HCV by antiviral therapy is associated with a considerable reduction in the incidence of HCC (11, 12). Nevertheless, recent studies have shown that HCC develops in 2.5–4.2% of patients after eradication of HCV by IFN therapy (13–15). It is therefore important to delineate important features of HCC that develop after the elimination of HCV as compared with those established during sustained HCV infection. Makiyama *et al.* (15) speculated that cancer cells already exist in the liver before HCV eradication by IFN treatment. The integration of HBV DNA because of past HBV infections (16) or occult HCV infections (17) may be linked to SVR-HCC. However, the molecular mechanism leading to the development of SVR-HCC remains obscure.

In the present study, we compared genetic alterations in surgically resected specimens of HCCs between patients with SVR and those with continuous HCV infection. Our results might contribute to a better understanding of the molecular changes in

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the liver of patients in whom HCC develops after the eradication of HCV.

Patients and methods

Patients

Thirteen consecutive patients who underwent surgical resection of HCC in Osaka City University Hospital after eradication of HCV by IFN monotherapy from 1998 June through 2007 July (SVR group) were studied (Table 1). One patient in the SVR group had two tumour nodules, both of which were examined. As a control, 44 HCV-RNA-positive patients with HCC were studied. Thus, 58 HCC samples and 57 noncancerous tissue samples were evaluated. One portion of each sample was frozen in liquid nitrogen immediately after resection and stored at − 80 °C until analysis. Total RNA and DNA were extracted from these portions by conventional methods as described previously (18). None of the patients had a history of exposure to aflatoxin B1, more than 30 g/day of alcohol intake, insulin administration, hereditary haemochromatosis or other liver diseases such as hepatitis B, autoimmune hepatitis and primary biliary cirrhosis. The activity of hepatitis and stage of fibrosis were determined according to a modified version of Desmet's classification in liver tissue specimens before IFN therapy and in noncancerous liver tissue obtained intra-operatively (19).

Table 1. Clinical characteristics of patients with sustained virological response-hepatocellular carcinoma and hepatitis C virus-hepatocellular carcinoma

	SVR-HCC	HCV-HCC	*P*-value
n	13	44	
Male/female	13/0	44/0	
Age	64.3 (55–73)	64.0 (34–79)	0.977
Anti-HCV(+)/ HCV-RNA(+)	13/0	44/44	
HBs antigen positivity	0	0	
IFN therapy	13	0	
ALT (IU/L)	35.0 (17–81)	73.2 (13–188)	0.0001
Diabetes mellitus With/without/ unknown	2/11/0	13/28/3	0.25
Alcohol habits Positive/negative/ unknown	5/8/0	23/17/4	0.23
Tumour differentiation Well/moderately/ poorly	0/4/10	5/20/19	0.066
Noncancerous liver Cirrhosis/noncirrhosis	4/9	20/24	0.34
Tumour diameter (mm) (average)	43.1 (12–125)	38.3 (10–180)	0.756
Extrahepatic metastasis	0	0	

ALT, alanine aminotransferase; HBs antigen, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; SVR, sustained virological reaction.

Sequencing the displacement-loop region of mitochondrial DNA

Each DNA sample (50 ng) was subjected to amplification by polymerase chain reaction (PCR) with the use of overlapping sets of primers to screen the entire mitochondrial genome. To avoid coamplification of nuclear pseudogenes, the primers were selected with the use of mitochondrial DNA (mtDNA)-depleted cells established as described previously (2, 20). PCR (an initial incubation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min) was performed in a final volume of 50 µl with a GeneAmp PCR system 9600 (Perkin-Elmer Life Sciences Japan, Tokyo, Japan). Aberrant PCR products were purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and sequenced with an Applied Biosystems DNA sequencer (Perkin-Elmer Life Sciences Japan) and a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Tokyo, Japan). The sequence of the displacement (D)-loop (nucleotides 100–600) was examined for all 57 patients with HCC. All mutations were confirmed by repeated DNA sequencing.

Direct sequencing for TP53

We directly sequenced exons 5 through 8 of TP53 genes, in which 98% of TP53 mutations are detected (21), in 58 tumours. One hundred nanograms of genomic DNA was subjected to 35 PCR cycles (94, 55 and 72 °C for 0.5, 0.5 and 1 min respectively) with rTaq DNA polymerase (TakaraBio Co. Ltd, Otsu, Japan). After the PCR products were purified with a QIAquick PCR purification kit, we sequenced the amplified products with a DNA sequencing system and a Dye Terminator Cycle Sequencing FS Ready Reaction kit.

Methylation-specific polymerase chain reaction

Bisulphite modification of genomic DNA was performed as described by Herman *et al.* (22). Briefly, 1 µg of DNA was denatured with NaOH, and 10 mM hydroquinone and 3 M sodium bisulphite were successively added to the mixture. The sample was incubated at 50 °C for 16 h. Modified DNA was purified with the use of Wizard DNA purification resin (Promega Corporation, Madison, WI, USA), followed by ethanol precipitation. DNA methylation patterns were determined by chemical modification of the unmethylated cytosines to uracil and subsequent PCR, using primers specific for either methylated or modified unmethylated DNA. The primers used in this study are shown in Table 2 (23, 24). The PCR amplification procedure has been described previously (5). Ten microlitres of each PCR product was loaded directly onto nondenaturing 2% agarose gels, stained with ethidium bromide and visualized under ultraviolet illumination.

Semiquantitative reverse-transcription polymerase chain reaction analysis

To investigate *p16* mRNA expression, we performed reverse-transcription PCR (RT-PCR) with total RNA from 35 tumours and 27 noncancerous lesions. Briefly, 1 µg of the RNA was used as a template to generate complementary DNA (cDNA) using random hexamers and reverse transcriptase. The cDNA was used for PCR amplification. Primer sequences were 5′-CCACCCCGC TTTCGTAGTTTT-3′ (upper primer) and 5′-TGCGAGGCTCG CAAGAAAT-3′ (lower primer) for *p16* and 5′-CCTCGCCTT TGCCGATCC-3′ (upper primer) and 5′-GGATCTTCATGAGG TAGTCAGTC-3′ (lower primer) for β-actin. The PCR procedure for *p16* consisted of one cycle at 95 °C for 12 min, 30 cycles at 95 °C for 30 s, 51 °C for 1 min and 72 °C for 30 s, and one cycle at

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Table 2. Primers used for methylation-specific polymerase chain reaction

Gene		Sequence
p16	Unmethylated	5′-TTATTAGAGGGTGGGGTGGATTGT-3′ (sense)
		5′-CAACCCCAAACCACAACCATAA-3′ (antisense)
	Methylated	5′-TTATTAGAGGGTGGGGCGGATCGC-3′ (sense)
		5′-GACCCCGAACCGCGACCGTAA-3′ (antisense)
p15	Unmethylated	5′-TGTGATGTGTTTGTATTTTGTGGTT-3′ (sense)
		5′-CCATACAATAACCAAACAACCAA-3′ (antisense)
	Methylated	5′-GCGTTCGTATTTTGCGGTT-3′ (sense)
		5′-CGTACAATAACCGAACGACCGA-3′ (antisense)
p14	Unmethylated	5′-TTTTTGGTGTTAAAGGGTGGTGTAGT-3′ (sense)
		5′-CACAAAAACCCTCACTCACAACAA-3′ (antisense)
	Methylated	5′-GTGTTAAAGGGCGGCGTAGC-3′ (sense)
		5′-AAAACCCTCACTCGCGACGA-3′ (antisense)
RB		5′-CTTTGTATAGCCCCGTTAAGTG-3′ (sense)
		5′-GTCATGAGGAATTAAACTGGGA-3′ (antisense)
PTEN	Unmethylated	5′-GTGTTGGTGGAGGTAGTTGTTT-3′ (sense)
		5′-ACCACTTAACTCTAAACCACAACCA-3′ (antisense)
	Methylated	5′-TTCGTTCGTCGTCGTCGTATTT-3′ (sense)
		5′-GCCGCTTAACTCTAAACCGCAACCG-3′ (antisense)

72 °C for 3 min. That for β-actin consisted of one cycle at 94 °C for 3 min, 24 cycles at 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s, and one cycle at 72 °C for 3 min. Ten microlitres of each PCR product was loaded directly onto nondenaturing 8% polyacrylamide gels, and the gels were stained with SYBR Greene (BioWhittaker Molecular Applications, Rockland, ME, USA) according to the manufacturer's protocol. The intensity of the bands was quantified by densitometry.

Statistical analysis

Age, tumour size, liver function and mtDNA mutations were compared between the two groups with the Mann–Whitney *U* test. Histological findings, diabetes mellitus, alcohol use, tumour differentiation, TP53 mutation and methylation status were compared between the two groups with the χ^2 test.

Ethical considerations

This study protocol complied with the ethical guidelines of the Declaration of Helsinki (1975) and was approved by the Ethics Committee of Osaka City University Graduate School of Medical.

Results

Histological findings in patients with sustained virological response

In patients with SVR, the period from the end of IFN treatment to hepatectomy for HCC ranged from 13 to 156 months. Histological examinations, performed in 11 of the 13 patients with SVR-HCCs, showed that the staging of hepatic fibrosis improved in five patients and the grade of hepatic activity improved in eight patients (Table 3).

Mitochondrial DNA mutations of hepatocellular carcinoma

Mutations of mtDNA were found in both HCC and noncancerous liver tissue. Previously, three mutation sites in mtDNA have been reported to be unique for the Japanese. Excluding these sites, we evaluated the average number of mtDNA mutations in D-loop lesions (Table 4). The average number of mtDNA mutations in D-loop lesions was 4.2 in 44 HCCs with HCV and 2.0 in 14 HCCs from SVR patients. The average number of mtDNA mutations in D-loop lesions was 2.8 in 44 noncancerous lesions with HCV and 1.3 in 13 noncancerous lesion from SVR patients. No specific mutation in mtDNA of SVR-HCC was found in the present study. The frequency of mtDNA mutations in HCC was significantly lower in SVR patients than in HCV patients ($P = 0.0021$). The frequency of mtDNA mutations was also lower in noncancerous livers of SVR patients ($P = 0.007$). In the present study, no regularity of mtDNA mutations was found in the D-loop region.

TP53 mutation analysis

TP53 mutations were detected in 12 (27.3%) of 44 HCCs with HCV (Table 4). In detail, TP53 was mutated in codon 123, TAT to TTC; codon 132, AAG to TTG; codon 133, ATG to TTG; codon 158, CGC to CTC; codon 189, GCC to GTC; codon 220, TAT to TGT; codon 246, ATG to GTG; codon 272, GAG to GTG; codon 275, TGT to TAT; and codon 271, CAT to CGT. Two cases were mutated by insertion in exons 5 and 8. The histological findings showed that HCCs with TP53 mutations consisted of seven moderately differentiated and five poorly differentiated HCCs. TP53 mutations were detected in two (14.3%) of 14 HCCs from the 13 patients in whom HCV was eradicated by IFN therapy. In detail, TP53 was mutated in codon 135, TGC to TGG and codon 242, TGC to TTC. The histological findings showed that HCCs with TP53 mutations in SVR patients consisted of two poorly differentiated HCCs.

Methylation pattern of hepatocellular carcinoma

In patients with HCV, hypermethylation of *p16, p15, p14, RB* and the *PTEN* promoter was, respectively, detected in 34 (77.3%), 13 (29.5%), 8 (18.2%), 12 (27.3%) and 11 (25.0%) of 44 HCCs and 13 (29.5%), 14 (31.8%), 4 (9.1%), 11 (25.0%) and 5 (11.4%) of 44 noncancerous liver samples (Fig. 1A). In patients with SVR, hypermethylation of *p16, p15, p14, RB* and the *PTEN* promoter was, respectively, detected in 14 (100%), 0 (0%), 0 (0%), 2 (14.3%) and 2 (14.3%) of 14 HCCs and 2 (15.4%), 0 (0%), 0 (0%), 2 (15.4%) and 0 (0%) of 13

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Table 3. Clinical course of patients with sustained virological response-hepatocellular carcinoma

Case	Pre-IFN therapy				Span for carcinogenesis after IFN therapy (months)	At operation		
	Genotype	HCV-RNA	*F* factor	*A* factor		*F* factor	*A* factor	BMI
56	1b	1 MEq	2	2	45	2	1	23.7
101	2a	+	3	2	19	4	2	23.7
149	2a	1.1 MEq	4	3	20	4	2	23.6
196	2b	+	2	2	41	1	2	23.4
198	2a	0.4 MEq	2	2	103	1	1	21.5
200	2a	1.1 MEq	2	2	13	2	1	24.6
221	2a	0.9 MEq	2	3	80	2	2	18.1
268	Unknown	+	Unknown	Unknown	144	1	1	20.3
269	2a	0.4 MEq	2	3	156	0	0	23.6
271	1b	+	4	1	156	3	1	28.1
325	1b	300 KIU	3	2	15	2	1	25.2
327	2b	160 KIU	3	3	36	4	2	26.8
328	1b	+	Unknown	Unknown	14	4	2	27.6

BMI, body mass index; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; IFN, interferon; SVR, sustained virological response.

Table 4. Comparison of mutation in the displacement-loop of mitochondrial DNA, mutation in TP53 and methylation between sustained virological response-hepatocellular carcinoma and hepatitis C virus-hepatocellular carcinoma

	SVR-HCC	HCV-HCC	*P*-value
Mean mutation number in D-loop of mtDNA	2.0	4.2	0.0021
TP53 mutation	14.3%	27.3%	0.322
Methylation			
p16	100.0%	77.3%	0.049
p15	0.0%	29.5%	0.021
p14	0.0%	18.2%	0.085
RB	14.3%	27.3%	0.322
PTEN	14.3%	25.0%	0.402

D-loop, displacement-loop; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; mtDNA, mitochondrial DNA; SVR, sustained virological response.

noncancerous liver samples (Fig. 1B). Methylation of *p14*, *p15*, *RB* and *PTEN* was thus slightly but not significantly more frequent in HCV-HCC than in SVR-HCC.

Expression of *p16* mRNA in hepatocellular carcinoma

Expression of *p16* mRNA was examined in 29 patients with HCV-HCC and six with SVR-HCC. In six SVR-HCCs with *p16* promoter methylation, *p16* mRNA expression was lower than that in the noncancerous liver (Fig. 2). In 29 HCV-HCCs with *p16* methylation, *p16* mRNA expression was lower than that in HCC without *p16* methylation.

Discussion

In agreement with previous studies, all patients with SVR-HCC were males in the present study (15), suggesting that sex-related factors have a role in SVR-HCC. We, therefore, studied male patients with HCV-HCC and matched subjects with SVR-HCC. First of all, mtDNA mutations were frequent in HCC as well as in noncancerous liver tissues from patients with HCV (2, 25). Chronic viral inflammation induces ROS production, followed by mtDNA damage in the liver, which is speculated to contribute to hepatocarcinogenesis (25). In contrast to mtDNA mutations, the frequency of mtDNA mutations was low in SVR liver. Histological examination of noncancerous liver tissue showed that persistent inflammation was minimal or absent in SVR patients. Nishikawa *et al.* reported that IFN therapy reduces the frequency of mtDNA mutations in the liver of patients with chronic hepatitis C. In their study, a reduced frequency of mtDNA mutations was detected only in patients whose transaminases were normalized by IFN therapy in association with HCV elimination (26). Our study also showed that the frequency of mtDNA mutations was reduced in the liver of SVR patients. In the present study, no patient with HCV-HCC received IFN. Therefore, we could not exactly clarify which factor was more closely related to fewer mtDNA mutations in SVR-HCC, IFN or HCV eradication. However, we speculate that chronic inflammation was not related to the development of HCC in SVR patients.

Destruction of tumour suppressor gene function is thought to be a critical step in carcinogenesis. Previous studies showed that TP53 mutations were detected in 27% (21) and 38.3% (27) of HCCs with viral infection. These high rates were apparently related to the late stage of hepatocarcinogenesis. In agreement with these previous reports, TP53 was mutated in seven moderately differentiated HCC and five poorly differentiated HCC (27.7%) in the 44 patients with HCV in our study. To our knowledge, no previous study has reported TP53 mutations in SVR-HCC. We found two TP53 mutations in 14 SVR-HCC, including dedifferentiated lesions. mtDNA damage induced by chronic viral hepatitis correlates with genomic injury. It was speculated that a decrease in mtDNA mutations followed loss of TP53 mutations. Although the small number of the SVR-HCCs examined in our study precludes firm conclusions, TP53 alterations might differ between SVR-HCC and HCV-HCC.

Next, we showed epigenetic alterations in both HCV-HCC and SVR-HCC. Previous studies have reported that *p16*, *p15*,

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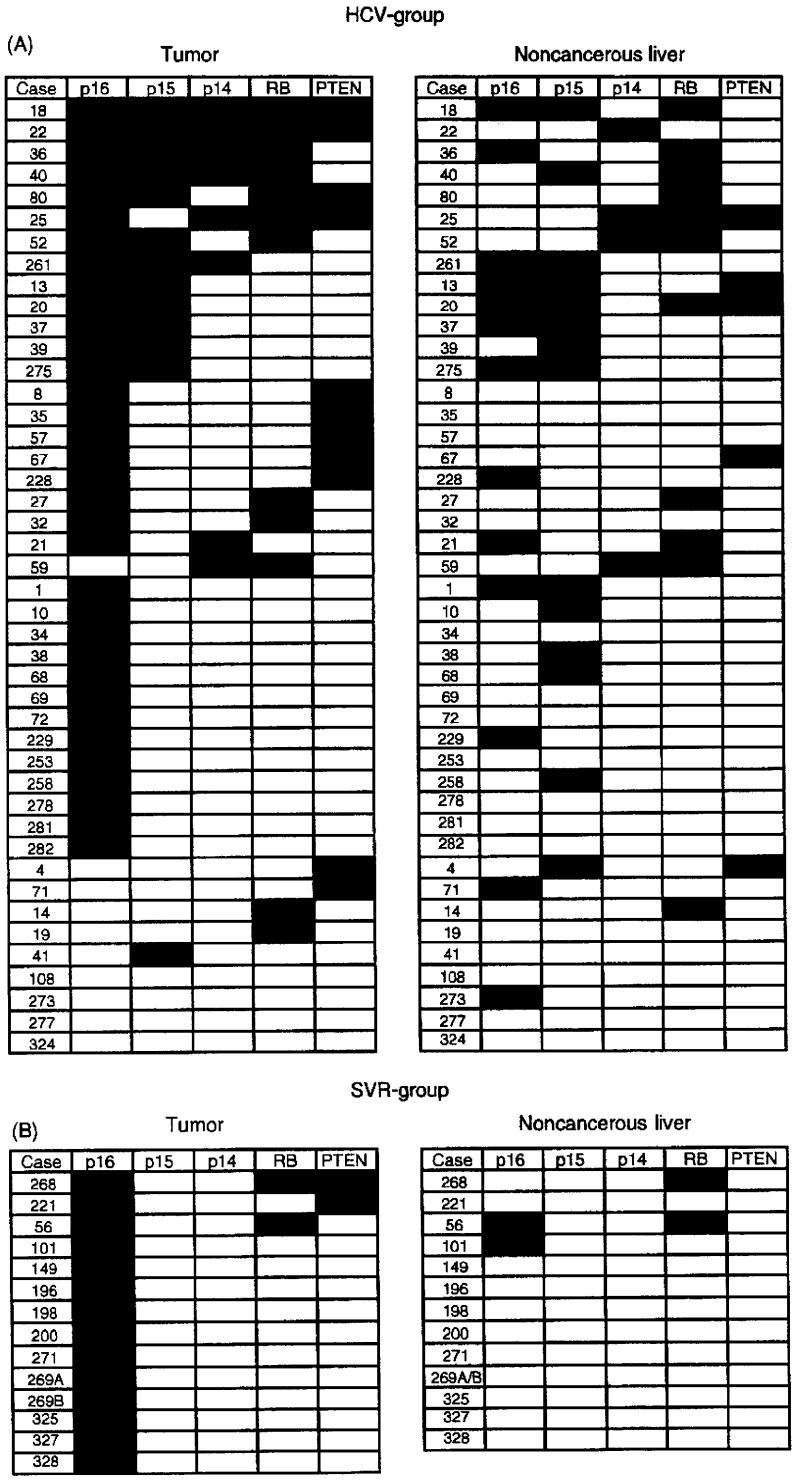

Fig. 1. Methylation patterns of *p16, p15, p14, RB* and *PTEN* promoter in 44 hepatocellular carcinomas (HCCs) and 44 noncancerous liver samples from the hepatitis C virus (HCV) group were examined by methylation-specific polymerase chain reaction (MSP) (A). Methylation patterns were also examined by MSP for 14 HCCs and 13 noncancerous liver samples from the sustained virological response (SVR) group (B). Black boxes indicate methylated sequences, whereas blank boxes indicate unmethylated sequences.

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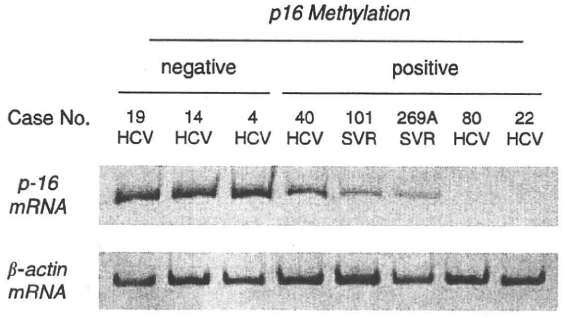

Fig. 2. Expression of *p16* mRNA in hepatocellular carcinoma (HCC). The promoter of *p16* was methylated in cases 40, 101, 269A, 80 and 22. In these tumours, *p16* expression was lower than that in HCC without methylation. β-actin expression was examined as a control. HCV, hepatitis C virus; SVR, sustained virological response.

p14, *RB* and *PTEN* are, respectively, methylated in 58–82% (4, 5, 28–32), 5–64% (5, 32–34), 0–36% (5, 32), 21% (5) and 17% (28) of HCCs from patients with viral infections. In our study, methylation of *p16*, *p15*, *p14*, *RB* and *PTEN* was, respectively, detected in 34 (77.3%), 13 (29.5%), eight (18.2%), 12 (27.3%) and 11 (25.0%) of 44 HCV-HCCs. Our findings are thus consistent with those of previous studies. In SVR-HCC, *p16* was methylated in all samples, whereas *RB* and *PTEN* were methylated in only two samples and methylation of *p15* and *p14* was not detected. This was a novel methylation profile that differed from that of SVR-HCC and HCV-HCC. We showed that promoter methylation of the *p16* gene, leading to the loss of *p16* expression, was frequently observed not only in HCV-HCC but also in SVR-HCC. These data suggested that aberrant *p16* methylation might contribute to the development of SVR-HCC.

Epidemiological studies have shown that past exposure to *Helicobacter pylori* is closely associated with an increased risk of gastric cancer and that most cases of *H. pylori*-negative gastric cancer have a history of exposure to *H. pylori* (35, 36). Maekita and colleagues reported that permanent methylation of specific CpG islands in gastric mucosae is associated with a heightened risk of gastric cancer in *H. pylori*-negative patients (37, 38). It was speculated that methylation of CpG islands in gastric stem cells led to a continuous high level of methylation in gastric mucosae (39). It was well known that HCV was spontaneously eradicated in 20% of patients with the acute infection (40). To our knowledge, there has been no report about HCC development in patients who had been cured in acute hepatitis. In the present study, *p16* was methylated in both HCC infected with HCV and HCC after eradication of HCV. We speculate that *p16* in hepatic stem cells might be methylated in the continuous presence of HCV. These cells with methylated *p16* might survive and grow after eradication of HCV by IFN therapy. Future studies should examine the methylation status of genes in successive liver specimens obtained before and after IFN therapy.

In conclusion, epigenetic alterations of some genes in SVR-HCC differed from those in HCV-HCC. Moreover, mutation of mtDNA was less common in SVR-HCC than in HCV-HCC. The present results suggest that the development of HCC in patients cured of HCV infection by IFN therapy might be associated with particular molecular alterations.

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References

1. Marrogi AJ, Khan MA, van Gijssel HE, *et al.* Oxidative stress and p53 mutations in the carcinogenesis of iron overload-associated hepatocellular carcinoma. *J Natl Cancer Inst* 2001; **93**: 1652–5.
2. Nishikawa M, Nishiguchi S, Shiomi S, *et al.* Somatic mutation of mitochondrial DNA in cancerous and noncancerous liver tissue in individuals with hepatocellular carcinoma. *Cancer Res* 2001; **61**: 1843–5.
3. Minouchi K, Kaneko S, Kobayashi K. Mutation of p53 gene in regenerative nodules in cirrhotic liver. *J Hepatol* 2002; **37**: 231–9.
4. Kaneto H, Sasaki S, Yamamoto H, *et al.* Detection of hypermethylation of the p16 (INK4A) gene promoter in chronic hepatitis and cirrhosis associated with hepatitis B or C virus. *Gut* 2001; **48**: 372–7.
5. Roncalli M, Bianchi P, Bruni B, *et al.* Methylation framework of cell cycle gene inhibitors in cirrhosis and associated hepatocellular carcinoma. *Hepatology* 2002; **36**: 427–32.
6. Moriya K, Fujie H, Shintani Y, *et al.* The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4**: 1065–7.
7. Majumder M, Ghosh AK, Steele R, *et al.* Hepatitis C virus NS5A physically associates with p53 and regulates p21/waf1 gene expression in a p53-dependent manner. *J Virol* 2001; **75**: 1401–7.
8. Larsson M, Babcock E, Grakoui A, *et al.* Lack of phenotypic and functional impairment in dendritic cells from chimpanzees chronically infected with hepatitis C virus. *J Virol* 2004; **78**: 6151–61.
9. McHutchison JG, Gordon SC, Schiff ER, *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485–92.
10. Manns MP, McHutchison JG, Gordon SC, *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958–65.
11. Bruno S, Stroffolini T, Colombo M, *et al.* Sustained virological response to interferon-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. *Hepatology* 2007; **45**: 579–87.
12. Yoshida H, Shiratori Y, Moriyama M, *et al.* Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. *Ann Intern Med* 1999; **131**: 174–81.
13. Toyoda H, Kumada T, Tokuda A, *et al.* Long-term follow-up of sustained responders to interferon therapy, in patients with chronic hepatitis C. *J Viral Hepat* 2000; **7**: 414–9.
14. Enokimura N, Shiraki K, Kawakita T, *et al.* Hepatocellular carcinoma development in sustained viral responders to interferon therapy in patients with chronic hepatitis C. *Anticancer Res* 2003; **23**: 593–6.
15. Makiyama A, Itoh Y, Kasahara A, *et al.* Characteristics of patients with chronic hepatitis C who develop hepatocellular carcinoma after a sustained response to interferon therapy. *Cancer* 2004; **101**: 1616–22.
16. Tamori A, Nishiguchi S, Shiomi S, *et al.* Hepatitis B virus DNA integration in hepatocellular carcinoma after interferon-induced

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