Table 3 Factors associated with SVR to combination therapy ($n = 220$; multivariate analysis)

Variable		Odds ratio (95% CI)	*P*
Sex	Male *vs* female	0.808 (0.365–1.789)	0.5985
Age (years)		1.015 (0.983–1.048)	0.3677
Baseline serum ALT (IU/L)		0.997 (0.992–1.002)	0.1973
Genotype	1 *vs* 2	0.074 (0.030–0.182)	<0.0001
Viral load (KIU/mL)		1.002 (1.001–1.004)	0.0002

ALT, alanine aminotransferase; CI, confidence interval; SVR, sustained virologic response.

Table 4 Treatment efficacy in patients aged ≥60 years

	Combination therapy ($n = 66$)	Monotherapy ($n = 47$)	*P*
Sex ratio (male/female)	38/28	30/17	0.5033
Baseline serum ALT(IU/L)	97.6 ± 62.0	100.0 ± 71.8	0.8536
Genotype (1/2)	54/12	34/13	0.2316
Activity (A0/A1/A2/A3)	1/22/19/4	1/18/25/0	0.1593
Fibrosis (F0/F1/F2/F3/F4)	2/20/11/9/3	2/16/20/7/0	0.1773
SVR rate (intention-to-treat)	31.8 (21/66)	10.6 (5/47)	0.0084
SVR rate (per-protocol)	40.4 (21/52)	10.6 (5/47)	0.0008
SVR/relapse/NR/discontinuation	21/24/7/14	5/23/15/4	<0.0001
Treatment discontinuation rate	21.2 (14/66)	8.5 (4/47)	0.0690

ALT, alanine aminotransferase; NR, non-response; SVR, sustained virologic response.

With combination therapy, the SVR rate was similar for all age groups. In patients ≥60 years with genotype 2 and a high viral load, the SVR rate was significantly higher with combination therapy than with monotherapy (83.3% *vs* 23.1%, $P = 0.0048$ by per-protocol analysis and by intention-to-treat analysis).

Adverse events

For 14 of 74 patients with dose reduction of ribavirin, ribavirin was reduced due to fatigue and anemic symptoms though the hemoglobin levels were above 10 g/dL, which is the level of dose reduction of this study. The combination therapy discontinuation rate was not statistically different between patients aged ≥60 years and those aged <60 years (Table 2). The combination therapy discontinuation rate was higher in combination therapy (21.2%) than in monotherapy (8.5%) among patients aged ≥60 years (Table 4). The reasons for discontinuation of the combination therapy and the times at which the therapy was discontinued are shown in Table 5. If discontinuation of treatment occurred we did not restart therapy after disappearance of the initial symptom or illness. Ribavirin discontinuation was higher in older patients ($P < 0.05$). A serious adverse effect occurred in one patient in each group: infarction of vessel in the retina in the older group and cerebral hemorrhage in the younger group.

Effect of dose reduction and discontinuation of ribavirin or IFN on the SVR rate

Ribavirin dose reduction and discontinuation rates are shown according to age group in Fig. 4. The total of dose reduction and discontinuation rates increased with age. The SVR of patients who completed treatment was 44.7% (51/114). Among patients who had dose reduction, the SVR was 36.5% (19/52). Among patients who discontinued treatment, the SVR was 18.5% (10/54). The SVR was not significantly different between those in whom the dose of ribavirin was reduced and those in whom it was not. Creatinine clearance in patients who needed dose reduction or discontinuation of ribavirin was worse than that in patients who did not (90.2 ± 20.9 mL/min *vs* 107.5 ± 24.2 mL/min, $P < 0.0001$). The SVR in those who completed full treatment was significantly higher than that in those who had reduced-dose IFN (39.5% *vs* 20%, $P = 0.0282$). The SVR in those who completed full treatment was significantly higher than that in those who had discontinued IFN (43.3% *vs* 5%, $P < 0.0001$).

Comparison between 24-week and 48-week treatment

Among the patients with HCV genotype 1, the SVR of 48-week treatment was significantly higher than that of 24-week treatment (48.1% *vs* 24.3%, $P = 0.0148$ by per-protocol analysis; 37.1% *vs* 19.4%, $P = 0.0265$ by intention-to-treat analysis). However, among the patients with HCV genotype 2, the SVR of the 48-week treatment was similar to that of the 24-week treatment (75.0% *vs* 85.0%, $P = 0.4884$ by per-protocol analysis; 75.0% *vs* 81.0%, $P = 0.6997$ by intention-to-treat analysis).

The IFN dose reduction rate for 48-week treatment was significantly higher than that of 24-week treatment (27.3% *vs* 13.1%, $P = 0.0212$). The treatment discontinuation rate for the 48-week course was not statistically different from the 24-week course (20.5% *vs* 17.6%, $P = 0.6621$).

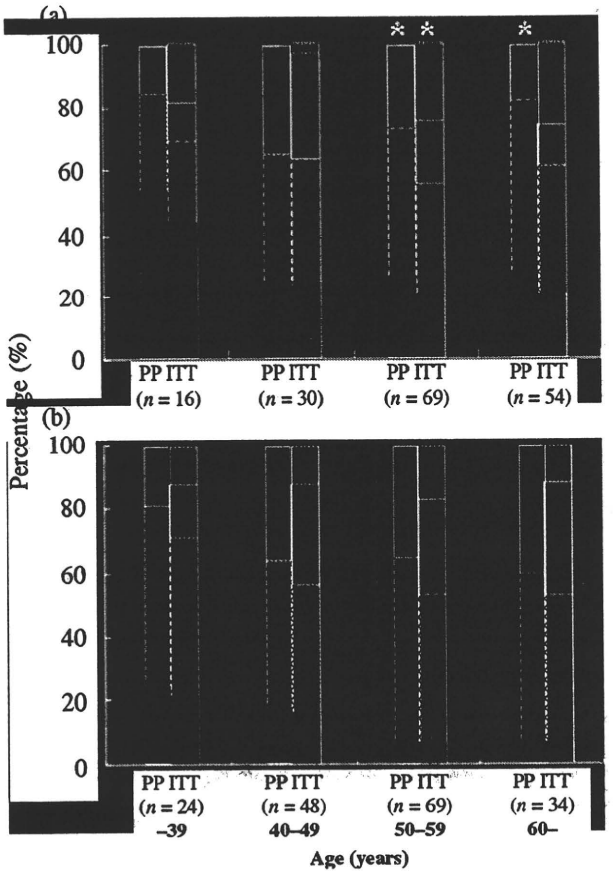

Figure 2 Virologic response to (a) combination therapy and (b) interferon (IFN) monotherapy according to age of patients with genotype 1 and a high viral load. Asterisks indicate significant differences *vs* the respective IFN monotherapy (* $P < 0.05$). (▥) Treatment discontinuation; (□) non-responder; (▨) relapse; (■) sustained virologic response. ITT, intention-to-treat analysis; PP, per-protocol analysis.

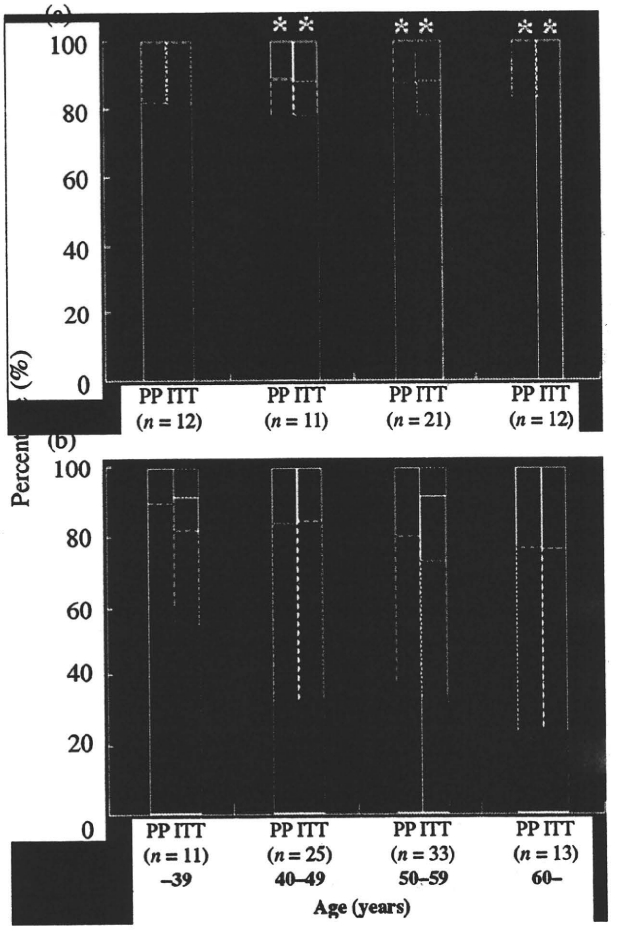

Figure 3 Virologic response to (a) combination therapy and (b) interferon (IFN) monotherapy according to age of patients with genotype 2 and high viral load. Asterisks indicate significant differences *vs* the respective IFN monotherapy (* $P < 0.05$). (▥) Treatment discontinuation; (□) non-responder; (▨) relapse; (■) sustained virologic response. ITT, intention-to-treat analysis; PP, per-protocol analysis.

Discussion

It is important to eradicate HCV by IFN to reduce the risk of hepatocellular carcinoma.[4,5] In addition, IFN reportedly reduces liver-related mortality in chronic hepatitis C patients aged >60 years.[16,17] However, these findings are based on studies of IFN monotherapy. The present study showed the effect of ribavirin and IFN in combination. Ribavirin has been used in combination with IFN to treat chronic hepatitis C, and this combination therapy has been reported to be more effective than IFN monotherapy for eradicating HCV.[7–10] However, ribavirin and IFN or pegylated IFN in combination produce a common adverse effect, that is, hemoglobin levels decrease in 20–36% of treated patients with chronic hepatitis C, necessitating dose reduction or discontinuation.[7,8,18,19]

It has been reported that there is no significant difference in the efficacy of IFN monotherapy between older and younger patients after standardization of their background clinical characteristics, suggesting that age itself does not influence the outcome of IFN monotherapy.[11,12] However, the efficacy and tolerability of combination therapy in the elderly patient has not been clarified. We therefore conducted a multi-institution study to evaluate the efficacy and tolerability of ribavirin plus IFN-α in older patients with chronic hepatitis C.

Multivariate analysis showed baseline viral load and genotype to be the only significant factors associated with SVR. Age was not associated with SVR. Many studies have shown baseline viral load and genotype to be significant factors associated with SVR.[8,19] Our results suggest that the SVR of patients aged ≥60 years is comparable to that of younger patients. Because the SVR differs according to genotype and viral load, we classified patients by genotype and compared the SVR rate for both combination therapy and IFN monotherapy. In patients aged ≥60 years, the SVR rate of combination therapy was significantly increased over that of IFN monotherapy (in patients with genotype 1 and a high

Table 5 Reasons for discontinuation of combination therapy

Patients aged < 60 years			Patients aged ≥ 60 years		
Reason	*n*	Weeks after starting treatment	Reason	*n*	Weeks after starting treatment
Cerebral hemorrhage	**1**	**4**	**Infarction in the retina**	**1**	**14**
Rash	5	1,1,5,22,25	Fatigue	4	4,12,12.14
Fatigue	5	6,12,20,20,21	Anemia	3	10,16, 22
Depression	2	4,10	Anorexia	2	1,19
Anorexia	2	21,23	Nervousness	1	2
Vomiting	1	2	Dizziness	1	6
Anemia	1	4	Vomiting	1	16
Worsening diabetes	1	16	Depression	1	18
Spontaneous pneumothorax	1	17			
Hypothyroidism	1	18			
Uterine cancer	1	20			
Thyroiditis	1	24			
Pancytopenia	1	37			

Bold, serious adverse effect.

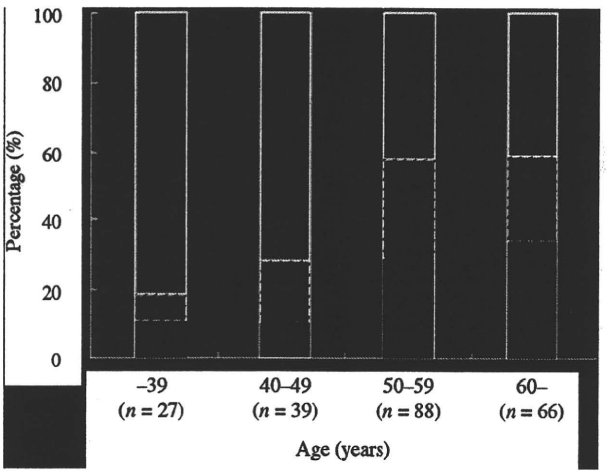

Figure 4 Ribavirin dose reduction and discontinuation rates according to age of patients ($n = 220$). (□) Completion: SVR 44.7% (51/114); (▨) dose reduction: SVR 36.5% (19/52); (■) treatment discontinuation: SVR 18.5% (10/54). SVR, sustained virologic response.

viral load by per-protocol analysis, 27.5% *vs* 6.7%, $P = 0.032$; in patients with genotype 2 and a high viral load by per-protocol analysis, 83.3% *vs* 23.1%, $P = 0.0048$; Figs 2,3). Moreover, the SVR rate among patients aged ≥60 years with HCV genotype 1 did not decrease with age. Neither did the SVR rate change with age for patients ≥60 years with genotype 2. Patients with genotype 2 achieved a high SVR rate of approximately 80% in all age categories. Adverse effects are thought to increase in elderly patients, but adverse effects necessitating discontinuation of IFN and ribavirin did not differ significantly between the older and younger patients (21.2% *vs* 14.9%). In addition, the severe adverse effects were not associated with age. These findings were similar to previously reported findings that there was no difference between young and elderly patients with respect to adverse effects.[11,12] The treatment discontinuation rate tended to be higher in combination therapy (14/66) than in monotherapy (4/47) among patients aged ≥60 years, but there was no significantly difference between the two groups. (Table 4). This is because there was a small number of patients in the monotherapy group. The reason for discontinuation of combination therapy in seven of 14 patients was ribavirin-related adverse effects such as general fatigue or anemia.

The ribavirin dose reduction and discontinuation rates increased with age, but the SVR rate did not differ significantly between patients with and without dose reduction who completed the treatment schedule (36.5% *vs* 44.7%). These findings are consistent with previously reported findings.[19] In patients aged ≥60 years with HCV genotype 1 and a high viral load, the SVR rate did not differ significantly between combination therapy and IFN monotherapy by intention-to-treat analysis, but it did differ significantly by per-protocol analysis. These findings indicate that rather than discontinuing treatment, we should continue as permitted by dose reduction of ribavirin. In groups 50–59 years and >60 years of age the rate of dose reduction and treatment discontinuation was similarly high. In contrast, in groups <50 years of age the rate was low. In the present study we focused on patients aged ≥60 years because 60 years is often used as a cut-off for older patients; if we had focused on patients ≥65 years the number of study patients would have decreased and the comparison would have been difficult. There were high dose-reduction and discontinuation rates in the patients aged ≥50 years, so we should consider dose modification for these patients in advance.

Careful monitoring and appropriate reduction of the ribavirin dose is required to circumvent the need for discontinuation in elderly patients.[20,21] Also, it will be necessary to be careful when treating elderly patients with other diseases commonly observed in this age group, such as diabetes or hypertension. The present study, however, was limited due to being a retrospective analysis and using of historical controls, therefore further prospective studies are needed.

In conclusion, combination therapy was shown to be of comparable efficacy for chronic hepatitis C between patients aged <60 years and those aged ≥60 years, although the rate of ribavirin

discontinuation was shown to be higher among the older patients than among the younger patients. The efficacy of combination therapy was shown to be greater than that of IFN monotherapy in older patients.

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References

1 Yoshizawa H. Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. *Oncology* 2002; **62** (Suppl. 1): 8–17.
2 Marcellin P, Boyer N, Gervais A *et al.* Long-term histologic improvement and loss of detectable intrahepatic HCV RNA in patients with chronic hepatitis C and sustained response to interferon-alpha therapy. *Ann. Intern. Med.* 1997; **127**: 875–81.
3 Shiratori Y, Imazeki F, Moriyama M *et al.* Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. *Ann. Intern. Med.* 2000; **132**: 517–24.
4 Ikeda K, Saitoh S, Arase Y *et al.* Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C. A long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. *Hepatology* 1999; **29**: 1124–30.
5 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.
6 Imai Y, Kawata S, Tamura S *et al.* Relation of interferon therapy and hepatocellular carcinoma in patients with chronic hepatitis C. Osaka Hepatocellular Carcinoma Prevention Study Group. *Ann. Intern. Med.* 1998; **129**: 94–9.
7 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.
8 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.
9 Poynard T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; **352**: 1426–32.
10 Lai MY, Kao JH, Yang PM *et al.* Long-term efficacy of ribavirin plus interferon alfa in the treatment of chronic hepatitis C. *Gastroenterology* 1996; **111**: 1307–12.
11 Bresci. G, Del Corso L, Romanelli AM, Giuliano G, Pentimone F. The use of recombinant interferon alfa-2b in elderly patients with anti-HCV-positive chronic active hepatitis. *J. Am. Geriatr. Soc.* 1993; **41**: 857–62.
12 Horiike N, Masumoto T, Nakanishi K *et al.* Interferon therapy for patients more than 60 years of age with chronic hepatitis C. *J. Gastroenterol. Hepatol.* 1995; **10**: 246–9.
13 Okamoto H, Mishiro S, Tokita H, Tsuda F, Miyakawa Y, Mayumi M. Superinfection of chimpanzees carrying hepatitis C virus of genotype II/1b with that of genotype III/2a or I/1a. *Hepatology* 1994; **20**: 1131–6.
14 Simmonds P, Alberti A, Alter HJ *et al.* A proposed system for the nomenclature of hepatitis C viral genotypes. *Hepatology* 1994; **19**: 1321–4.
15 Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. *Hepatology* 1996; **24**: 289–93.
16 Imai Y, Kasahara A, Tanaka H *et al.* Interferon therapy for aged patients with chronic hepatitis C: improved survival in patients exhibiting a biochemical response. *J. Gastroenterol.* 2004; **39**: 1069–77.
17 Yoshida H, Arakawa Y, Sata M *et al.* Interferon therapy prolonged life expectancy among chronic hepatitis C patients. *Gastroenterology* 2002; **123**: 483–91.
18 Sulkowski MS, Wasserman R, Brooks L, Ball L, Gish R. Changes in haemoglobin during interferon alpha-2b plus ribavirin combination therapy for chronic hepatitis C virus infection. *J. Viral Hepat.* 2004; **11**: 243–50.
19 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* 2002; **347**: 975–82.
20 Takaki S, Tsubota A, Hosaka T *et al.* Factors contributing to ribavirin dose reduction due to anemia during interferon alfa2b and ribavirin combination therapy for chronic hepatitis C. *J. Gastroenterol.* 2004; **39**: 668–73.
21 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; **123**: 1061–9.

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Role of tumor markers in assessment of tumor progression and prediction of outcomes in patients with hepatocellular carcinoma

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The efficacies of tumor markers, alfa-fetoprotein (AFP), *Lens culinaris* agglutinin A-reactive fraction of alfa-fetoprotein (AFP-L3), and des-gamma-carboxy prothrombin (DCP) were evaluated for assessment of progression of hepatocellular carcinoma (HCC) and patient prognosis. The prevalence of elevated levels of each tumor marker increased with progression of tumor stage for all three markers among patients with HCC. Survival was poorer among patients with elevated levels of tumor markers than among those without elevated levels. Evaluation of tumor progression with tumor markers was based only on the results of laboratory tests. The tests are objective, simple to perform, and easy to repeat, and therefore, may be useful to supplement conventional tumor staging for the evaluation of tumor progression and prediction of patient outcome.

Key words: hepatocellular carcinoma, AFP, AFP-L3, DCP, progression, prognosis

Hepatocellular carcinoma (HCC) is a common cause of death in patients with chronic hepatitis and cirrhosis.[1,2] It is one of the most important malignancies in Japan; the incidence of HCC has increased over the last 30 years and has more than doubled in the last 10 years. HCC is currently the third leading cause of cancer-related death in Japan.[3]

Assessment of the progression of HCC is based on tumor morphology such as tumor size, number of tumors, and portal vein thrombosis. These factors are usually evaluated by imaging or pathologic examination. However, the sensitivity of imaging examination for evaluation of tumor progression varies and is related to the imaging modalities, the examiner's skill, and the imaging apparatuses. We often encounter discrepancies on the size or number of HCCs measured with different imaging modalities (Fig. 1). Another difficulty in the evaluation of tumor progression is discrepancy between imaging findings and pathologic results from the resected specimen, especially with respect to vascular invasion. It is not possible to evaluate microscopic vascular invasion by means of imaging studies, which likely leads to underestimation of the degree of vascular invasion. Moreover, it is often difficult to accurately evaluate tumor progression by imaging studies in cases of recurrent HCC. Patients who have undergone repeated treatments may have necrotized tumors from locoregional ablative therapy (LAT) such as radiofrequency ablation (RFA), or tumors that have retained lipiodol after transcatheter arterial chemoembolization (TACE).

Several tumor markers for HCC have been reported.[4–9] Of these, alfa-fetoprotein (AFP), *Lens culinaris* agglutinin A-reactive fraction of AFP (AFP-L3), and des-gamma-carboxy prothrombin (DCP) are currently used in clinical practice in Japan. These markers were originally used for detection and diagnosis of HCC in routine clinical settings;[4–6] however, the efficacies of these tumor markers for this purpose are not satisfactory. AFP is the most common tumor marker for HCC, but there are many patients who show elevated AFP in the absence of HCC. AFP levels are elevated in up to 20% of patients with chronic hepatitis and in 20–60% of patients with cirrhosis, even in the absence of HCC.[10] In contrast,

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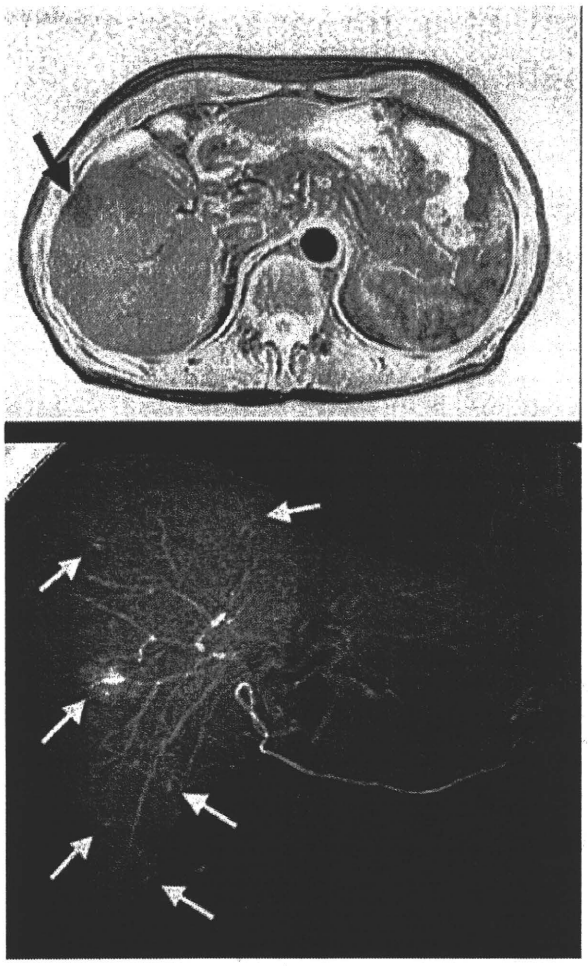

Figure 1 (a) Dynamic magnetic resonance imaging study of a 59-year old, male outpatient revealed a solitary tumor in the right lobe of the liver (white arrow). However, (b) digital subtraction arteriography performed after hospitalization showed multiple small hepatocellular carcinoma nodules throughout the right lobe (black arrows).

AFP-L3 has reportedly high specificity for HCC, but its sensitivity is low. Other investigators have reported that elevation of these tumor markers, especially of AFP-L3 and DCP, may be indicative of events related to tumor progression, such as invasion of the portal vein by HCC or an increase in the intratumoral arterial blood supply of HCC.[11–15] In a recent study, we showed that a combination of these tumor markers is reflective of progression of HCC and accurately predicts patient survival.[16] We found that tumor markers AFP-L3 and DCP reflect different features of tumor progression, and that the number of elevated tumor markers can be used to predict patient survival.

In this paper, we describe the role of tumor markers in the assessment of tumor progression and prediction of patient outcome.

MEASUREMENT OF TUMOR MARKERS AND CUT-OFF LEVELS

THE THREE TUMOR markers were measured routinely at the time of initial HCC diagnosis. The serum AFP level was determined by enzyme-linked immunosorbent assay with a commercially available kit (ELISA-AFP, International Reagents, Kobe, Japan). Serum AFP-L3 was measured by lectin-affinity electrophoresis coupled with antibody-affinity blotting (AFP Differentiation Kit L, Wako Pure Chemical Industries, Osaka, Japan) and was expressed as a percentage (AFP-L3 = AFP-L3 level/total AFP level × 100).[5,17] The serum DCP level was determined by means of sensitive enzyme immunoassay (Eitest PIVKA-II kit, Eisai Co., Tokyo, Japan) according to the manufacturer's instructions.[6,18,19] In our paper, values of 400 ng/mL, 15%, and 100 mAU/mL were used as cut-off values to establish elevation of AFP, AFP-L3, and DCP, respectively, according to previous reports.[20–22]

Tumor progression as shown by imaging findings was assessed on the basis of the TNM classification of the Liver Cancer Study Group of Japan.[23] In most cases, the maximum diameter of the tumor was determined with B-mode ultrasonography (US). Vascular invasion was assessed with dynamic computed tomography (CT) and angiography. Lymph node invasion and distant metastases were assessed with ultrasonographic, dynamic CT, and chest X-ray screenings. Bone scintigraphy or brain CT was performed if suggestive symptoms were present.

ELEVATION OF TUMOR MARKERS AND TUMOR PROGRESSION

THE PERCENTAGE OF patients with elevation of each tumor marker according to the progression of tumor stage is shown in Figure 2. The percentage of patients with elevated levels of AFP, AFP-L3, and DCP increased in parallel with increases in tumor stage. These findings indicate that levels of tumor markers of HCC increase with progression of the tumor and that mea-

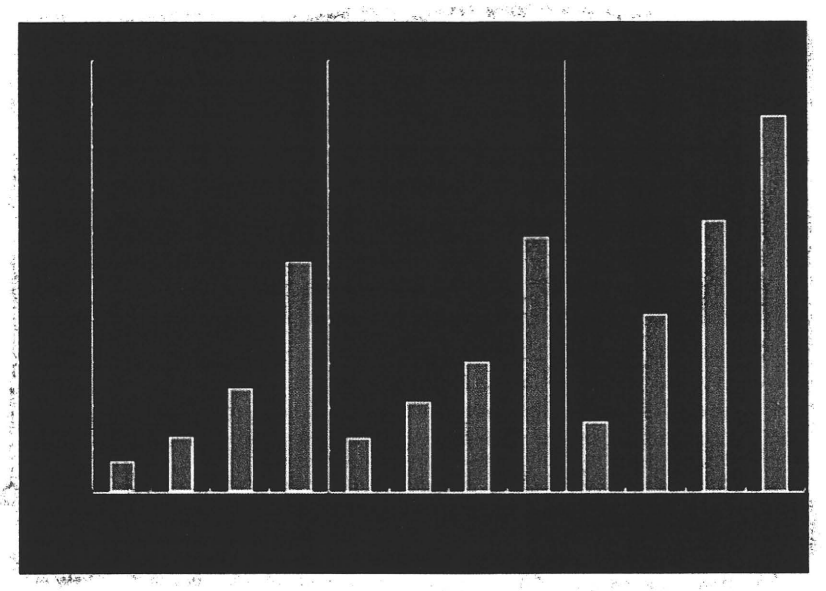

Figure 2 Percentage of patients with elevated level of tumor markers AFP, AFP-L3, and DCP. For all three tumor markers, the percentage of patients with elevated level gradually increased in parallel with the progression of tumor stage (I–IV). AFP, alfa-fetoprotein; AFP-L3, *Lens culinaris* agglutinin A-reactive fraction of alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin.

surement of these markers would be useful also for evaluation of HCC progression.

ELEVATION OF TUMOR MARKERS AND PATIENT SURVIVAL

THE 1-, 3-, 5-, 8-, and 10-year survival rates of patients with HCC according to each tumor marker are shown in Table 1. For all three tumor markers, the rate of survival of patients with elevated tumor marker level is lower than that of patients without elevated level. The difference in survival was significant according to the elevation of AFP, AFP-L3, and DCP ($P < 0.0001$ for all three tumor markers).

USE OF TUMOR MARKERS TO MONITOR CLINICAL COURSES OF PATIENTS WITH HCC

EVALUATION OF TUMOR stage is often difficult in patients with recurrent HCC who have received repeated treatments for HCC, such as LAT including percutaneous ethanol injection (PEIT), percutaneous microwave thermocoagulation (PMCT), RFA, and TACE. US, CT, or magnetic resonance imaging studies usually reveal a mixture of necrotized tumor tissue due to previous LAT and tumors retaining lipiodol after previous TACE. Accurate assessment of tumor stage in these cases requires careful evaluation of imaging results with distinguishing viable tumors from treated tumors and identifying local recurrences (Figs 3,4). In addition, fre-

Table 1 Survival rates according to level of tumor markers at the time of initial diagnosis of hepatocellular carcinoma

	Survival rate (%)				
	1 year	3 years	5 years	8 years	10 years
AFP (≤400 ng/mL) ($n = 2076$)	88.6	64.8	45.3	20.4	14.1
AFP (>400 ng/mL) ($n = 524$)	62.6	40.7	25.7	0	0
AFP-L3 (≤15%) ($n = 1899$)	89.8	67.7	46.4	21.6	14.9
AFP-L3 (>15%) ($n = 721$)	66.2	38.5	27.9	11.1	0
DCP (≤100 mAU/mL) ($n = 1347$)	92.4	73.9	52.6	24.5	15.8
DCP (>100 mAU/mL) ($n = 1253$)	73.8	44.8	28.9	12.7	12.7

AFP, alfa-fetoprotein; AFP-L3, *Lens culinaris* agglutinin A-reactive alfa-fetoprotein; DCP, des-gamma-carboxy prothrombin.

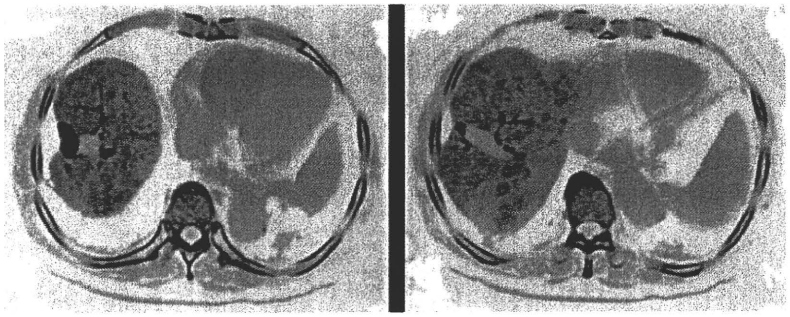

Figure 3 (a, b) Enhanced computed tomography images of recurrent HCC in a 63-year-old man. At the time of evaluation of recurrent HCC, many small, viable HCC nodules were mixed with tumor tissue necrotized by radiofrequency ablation and tumors retaining lipiodol after transcatheter arterial chemoembolization. Of the three tumor markers, only alfa-fetoprotein showed increased levels at the time of this imaging examination. This patient has survived for more than 4 years.

quent monitoring of patients by radiologic methods increases the exposure of patients to radiation.[24] In contrast, assessment of tumor progression with tumor markers can be done frequently with simple blood tests, and the results are not influenced by previous treatments. Monitoring of patients with HCC by means of imaging studies is necessary in the management of patients with HCC; however, monitoring these patients in combination with several tumor markers that can be done more easily and frequently would play a role as a supplemental tool for follow-up (Fig. 5).

LIMITATIONS OF TUMOR MARKERS

ONE IMPORTANT LIMITATION of the assessment of tumor progression with tumor markers is that it can not be used for planning of treatment or treatment itself. Treatment planning and treatment procedures always require imaging study, although data pertaining to tumor markers may provide additional information that would influence the choice of treatment options. Another disadvantage is that tumor marker level is influenced by drugs such as vitamin K and warfarin. Therefore, the results of tumor marker analyses must be interpreted carefully.

CONCLUSION

TUMOR MARKERS HAVE potential as modalities for assessment of the progression of HCC, in addition to their use for the detection and diagnosis of HCC. Although tumor markers can not replace the results of imaging or pathology studies, tumor markers are advantageous in terms of objectivity and simplicity. Evaluation of tumor progression with tumor markers may, therefore, be useful for global comparisons. In addition, tumor marker levels can be measured with stored serum samples, allowing comparison of tumor progression between patients at different times with the same standard. Although most currently available standards to evaluate tumor progression do not contain tumor markers, measurement of levels of those markers will provide additional important information for the management of patients with HCC.

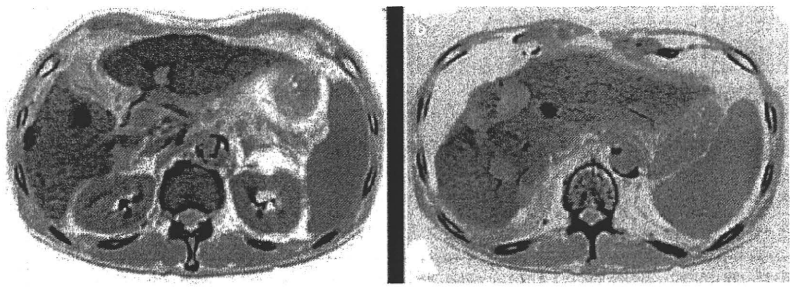

Figure 4 (a, b) Recurrent HCC in a 56-year-old man. Computed tomography images appear similar to those in Figure 3; however, all three tumor markers were elevated in this patient, and he died within 5 months of this imaging examination.

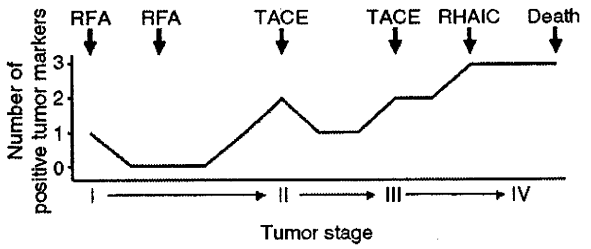

Figure 5 Changes in the number of elevated tumor markers during the clinical course of a patient with HCC. The number of elevated tumor markers increased with recurrence of HCC and tumor progression, and decreased with treatment. RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; RHAIC, repeated hepatic arterial infusion chemotherapy.

CONFLICT OF INTEREST

NO CONFLICT OF interest statement has been received from the authors.

REFERENCES

1 Di Bisceglie AM, Goodman ZD, Ishak KG, Hoofnagle JH, Melpolder JJ, Alter HJ. Long-term clinical and histological follow-up of chronic posttransfusion hepatitis. *Hepatology* 1991; **14**: 969–74.

2 Bruix J, Sherman M, Llovet JM *et al.* Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona EASL Conference. *J Hepatol* 2001; **35**: 421–30.

3 Yoshizawa H. Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. *Oncology* 2002; **62**: S8–17.

4 Oka H, Tamori A, Kuroki T, Kobayashi K, Yamamoto S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. *Hepatology* 1994; **19**: 61–6.

5 Taketa K, Endo Y, Sekiya C *et al.* A collaborative study for the evaluation of lectin-reactive alpha-fetoprotein in early detection of hepatocellular carcinoma. *Cancer Res* 1993; **53**: 5419–23.

6 Mita Y, Aoyagi Y, Yanagi M, Suda T, Suzuki Y, Asakura H. The usefulness of determining des-gamma-carboxy prothrombin by sensitive enzyme immunoassay in the early diagnosis of patients with hepatocellular carcinoma. *Cancer* 1998; **82**: 1643–8.

7 Hippo Y, Watanabe K, Watanabe A *et al.* Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. *Cancer Res* 2004; **64**: 2418–23.

8 Tsai JF, Jeng JE, Chuang LY *et al.* Serum insulin-like growth factor-II as a serologic marker of small hepatocellular carcinoma. *Scand J Gastroenterol* 2005; **40**: 68–75.

9 Marrero JA, Lok AS. Newer markers for hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S113–9.

10 Fujiyama S, Tanaka M, Maeda S, Ashihara H, Hirata R, Tomita K. Tumor markers in early diagnosis, follow-up and management of patients with hepatocellular carcinoma. *Oncology* 2002; **62**: S57–63.

11 Koike Y, Shiratori Y, Sato S *et al.* Des-gamma-carboxy prothrombin as a useful predisposing factor for the development of portal venous invasion in patients with hepatocellular carcinoma: a prospective analysis of 227 patients. *Cancer* 2001; **91**: 561–9.

12 Okuda H, Nakanishi T, Takatsu K *et al.* Serum levels of des-gamma-carboxy prothrombin measured using the revised enzyme immunoassay kit with increased sensitivity in relation to clinicopathological features of solitary hepatocellular carcinoma. *Cancer* 2000; **88**: 544–9.

13 Aoyagi Y, Isokawa O, Suda T, Watanabe M, Suzuki Y, Asakura H. The fucosylation index of alpha-fetoprotein as a possible prognostic indicator for patients with hepatocellular carcinoma. *Cancer* 1998; **83**: 2076–82.

14 Kumada T, Nakano S, Takeda I *et al.* Clinical utility of Lens culinaris agglutinin-reactive alpha-fetoprotein in small hepatocellular carcinoma: special reference to imaging diagnosis. *J Hepatol* 1999; **30**: 125–30.

15 Tada T, Kumada T, Toyoda H *et al.* Relationship between *Lens culinaris* agglutinin-reactive alpha-fetoprotein and pathologic features of hepatocellular carcinoma. *Liver Int* 2005; **25**: 848–53.

16 Toyoda H, Kumada T, Kiriyama S *et al.* Prognostic significance of simultaneous measurement of three tumor markers in patients with hepatocellular carcinoma. *Clin Gastroenterol Hepatol* 2006; **4**: 111–7.

17 Yamashita F, Tanaka M, Satomura S, Tanikawa K. Prognostic significance of Lens culinaris agglutinin A-reactive alpha-fetoprotein in small hepatocellular carcinoma. *Gastroenterology* 1996; **111**: 996–1001.

18 Okuda H, Nakanishi T, Takatsu K *et al.* Measurement of serum levels of des-gamma-carboxy prothrombin in patients with hepatocellular carcinoma by a revised enzyme immunoassay kit with increased sensitivity. *Cancer* 1999; **85**: 812–8.

19 Nomura F, Ishijima M, Kuwa K, Tanaka N, Nakai T, Ohnishi K. Serum des-gamma-carboxy prothrombin levels determined by a new generation of sensitive immunoassays in patients with small-sized hepatocellular carcinoma. *Am J Gastroenterol* 1999; **94**: 650–4.

20 The Cancer of the Liver Italian Program (CLIP) Investigators. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients. *Hepatology* 1998; **28**: 751–5.

21 Shimizu K, Taniichi T, Satomura S, Matsuura S, Taga H, Taketa K. Establishment of assay kits for determination of microheterogeneities of alpha-fetoprotein using lectin-affinity electrophoresis. *Clin Chim Acta* 1993; **214**: 3–12.
22 Weitz IC, Liebman HA. Des-gamma-carboxy (abnormal) prothrombin and hepatocellular carcinoma: a critical review. *Hepatology* 1993; **18**: 990–7.
23 Liver Cancer Study Group of Japan. *The General Rules for the Clinical and Pathological Study of Primary Liver Cancer*. English edn. Tokyo, Japan: Kanehara & Co., 2003.
24 Berrington de Gonzalez A, Darby S. Risk of cancer from diagnostic X-rays: estimates for the UK and 14 other countries. *Lancet* 2004; **363**: 345–51.

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HEPATOLOGY

Efficacy of antiviral therapy with lamivudine after initial treatment for hepatitis B virus-related hepatocellular carcinoma

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Key words
hepatitis B virus, hepatocellular carcinoma, lamivudine, recurrence, survival.

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Abstract

Aim: The aim of this study was to determine whether antiviral therapy with lamivudine is beneficial in patients after initial treatment for hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC).

Methods: Forty-nine consecutive patients with HBV-related HCC completely treated by hepatic resection or radiofrequency ablation were retrospectively enrolled in this study. Comparison was made between 16 patients who received lamivudine therapy at a dose of 100 mg/day after treatment for HCC (lamivudine group) and 33 patients who did not (control group) in terms of changes in remnant liver function, HCC recurrence and survival.

Results: Cumulative recurrence rates of HCC did not significantly differ between the two groups ($P = 0.622$). However, median Child–Pugh score at the time of HCC recurrence was significantly different; 5 (range 5–6) in the lamivudine group versus 7 (range 5–12) in the control group ($P = 0.005$). All patients in the lamivudine group were able to receive curative treatment for recurrent HCC. In contrast, 10 of 15 patients in the control group were unable to receive curative optimal therapy for recurrent HCC due to deterioration of remnant liver function. The cumulative survival rates of patients in the lamivudine group tended to be higher than those of patients in the control group ($P = 0.063$).

Conclusion: It is suggested that lamivudine therapy is beneficial for patients after initial treatment for HBV-related HCC because it contributes to improving remnant liver function, thus decreasing the risk of liver failure and increasing the chances of receiving available treatment modalities for recurrent HCC.

Introduction

Hepatocellular carcinoma (HCC) has a characteristically high rate of recurrence, including intrahepatic and multicentric recurrences, even if treatment for HCC results in complete curative response, due to the underlying chronic liver disease.[1–3] The frequent recurrence of HCC contributes to short survival because repeated curative treatment is often difficult due to deterioration of liver function. There are several established treatment options for HCC. Generally, hepatic resection, percutaneous ethanol injection therapy (PEIT), percutaneous microwave coagulation therapy (PMCT), radiofrequency ablation (RFA) and cadaveric or living-related liver transplantation are recognized as curative treatment options for HCC. However, patients with HCC can not always receive optimal treatment, as treatment choice is limited by remnant liver function.[4–6] Treatment options in patients with cirrhosis are often restricted, and many patients cannot receive optimal curative treatments, as such treatments may lead to severe hepatic decompensation. In order to have optimal treatment options when HCC recurs, it is very important that remnant liver function is improved or well maintained after treatment for initial HCC.

Several studies have recently reported that lamivudine, a nucleoside analog that inhibits the reverse transcriptase activity of viral DNA polymerase, has been useful for hepatitis B virus (HBV)-infected patients; not only for patients with chronic hepatitis but also those with decompensated liver cirrhosis.[7–13] These studies reported that lamivudine treatment consistently reduced HBV replication, and thus might improve remnant liver function, prevent liver failure and prolong survival.

The prevention of recurrent HCC in patients after curative treatment is important in order to improve prognosis.[14,15] Recently, there have been reports regarding the effects of lamivudine on the prevention of initial HCC.[16,17] Liaw *et al.* recently documented that lamivudine reduced not only the risk of hepatic decompensation but also the risk of initial HCC among patients with chronic HBV

and advanced hepatic fibrosis.[16] To our knowledge, however, there are very few reports that have documented whether lamivudine therapy is beneficial with regard to the incidence of recurrent HCC or survival after initial HCC treatment.[18]

In the present study, we retrospectively evaluated the efficacy of antiviral therapy with lamivudine in patients judged as having complete curative response to initial treatment for HBV-related HCC by comparing changes in remnant liver function, incidence of HCC recurrence and survival between patients who started lamivudine therapy after initial HCC treatment and those who did not.

Methods

Between December 1998 and December 2004, a total of 105 patients with chronic HBV infection were diagnosed as having initial HCC (not recurrence) and were treated at the Department of Gastroenterology, Nagoya University School of Medicine or the Department of Gastroenterology, Ogaki Municipal Hospital. All patients were positive for hepatitis B surface antigen (HBsAg) and were not positive for hepatitis C virus antibody. Serum HBV-DNA was detected in all but three patients.

The inclusion criteria for this study were as follows: (i) patients who did not receive lamivudine therapy prior to diagnosis of initial HCC; (ii) patients who underwent hepatic resection or RFA for initial HCC treatment; and (iii) patients who were judged as having complete curative response 1 month after initial HCC treatment. Dynamic computed tomography (CT) was performed at 1 month after initial treatment of HCC in all patients in order to assess the therapeutic effects; no enhancement in the treated area was considered to indicate complete curative response.

Of 105 patients, 49 patients meeting the inclusion criteria were enrolled. They comprised 41 men and 8 women (mean age, 60.6 ± 9.2 years). Thirty-one patients who underwent hepatic resection were histologically confirmed as having HCC, and 18 patients who received RFA were diagnosed as having HCC by ultrasonography (US), dynamic CT, arterial angiography and angiography-assisted CT. None of the 49 patients exhibited extrahepatic metastasis and/or vessel invasion. HCC Stage was determined according to the criteria of the Liver Cancer Study Group of Japan.[19] None of the patients received any antiviral drugs, such as interferon, for at least 1 year prior to lamivudine administration.

Of the 49 patients, 16 received lamivudine (Zeffix, Glaxo-Smith-Kline, UK) at a dose of 100 mg/day (lamivudine group) for as long as possible. The remaining 33 patients did not receive lamivudine (control group).

Biochemical tests, including alanine aminotransferase (ALT), prothrombin time (PT), albumin, total bilirubin and platelet count, were performed using standard methods. HBsAg, hepatitis B envelop antigen (HBeAg) and hepatitis B envelop antibody (HBeAb) were determined by enzyme immunoassay. HBV-DNA was quantified by PCR assay (Amplicor HBV monitor assay, Roche Diagnostics, Manheim, Germany). The lower limit of the assay was 2.6 log copies/mL. These parameters were measured every 1–3 months during lamivudine treatment.

All patients were followed primarily with abdominal US and liver function tests, as well as measurement of tumor markers, serum α-fetoprotein and des-gamma-carboxy prothrombin, at 1- to 3-month intervals after initial treatment for HCC. When suspicious findings on US or tumor markers were detected, dynamic CT was performed in order to examine recurrent HCC. Angiography-assisted CT was performed whenever possible.

Data analyses were performed using the JMP statistical software package, version 4.0 (SAS Institute, Cary, NY, USA.). Continuous data are expressed as mean values ± SD. HBV-DNA levels and Child–Pugh scores are given as median values with ranges. For qualitative variables, chi-squared test or Fisher's exact probability test was performed where appropriate. For continuous variables, Student's *t*-test or Mann–Whitney *U*-test was performed where appropriate. Cumulative recurrence and survival rates were calculated by the Kaplan–Meier method from the date of initial HCC treatment, and differences between two groups were compared by log-lank test. A *P*-value of <0.05 was considered statistically significant.

This study was approved by the ethics committees of both hospitals and was performed in compliance with the Helsinki declaration.

Results

Background characteristics

Background characteristics at the time of initial HCC treatment for the lamivudine and control groups are summarized in Table 1. There were no significant differences among the two groups with regard to age, sex, HBeAg, ALT, PT, albumin, total bilirubin, platelet count, presence of ascites, hepatic encephalopathy, Child–Pugh score, stage of initial HCC, initial HCC treatment and follow-up period. However, there was a significant difference with respect to HBV-DNA among the two groups. Median HBV-DNA levels in the lamivudine group (6.2 log copies/mL, range 2.8–8.3) were significantly higher than those in the control group (4.1 log copies/mL, range 2.6–7.1) ($P = 0.003$).

Comparison of cumulative HCC recurrence rates between lamivudine and control groups

Of the 49 patients, 22 (7 in the lamivudine group and 15 in the control group) experienced HCC recurrence during the follow-up period. The mean period until recurrence from initial treatment was 26.3 ± 21.6 months (range 4.7–56.9) in the lamivudine group and 18.7 ± 9.1 months (range 6.0–36.3) in the control group ($P = 0.250$). The sites of recurrent HCC in all 22 patients were areas of the liver distant from those initially treated for HCC.

The cumulative HCC recurrence rates in the two groups are shown in Fig. 1. Overall, cumulative HCC recurrence rates at 1, 2 and 3 years were 15.8%, 35.3% and 47.3%, respectively. The cumulative HCC recurrence rates at 1, 2 and 3 years in the lamivudine group were 13.5%, 35.1% and 35.1%, respectively, while those in the control group were 13.4%, 39.2% and 53.2%, respectively. There were no significant differences regarding the recurrence rates of HCC between the two groups ($P = 0.622$).

Biochemical and virological response, serological status and emergence of YMDD mutants in lamivudine group

In the lamivudine group, the mean lamivudine treatment period was 22.7 ± 14.2 months (range 6.3–54.8). Serial changes in bio-

Table 1 Background characteristics at the time of initial HCC treatment for the lamivudine and control groups

Characteristic	Lamivudine group ($n = 16$)	Control group ($n = 33$)	*P*-value
Age (years)	59.8 ± 7.8	61.1 ± 9.8	NS
Sex (men/women)	14/2	27/6	NS
HBeAg (+/–)	4/12	2/27	NS
HBV-DNA (log copies/mL)	6.2 (2.8–8.3)	4.1 (2.6–7.1)	0.003
ALT (IU/L)	56.6 ± 25.7	54.2 ± 44.8	NS
Total bilirubin (mg/dL)	0.8 ± 0.3	0.9 ± 0.4	NS
Albumin (g/dL)	3.7 ± 0.6	3.7 ± 0.5	NS
Prothrombin time (%)	85.3 ± 15.4	85.0 ± 15.1	NS
Platelet count ($\times 10^4$/mL)	10.8 ± 4.2	12.1 ± 5.4	NS
Ascites (+/–)	14/2	32/1	NS
Hepatic encephalopathy (+/–)	15/1	32/1	NS
Child–Pugh score	5 (5–10)	5 (5–8)	NS
Stage of initial HCC (I/II/III)	12/3/1	13/16/4	NS
Initial treatment for HCC (Ope/RFA)	13/3	18/15	NS
Follow-up period (month)	38.0 ± 21.6	32.6 ± 18.9	NS

ALT, alanine aminotransferase; HBeAg, hepatitis B envelop antigen; HBV-DNA hepatitis B virus DNA; HCC, hepatocellular carcinoma; NS, not significant; Ope, operation; RFA, radiofrequency ablation.
Values shown as mean ± SD, number of patients, or median (range).

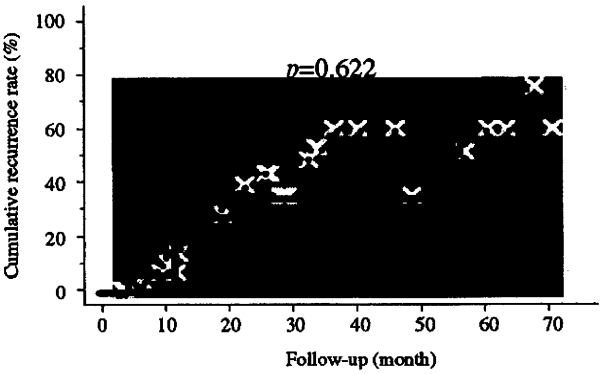

Figure 1 Comparison of cumulative hepatocellular carcinoma recurrence rates in (—) the lamivudine group, $n = 16$, and (- - -) the control group, $n = 33$.

chemical data, including ALT, total bilirubin, albumin, PT, platelet counts and serial changes in HBV-DNA levels before, and at 1, 3, 6, 9 and 12 months after lamivudine administration were investigated and are summarized in Table 2 (including 2 patients with less than 1 year of lamivudine administration). In comparison with those at the start of lamivudine administration, albumin levels gradually but significantly increased, and ALT levels gradually but significantly decreased. Total bilirubin, PT and platelet levels did not show any significant changes.

HBV-DNA levels decreased significantly when compared with those before the start of lamivudine administration. Two of four patients in the lamivudine group who were positive for HBeAg at the start of lamivudine administration exhibited seroconversion to HBeAb positivity. The emergence of YMDD mutants was observed in five of 16 patients in the lamivudine group (31.6%). Of these, three patients maintained stable liver function, while two exhibited breakthrough hepatitis, which was controlled after adefovir dipivoxil administration at a dose of 10 mg/day. There were no serious adverse effects during lamivudine therapy.

We compared the serial changes in ALT levels and HBV-DNA levels during the year of lamivudine administration between seven patients with HCC recurrence and nine patients without HCC recurrence in the lamivudine group. Serial changes are shown in Fig. 2; there were no significant differences among serial changes in ALT levels and HBV-DNA levels ($P = 0.832$ and $P = 0.290$, respectively).

Comparison of data at the time of initial HCC and at the time of recurrent HCC for the patients with recurrent HCC

We compared ALT levels, HBV-DNA levels and Child–Pugh scores at the time of initial HCC and recurrent HCC in 22 patients with recurrent HCC.

Data for the seven patients in the lamivudine group with recurrent HCC are summarized in Table 3. Mean ALT levels decreased significantly from 56.1 ± 25.3 IU/L (range 32–104) at the time of initial HCC to 36.3 ± 8.1 IU/L (range 22–48) at the time of recurrent HCC ($P = 0.028$). Median HBV-DNA levels were 6.3 log copies/mL (range 4.2–8.3) at the time of initial HCC, while those at the time of recurrent HCC were undetectable (<2.6 log copies/mL) ($P = 0.018$). Median Child–Pugh scores were not significantly different; 5 (range 5–10) at initial HCC versus 5 (range 5–6) at the time of recurrent HCC. All seven patients were able to receive curative treatment for recurrent HCC and experienced complete therapeutic response.

Data for the 15 patients in the control group with recurrent HCC are summarized in Table 4. With regard to mean ALT levels and median HBV-DNA levels, there were no significant differences between those at the time of initial HCC and those at the time of recurrent HCC. Median Child–Pugh scores increased significantly from 5 (range 5–8) at the time of initial HCC to 7 (range 5–12) at the time of recurrent HCC ($P = 0.013$). Five of 15 patients were able to receive curative treatment (RFA) for recur-

Table 2 Serial changes in biochemical data and HBV-DNA levels before, and at set time points after lamivudine administration

Variable	Pretreatment	1 month	3 months	6 months	9 months	12 months
ALT (IU/L)	56.6 ± 25.7	58.9 ± 38.3	44.1 ± 35.9*	39.9 ± 21.9*	38.7 ± 21.1*	35.9 ± 15.6*
Total bilirubin (mg/dL)	0.88 ± 0.30	0.95 ± 0.58	0.92 ± 0.35	0.86 ± 0.40	0.86 ± 0.28	0.99 ± 0.40
Albumin (g/dL)	3.66 ± 0.63	3.69 ± 0.43	3.82 ± 0.39	3.83 ± 0.35	3.89 ± 0.35	3.97 ± 0.28*
Prothrombin time (%)	85.3 ± 15.4	88.8 ± 14.3	90.6 ± 15.0	85.6 ± 12.3	90.0 ± 14.1	88.3 ± 16.9
Platelet count (x10^4/mL)	10.8 ± 4.2	11.0 ± 4.1	10.4 ± 3.8	9.8 ± 3.3	10.0 ± 4.5	10.4 ± 3.6
HBV-DNA (log copies/mL)	6.2 (2.8–8.3)	4.1 (2.6–6.2)*	2.6 (2.6–4.0)*	2.6 (2.6–3.6)*	2.6 (2.6–3.2)*	2.6 (2.6–3.7)*

*$P <$ 0.05 *vs* pretreatment data. ALT, alanine aminotransferase; HBV-DNA, hepatitis B virus DNA.
Values shown as mean ± SD, or median (range).

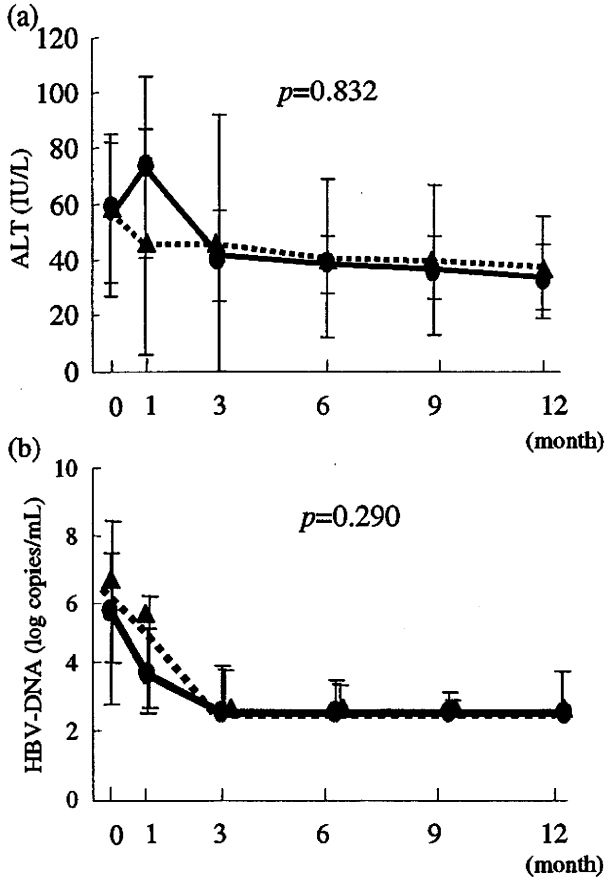

Figure 2 Serial changes in (a) alanine aminotransferase (ALT) levels and (b) hepatitis B virus (HBV)-DNA levels during the course of lamivudine administration in (●, —) seven patients with recurrent hepatocellular carcinoma (HCC) and (▲, - - -) nine patients without recurrent HCC.

rent HCC, but the remaining 10 patients were not able to receive optimal curative therapy, hepatic resection or RFA, for recurrent HCC due to deterioration of remnant liver function. Of the seven patients receiving transcatheter arterial embolization (TAE) for recurrent HCC, four received TAE that was not satisfactorily performed due to deterioration of remnant liver function. The remaining three patients were unable to receive any treatment for recurrent HCC due to poor liver function.

Data at the time of recurrent HCC comparing the lamivudine group and the control group are summarized in Table 5. When compared with data for the control group, median HBV-DNA levels were significantly reduced ($P = 0.023$) and median Child–Pugh scores were significantly lower ($P = 0.005$) in the lamivudine group. With regard to treatment for recurrent HCC, patients in the lamivudine group were significantly more able to receive curative treatments, such as hepatic resection or RFA ($P = 0.014$). There were no significant differences in mean ALT levels and stage of recurrent HCC.

Comparison of cumulative survival rates between lamivudine and control groups

The mean follow-up period was 38.0 ± 21.6 months (range 9.2–78.2) for the lamivudine group and 32.6 ± 18.9 months (range 0.5–75.7) for the control group ($P = 0.378$). The cumulative survival rates in the two groups are shown in Fig. 3. All 16 patients in the lamivudine group remained alive during the follow-up period. However, six of 33 patients in the control group died. The cumulative survival rates at 1, 2 and 3 years in the control group were 13.4%, 39.2% and 53.2%, respectively.

There were no significant differences with regard to survival rate between the two groups; however, the survival rates in the lamivudine group tended to be higher than those in the control group ($P = 0.063$). As for cause of death, in the control group, three patients died of progressive liver failure and the other three patients died of HCC progression.

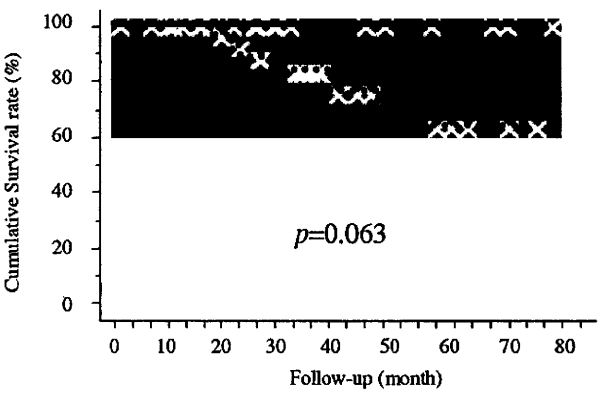

Figure 3 Comparison of cumulative survival rates in the (—) the lamivudine group, $n = 16$, and (- - -) the control group, $n = 33$.

Table 3 Comparison of data at initial and recurrent HCC for the 7 patients in the lamivudine group with recurrent HCC

Variable	Initial HCC	Recurrent HCC	*P*-value
HBV-DNA (log copies/mL)	6.3 (4.2–8.3)	<2.6	0.018
ALT (IU/L)	56.1 ± 25.3 (32–104)	36.3 ± 8.1 (22–48)	0.028
Child–Pugh score	5 (5–10)	5 (5–6)	NS
HCC stage (I/II/III)	5/1/1	4/3/0	NS
Treatment for recurrent HCC (Ope/RFA/TAE/None)	5/2/0/0	2/5/0/0	NS

ALT, alanine aminotransferase; HBV-DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; NS, not significant; Ope, operation; RFA, radiofrequency ablation; TAE, transcatheter arterial embolization.
Values shown as mean ± SD, number of patients, or median (range).

Table 4 Comparison of data at initial and recurrent HCC for the 15 patients in the control group with recurrent HCC

Variable	Initial HCC	Recurrent HCC	*P*-value
HBV-DNA (log copies/mL)	4.9 (3.2–6.4)	4.2 (2.6–6.1)	NS
ALT (IU/L)	66.1 ± 49.2 (17–207)	58.4 ± 41.2 (18–160)	NS
Child–Pugh score	5 (5–8)	7 (5–12)	0.013
HCC stage (I/II/III)	5/6/4	7/8/0	NS
Treatment for recurrent HCC (Ope/RFA/TAE/None)	5/10/0/0	0/5/7/3	0.001

ALT, alanine aminotransferase; HBV-DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; NS, not significant; Ope, operation; RFA, radiofrequency ablation; TAE, transcatheter arterial embolization.
Values shown as mean ± SD, number of patients, or median (range).

Table 5 Comparison of data at the time of recurrent HCC in the lamivudine and control groups ($n = 22$)

Variable	Lamivudine ($n = 7$)	Control ($n = 15$)	*P*-value
HBV-DNA (log copies/mL)	<2.6	4.2 (2.6–6.1)	0.023
ALT (IU/L)	36.3 ± 8.1 (22–48)	58.4 ± 41.2 (18–160)	NS
Child–Pugh score	5 (5–6)	7 (5–12)	0.005
HCC stage (I/II/III)	4/3/0	7/8/0	NS
Treatment for recurrent HCC (Ope/RFA/TAE/None)	2/5/0/0	0/5/7/3	0.014

ALT, alanine aminotransferase; HBV-DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; NS, not significant; Ope, operation; RFA, radiofrequency ablation; TAE, transcatheter arterial embolization.
Values shown as mean ± SD, number of patients, or median (range).

Discussion

There have been several studies investigating whether antiviral therapy with interferon is useful in reducing the incidence of HBV-related HCC.[3,20–23] Some of these studies reported that interferon was beneficial for preventing HCC only in patients achieving sustained suppression of HBV replication. Lin *et al.* conducted a randomized controlled trial in order to evaluate the effectiveness of interferon alpha with 101 HBeAg-positive Taiwanese men.[20] They found that HCC occurred in one (1.5%) of the 67 treated patients and four (12%) untreated patients ($P = 0.047$) over a period of 1–12 years. Multivariate analysis revealed that interferon therapy, preexisting cirrhosis and patient age at entry were significant independent factors for HCC development. In contrast, other reports have demonstrated that the incidence of HCC in patients undergoing interferon therapy does not significantly vary between responders and non-responders.[21,23] Thus, the effects of interferon on the prevention of HCC have not yet been adequately clarified, possibly because the therapeutic efficacy of interferon is low.

There have been some recent reports regarding the efficacy of lamivudine treatment in reducing the incidence of HCC.[16,17] Liaw *et al.* conducted a prospective randomized controlled trial in order to evaluate the efficacy of lamivudine therapy in HBV patients, with incidence of HCC as an endpoint.[16] They reported that among 651 HBsAg-positive Asian patients, HCC occurred in 17 (3.9%) lamivudine-treated patients and 16 (7.4%) placebo controls, with a hazard ratio of 0.49 ($P = 0.047$) during a median follow-up of 32 months (range 0–42). They concluded that lamivudine treatment in chronic HBV patients not only reduces the incidence of hepatic decompensation but also reduces the incidence of HCC.

With regard to the incidence of recurrent HBV-related HCC, Lin *et al.* also conducted a prospective randomized controlled study in order to evaluate the effectiveness of interferon alpha with 16 HBV patients after medical ablation therapy for primary tumors.[3] They found that HCC recurred in four of four (100%) untreated patients and in four of 12 (33.3%) interferon alpha-treated patients ($P = 0.0384$). They concluded that interferon alpha therapy may reduce HCC recurrence after medical ablation for primary HCC, although the sample size was too small to reach a firm conclusion.

There are few reports regarding the effects of lamivudine therapy on recurrent HCC.[18] In the present study, the cumulative recurrence rates of HCC after initial and complete treatment for HCC did not significantly differ between the lamivudine group and the control group. These results are consistent with the recurrence rates recently reported by Piao *et al.*[18] In the present study, among 16 patients receiving lamivudine, serial changes in ALT and HBV-DNA levels in seven patients with recurrent HCC were similar to those in nine patients without recurrent HCC. These results indicate that lamivudine treatment after initial HCC treatment reduces both ALT levels and HBV-DNA levels, but is not associated with the incidence of recurrent HCC. This is not consistent with previous reports that have documented antiviral therapy contributing to reduced incidences of HCC.[14,20,22,24] However, the present study is insufficient to support a conclusion on whether lamivudine therapy is useful in preventing recurrent HCC.

In the present study, in order to evaluate whether lamivudine contributes to preventing the incidence of recurrent HCC as accurately as possible, we selected 49 consecutive patients successfully treated for initial HCC by hepatic resection or RFA. In patients in whom HCC recurred, the locations of recurrent HCC were all distant from the site of primary HCC, but it was actually difficult to distinguish whether recurrent HCC was derived from intrahepatic metastasis or multicentric occurrence. Previous reports have documented that within a few years of HCC treatment, undetectable intrahepatic metastases are already present at distant sites of the liver.[25] In the present study, 19 of 49 patients (47.3%) had recurrence within 3 years. Our study therefore might have included patients with recurrent HCC due to intrahepatic metastasis. This may be one of the reasons for a lack of differences regarding recurrence rates between the lamivudine and control groups. Further studies with larger numbers of patients and longer follow-up periods are necessary to clarify whether lamivudine is able to prevent HCC. Such studies may clarify whether decreasing ALT and HBV-DNA levels during lamivudine administration contribute to reduced incidence of multicentric HCC recurrence.

To our knowledge, this is the first report regarding liver function and HBV-DNA levels at the time of HCC recurrence. In the lamivudine group, median Child–Pugh scores at the time of HCC recurrence were significantly lower than those in the control group. In the lamivudine group, due to good remnant liver function, all seven patients with recurrence were able to receive optimal and curative treatment, such as hepatic resection or RFA. As a result, all experienced good local control of recurrent HCC. We believe that these results account for the improvement in cumulative survival rates among patients in the lamivudine group. All seven patients with recurrent HCC in the lamivudine group had serum HBV-DNA levels of less than 2.6. In contrast, in the control group, some patients were not able to receive curative treatment at the time of HCC recurrence due to their poor remnant liver function, and three patients were unable to receive any treatment. Our most important finding was that remnant liver function in patients with lamivudine was restored or well maintained at the time of recurrent HCC, and this allowed patients to receive curative treatment, resulting in improved survival.

In the present study, there were no serious adverse effects as a result of lamivudine therapy. The most significant problem associated with lamivudine therapy is the emergence of YMDD mutants.[26,27] These induce a relapse of hepatitis (breakthrough hepatitis) and can result in fatal liver failure. Adefovir dipivoxil and entecavir have recently been introduced in an effort to treat breakthrough hepatitis.[26,27] In the present study, the emergence of YMDD mutants was observed in five of 16 patients in the lamivudine group, and two of these developed breakthrough hepatitis. However, after administration of adefovir dipivoxil, breakthrough hepatitis was successfully controlled. None of the five patients developed recurrent HCC. Because new and emerging antiviral agents are increasingly available, beginning lamivudine administration after initial HCC treatment can be recommended for patients with active viral status or ALT elevation in order to prevent deterioration of remnant liver function.

In conclusion, we speculate that lamivudine therapy is beneficial for patients after initial treatment for HBV-related HCC because it contributes to improving remnant liver function, thus decreasing the risk of liver failure and increasing the chances of receiving available treatment modalities for recurrent HCC. With regard to impact on prevention of recurrent HCC, we did not observe any differences between patients who received lamivudine therapy and those who did not. However, the present study had a small sample, a short follow-up period and was retrospective. Therefore, confirmation of our findings requires further studies with larger numbers of patients and longer follow-up periods.

References

1 Kumada T, Nakano S, Takeda I *et al.* Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. *Hepatology* 1997; **25**: 87–92.

2 Ou DP, Yang LY, Huang GW, Tao YM, Ding X, Chang ZG. Clinical analysis of the risk factors for recurrence of HCC and its relationship with HBV. *World J. Gastroenterol.* 2005; **11**: 2061–6.

3 Lin SM, Lin CJ, Hsu CW *et al.* Prospective randomized controlled study of interferon-alpha in preventing hepatocellular carcinoma recurrence after medical ablation therapy for primary tumors. *Cancer* 2004; **100**: 376–82.

4 Blum HE. Hepatocellular carcinoma: therapy and prevention. *World J. Gastroenterol.* 2005; **11**: 7391–400.

5 Toyoda H, Kumada T, Kiriyama S *et al.* Changes in the characteristics and survival rate of hepatocellular carcinoma from 1976 to 2000: analysis of 1365 patients in a single institution in Japan. *Cancer* 2004; **100**: 2415–21.

6 Bruix J, Sherman M, Llovet JM *et al.* Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J. Hepatol.* 2001; **35**: 421–30.

7 Papatheodoridis GV, Dimou E, Dimakopoulos K *et al.* Outcome of hepatitis B e antigen-negative chronic hepatitis B on long-term nucleos(t)ide analog therapy starting with lamivudine. *Hepatology* 2005; **42**: 121–9.

8 Dienstag JL, Goldin RD, Heathcote EJ *et al.* Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003; **124**: 105–17.
9 Yao FY, Terrault NA, Freise C, Maslow L, Bass NM. Lamivudine treatment is beneficial in patients with severely decompensated cirrhosis and actively replicating hepatitis B infection awaiting liver transplantation: a comparative study using a matched, untreated cohort. *Hepatology* 2001; **34**: 411–16.
10 Bae SH, Yoon SK, Choi JY *et al.* Timing of lamivudine administration according to Child class in patients with decompensated cirrhosis. *J. Gastroenterol. Hepatol.* 2005; **20**: 1527–32.
11 Tseng PL, Lu SN, Tung HD, Wang JH, Changchien CS, Lee CM. Determinants of early mortality and benefits of lamivudine therapy in patients with hepatitis B virus-related decompensated liver cirrhosis. *J. Viral. Hepat.* 2005; **12**: 386–92.
12 Villeneuve JP, Condreay LD, Willems B *et al.* Lamivudine treatment for decompensated cirrhosis resulting from chronic hepatitis B. *Hepatology* 2000; **31**: 207–10.
13 Kapoor D, Guptan RC, Wakil SM *et al.* Beneficial effects of lamivudine in hepatitis B virus-related decompensated cirrhosis. *J. Hepatol.* 2000; **33**: 308–12.
14 Lok AS. Prevention of hepatitis B virus-related hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S303–9.
15 Kubo S, Hirohashi K, Tanaka H *et al.* Effect of viral status on recurrence after liver resection for patients with hepatitis B virus-related hepatocellular carcinoma. *Cancer* 2000; **88**: 1016–24.
16 Liaw YF, Sung JJ, Chow WC *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N. Engl. J. Med.* 2004; **351**: 1521–31.
17 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.
18 Piao CY, Fujioka S, Iwasaki Y *et al.* Lamivudine treatment in patients with HBV-related hepatocellular carcinoma—using an untreated, matched control cohort. *Acta Med. Okayama* 2005; **59**: 217–24.
19 Liver Cancer Study Group of Japan. *Classification of Primary Liver Cancer*, 1st English edn. Tokyo: Kanehara & Company, 1997.
20 Lin SM, Sheen IS, Chien RN, Chu CM, Liaw YF. Long-term beneficial effect of interferon therapy in patients with chronic hepatitis B virus infection. *Hepatology* 1999; **29**: 971–5.
21 Lampertico P, Del Ninno E, Vigano M *et al.* Long-term suppression of hepatitis B e antigen-negative chronic hepatitis B by 24-month interferon therapy. *Hepatology* 2003; **37**: 756–63.
22 van Zonneveld M, Honkoop P, Hansen BE *et al.* Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. *Hepatology* 2004; **39**: 804–10.
23 Camma C, Giunta M, Andreone P, Craxi A. Interferon and prevention of hepatocellular carcinoma in viral cirrhosis: an evidence-based approach. *J. Hepatol.* 2001; **34**: 593–602.
24 Chen CJ, Yang HI, Su J *et al.* Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65–73.
25 Sakon M, Umeshita K, Nagano H *et al.* Clinical significance of hepatic resection in hepatocellular carcinoma: analysis by disease-free survival curves. *Arch. Surg.* 2000; **135**: 1456–9.
26 Liaw YF. Management of YMDD mutations during lamivudine therapy in patients with chronic hepatitis B. *J. Gastroenterol. Hepatol.* 2002; **17**: S333–7.
27 Lampertico P, Vigano M, Manenti E, Iavarone M, Lunghi G, Colombo M. Adefovir rapidly suppresses hepatitis B in HBeAg-negative patients developing genotypic resistance to lamivudine. *Hepatology* 2005; **42**: 1414–19.

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Protein kinase C δ regulates the phosphorylation of heat shock protein 27 in human hepatocellular carcinoma

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Abstract

We have recently reported that attenuated phosphorylation of heat shock protein (HSP) 27 correlates with tumor progression in patients with hepatocellular carcinoma (HCC). In the present study, we investigated what kind of kinase regulates phosphorylation of HSP27 in human HCC-derived HuH7 cells. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 1-oleoyl-2-acetylglycerol, direct activators of protein kinase C (PKC), markedly strengthened the phosphorylation of HSP27. Bisindorylmaleimide I, an inhibitor of PKC, suppressed the TPA-induced levels of HSP27 phosphorylation in addition to its basal levels. Knock down of PKCδ suppressed HSP27 phosphorylation, as well as p38 mitogen-activated protein kinase (MAPK) phosphorylation. SB203580, an inhibitor of p38 MAPK, suppressed the TPA-induced HSP27 phosphorylation. Our results strongly suggest that activation of PKCδ regulates the phosphorylation of HSP27 via p38 MAPK in human HCC.

Keywords: PKCδ; HSP27; Phosphorylation; Protein kinase; Hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is characterized by its high incidence in hepatitis virus-associated liver disease, reaching approximately 3% in hepatitis B virus-infected cirrhotic patients and 7% in hepatitis C virus-infected cirrhotic patients (Ikeda et al., 1993; Shiratori et al., 2001). Moreover, the incidence of post-therapeutic recurrence is approximately 20% to 25% a year in cirrhotic patients who have already undergone curative treatment of the primary HCC (Kumada et al., 1997; Koda et al., 2000). Thus, overall survival of patients with HCC is still unsatisfactory even after hepatectomy. Therefore, it is required to clarify the further exact mechanism of HCC carcinogenesis.

Heat shock proteins (HSP) are produced by cells exposed to biological stressors such as heat and chemicals (Shimada et al., 1998). HSPs are classified as high-molecular-weight HSPs, such as HSP70, HSP90, and HSP110, or low-molecular-weight HSPs, which have molecular masses from 10 to 30 kDa. High-molecular-weight HSPs act as molecular chaperones in protein folding, oligomerization, and translocation (Benjamin and McMillan, 1998). Though the functions of low-molecular-weight HSPs, such as HSP27 and αB-crystallin, are not as well-characterized as those of the high-molecular-weight HSPs, it is thought that they may also have chaperone functions (Benjamin and McMillan, 1998). It is recognized that HSP27 activity is regulated by post-translational modifications such as phosphorylation (Welch, 1985; Benjamin and McMillan, 1998). Mouse HSP27 is phosphorylated at two serine residues (Ser-15 and Ser-82), whereas human HSP27 is phosphorylated at three serine residues (Ser-15, Ser-78, and Ser-82) (Benjamin and McMillan, 1998). It was also reported that phosphorylated HSP27 is translocated from the cytosol to the nucleus in hippocampal progenitor cells, and prevents apoptosis (Geum et al., 2002). In a recent study (Yasuda et al., 2005), we have shown

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that the levels of phosphorylated HSP27 were correlated inversely with tumor stage by TNM classification in patients with HCC. It has been reported that HSP27 phosphorylation is catalyzed by the mitogen-activated protein kinase (MAPK) superfamily (p38 MAPK, phospho-stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p44/p42 MAPK) (Kyriakis and Avruch, 1996; Guay et al., 1997; Benjamin and McMillan, 1998). In addition, it has been reported that p38 MAPK and p44/p42 MAPK are activated in HCC and contribute to the acceleration of the cell cycle (Ito et al., 1998; Iyoda et al., 2003).

Protein kinase C (PKC) is reportedly an upstream regulator of MAPK superfamily cascade (Noguchi et al., 1993; Tanaka et al., 2003; Tokuda et al., 2003). PKC is a Ser/Thr protein kinase family with multiple isoforms, its isoforms have been classified into three groups, classical PKC (α, β, γ), novel PKC (δ, ε, η, θ), and atypical PKC (ζ, ι/λ) (Saito et al., 2001). To date, these PKC isotypes are believed to play distinct regulatory roles. Regarding about the low-molecular-weight HSPs, PKC-dependent phosphorylation of low-molecular-weight HSPs by phorbol-esters has been previously described in Hela cells and MCF-7 cells (Arrigo, 1990; Faucher et al., 1993). In addition, it has been demonstrated that Ca^{2+}-independent PKCδ is superior in its ability to phosphorylate low-molecular-weight HSPs compared with a panel of other PKC isoforms in vitro (Maizels et al., 1998). Furthermore, the detection of phosphorylated low-molecular-weight HSPs in the rat corpora lutea of late pregnancy is reportedly associated with abundant and activated PKCδ (Maizels et al., 1998). However, the kinases that regulate phosphorylation of HSP27 in human HCC have not yet been clarified. In the present study, we investigated what kind of kinase regulates phosphorylation of HSP27 in human HCC. Our results strongly suggest that activation of PKCδ regulates the phosphorylation of HSP27 via p38 MAPK in human HCC.

Materials and methods

Materials

12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Oleoyl-2-acetylglycerol (OAG) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). PD98059, bisindolylmaleimide I, and SB203580 were obtained from Calbiochem-Novabiochem (La Jolla, CA). HSP27 antibodies, phospho-HSP27 (Ser-15) antibodies, and phospho-HSP27 (Ser-78) antibodies were purchased from Stressgen Biotechnologies. (Victoria, British Columbia, Canada). Phospho-HSP27 (Ser-82) was purchased from Biomol Research Laboratories. (Plymouth Meeting, PA). β-actin antibodies were purchased from Sigma. Phospho-Raf-1 antibodies, phospho-MEK1/2 antibodies, phospho-p44/p42 MAPK antibodies and p44/p42 MAP kinase antibodies, phospho-p38 MAPK antibodies, p38 MAPK antibodies, phospho-SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-PKC (pan) (βII Ser-660) antibodies, phospho-PKCδ (Thr-505) antibodies, PKCδ antibodies, and phospho-PKCθ (Thr-538) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). ECL Western blot detection system was purchased from Amersham Japan (Tokyo, Japan). The PKCδ siRNA (Silencer® Validated siRNA), PKCε siRNA (Silencer® Pre-designed siRNA) and non-specific control siRNA (Silencer® Negative control #1 siRNA) were obtained from Ambion, Inc. (Austin, TX). siLentFect was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). Other materials and chemicals were obtained from commercial sources. TPA, PD98059, bisindolylmaleimide I, and SB203580 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect assay for HSP27 phosphorylation.

Cell culture

Human HCC-derived HuH7, which were originated from well-differentiated HCC tissues, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HuH7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 90-mm diameter dishes in DMEM containing 10% FCS. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. When indicated, the cells were pre-treated with respective inhibitors and then stimulated with TPA or OAG for the indicated periods. Cell viability was estimated by the trypan blue dye exclusion method. Experiments were performed in triplicate.

Western blotting analysis

The cultured cells were washed twice with phosphate-buffered saline. The cultured cells were then lysed, homogenized and sonicated in lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 ×*g* for 10 min at 4 °C. The linear range of loading volume in Western blotting analysis was tested with serially diluted protein samples. Protein samples (10 μg) were loaded equally to SDS-polyacrylamide gel electrophoresis (PAGE) in respective experiments (except for total HSP27). For the detection of total HSP27, 2.5 μg of proteins were subjected to the each well of the gel. SDS-PAGE was performed by Laemmli (1970) in polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996). Band intensities visualized on X-ray film were determined by integrating the optical density over the band area (band volume) with NIH image software.

siRNA protocol

Transfection was performed according to the manufacturer's protocol (Bio-Rad). Briefly, 5 μl of siLentFect and finally 10 nM siRNA were diluted with FCS-free DMEM, pre-incubated at room temperature for 20 min and then added to the culture medium containing 10% FCS. Cells were incubated at 37 °C for 48 h with siRNA–siLentFect complexes and subsequently harvested for preparation of Western blotting analysis.

Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $p < 0.05$ was considered significant. All data are presented as the mean+S.E. of triplicate determinations. Each experiment was repeated three times with similar results.

Results

Comparisons between phosphorylated levels of p44/p42 MAPK and HSP27 in HuH7 cells

It is recognized that HSP27 phosphorylation is catalyzed by the MAP kinase superfamily (p38 MAPK, SAPK/JNK, and p44/p42 MAPK) (Kyriakis and Avruch, 1996; Guay et al., 1997; Benjamin and McMillan, 1998). It has been reported that p44/p42 MAPK is constantly activated in HCC (Ito et al., 1998). Therefore, we first examined the relationship between p44/p42 MAPK and HSP27 phosphorylation in HuH7 cells. The expression of HSP27 and its phosphorylated form (Ser-78 and Ser-82) were detectable in HuH7 cells (Fig. 1A). In addition, p44/p42 MAPK were constitutively phosphorylated in HuH7 cells (Fig. 1B). To elucidate whether p44/p42 MAPK is involved in the phosphorylation of HSP27 in HuH7 cells, we examined the effect of PD98059, a specific inhibitor of MEK1/2 (Alessi et al., 1995), on the phosphorylated levels of HSP27. Though PD98059 suppressed the phosphorylation of p44/p42 MAPK dose dependently in the range between 10 and 50 μM, the levels of HSP27 phosphorylation were not affected (Fig. 1C).

Effect of PKC activation on the HSP27 phosphorylation in HuH7 cells

It is well-recognized that PKC is an upstream regulator of Raf-1-MEK1/2-p44/p42 MAPK cascade (Noguchi et al., 1993). Thus, we investigated whether PKC is activated in HuH7 cells. PKC activity is controlled by three distinct phosphorylation events (specifically, the threonine 500 in the activation loop, the threonine 641 autophosphorylation site, and the serine 660 hydrophobic site at the carboxy terminus of PKCβII are phosphorylated in vivo) (Keranen et al., 1995). Since we have preliminary confirmed that phospho-PKC (pan) (βII Ser-660) antibodies can detect PKCα/β and PKCε by using the respective antibodies, we used phospho-PKC (pan) (βII Ser-660) antibodies to detect them. PKCα/β and PKCδ were markedly phosphorylated in HuH7 cells (Fig. 2A). To elucidate whether PKC is involved in the phosphorylation of HSP27 in these cells, we examined the effect of bisindolylmaleimide I, an inhibitor of classical PKC and novel PKC (Toullec et al., 1991), on the basal levels of HSP27 phosphorylation. Bisindolylmaleimide I decreased the phosphorylated levels of HSP27 dose dependently in the range between 10 and 50 μM (Fig. 2B). It is well-known that both classical and novel PKC are activated by phorbol-esters such as TPA (Nishizuka, 1991). We next investigated the effect of TPA (Nishizuka, 1991), a direct activator of PKC on the phosphorylated levels of HSP27 in HuH7 cells.

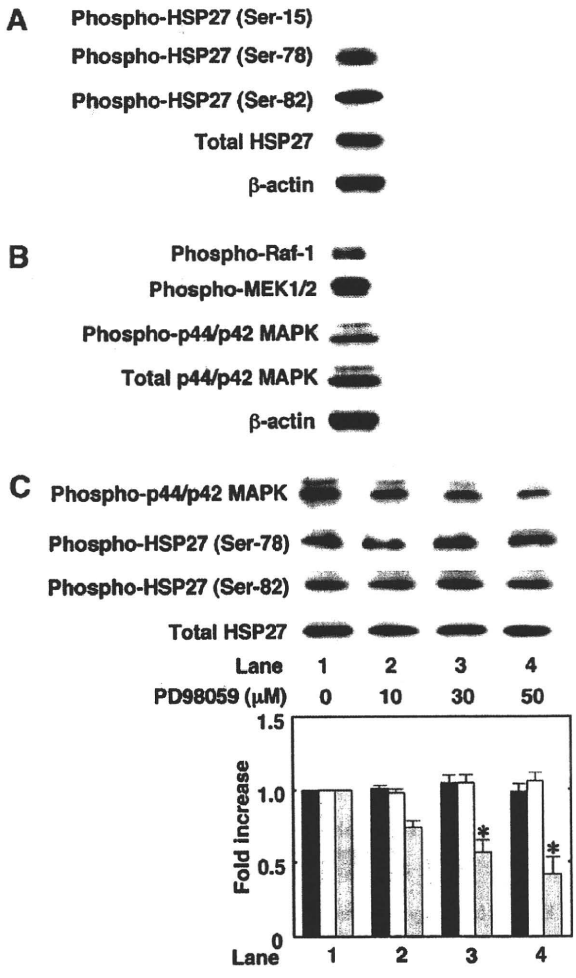

Fig. 1. The levels of HSP27 phosphorylation and the phosphorylated levels of Raf-1-MEK1/2-p44/p42 MAPK cascade, and effect of PD98059 on HSP27 phosphorylation in HuH7 cells. HuH7 were cultured in DMEM containing 10% FCS for 7 days. After 7 days, the medium was exchanged for FCS-free DMEM. The cells were immediately used for experiments. (A) The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-HSP27 (Ser-15), phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82), total HSP27 and β-actin. (B) The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-Raf-1, phospho-MEK1/2, phospho-p44/p42 MAPK, total p44/p42 MAPK and β-actin. (C) The cultured cells were pre-treated with various doses of PD98059 for 60 min, and then washed twice and collected. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-p44/p42 MAPK, phospho-HSP27 (Ser-78), phospho-HSP27 (Ser-82) and total HSP27. The phosphorylated levels were normalized by the levels of total HSP27. The histogram shows the fold increase of levels of phospho-HSP27 (Ser-82) (black bars), phospho-HSP27 (Ser-78) (white bars) and phospho-p44/p42 MAPK (gray bars) in PD98059-treated cells versus those of PD98059-untreated cells. Each value represents the mean±S.E. of triplicate determinations from three independent experiments. Representative results from triplicate independent experiments with similar results are shown. $^*p<0.05$, compared to the value of control.

TPA significantly strengthened the phosphorylated levels of HSP27 in a time-dependent manner (Fig. 2C). The phosphorylated levels reached a peak at 60 min after the TPA-stimulation. TPA stimulated the phosphorylation of HSP27 dose dependently in the range between 0.01 and 0.1 μM, the maximum effect was