Table 3 Risk factors for distant recurrence after TACE

Variables	Univariate		Multivariate	
	Risk ratio (95% CI)	*P* value	Risk ratio (95% CI)	*P* value
AFP (>10 ng/mL)	1.22 (0.40–3.85)	0.721		
DCP (>28 mAU/mL)	1.19 (0.37–3.83)	0.765	1.73 (0.44–7.41)	0.425
Age (≥75 years)	2.07 (0.67–7.67)	0.211	4.30 (0.88–32.60)	0.073
Tumor number (≥2)	3.98 (1.02–13.50)	0.047*	10.64 (1.76–93.75)	0.010*
Child-Pugh score (≥7)	0.70 (0.21–3.21)	0.611	0.71 (0.16–4.21)	0.688
Tumor size (≥20 mm)	1.09 (0.33–3.34)	0.879	1.50 (0.33–7.08)	0.594
Lipiodol uptake (heterogeneous)	1.99 (0.61–6.53)	0.250	1.32 (0.08–15.76)	0.834
Sex	2.30 (0.76–7.66)	0.138		
HCV	0.45 (0.11–3.04)	0.360	0.64 (0.08–7.19)	0.688
HBV	0.49 (0.12–3.27)	0.405		
Alcohol	0.60 (0.19–1.83)	0.365		
Location (within 10 mm from the liver surface)	0.54 (0.17–1.84)	0.304	0.78 (0.04–14.50)	0.864

Abbreviations are the same as those listed in Table 2 footnote

* Significant value

6 months, and 1 year at our institution during the same period were 2.8, 9.6, and 24.5%, respectively.

Heterogeneous Lipiodol uptake and high serum DCP were significantly correlated with local recurrence, whereas the presence of multiple tumors was significantly correlated with intrahepatic distant recurrence.

The risk factors for recurrence after TACE in patients with HCC have been described in several reports. These include the extent of Lipiodol uptake, location of HCC, size of HCC, tumor markers, viral markers, number of HCCs, age, and liver function [11–20]. However, most of these studies were on TACE for advanced HCC. In the present study, we examined the risk factors for early-stage HCC and revealed that only the extent of Lipiodol uptake and the serum DCP level were correlated with local recurrence, which is the most common type of recurrence after TACE. In early-stage HCCs, most of the tumors might be highly differentiated and less invasive, so that the tumors can be controlled merely by complete obstruction of their blood supply. Thus, only high DCP would be an additional risk factor, because HCC with high DCP showed a poorer differentiation grade than HCC with low DCP [22]. Our results suggest that it is better to treat early-stage HCC showing high serum DCP as well as incomplete Lipiodol uptake not only with TACE but also with additional locoregional treatment if possible.

Of note, we found that intrahepatic distant recurrence was observed in patients with multiple tumors, indicating that some of these tumors were intrahepatic metastases and that undetectable small HCCs might have already existed before the TACE was performed.

A meta-analysis showed that chemoembolization could improve the survival of well-selected patients with unresectable HCC [23]; in addition, there is a report that chemoembolization had an effect on HCC even in patients with poor liver function [24]. However, the efficacy of TACE for treating HCC at an early stage has not been well elucidated. Although we could not show a survival benefit of TACE in early-stage HCC, to the best of our knowledge, this is the first report about the outcome of TACE for HCC with 3 tumors or fewer of up to 3 cm.

While new technologies such as artificial pleural effusion and ascites, or real-time virtual sonography (RVS), have increased the number of patients eligible for RFA, the age of HCC patients is gradually increasing, so that, when considering the treatment guideline algorithm for HCC, more patients would be excluded from such treatment owing to the presence of complications and poor performance status, among other factors. Eventually, we may not be able to avoid the selection of palliative TACE instead of RFA and operation. This study helped us to decide whether additional treatment should be considered in patients with difficult conditions according to the treatment algorithms .

In conclusion, palliative TACE could be effective for HCC with 3 tumors or fewer of up to 3 cm. Lipiodol uptake, serum DCP, and the number of tumors (≥2) are the most important risk factors for recurrence in these HCCs treated with palliative TACE. Patients showing heterogeneous Lipiodol uptake after TACE should not be left untreated if at all possible.

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Conflict of interest None.

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HEPATOLOGY

Predicting the treatment effect of sorafenib using serum angiogenesis markers in patients with hepatocellular carcinoma

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Abstract

Background and Aim: Sorafenib, the first agent demonstrated to have efficacy to improve the survival of patients with advanced hepatocellular carcinoma (HCC), is an active multikinase inhibitor affecting angiogenesis and tumor proliferation. We analyzed cytokines related to angiogenesis or cell proliferation, and tried to determine their utility as biomarkers of sorafenib treatment effect for HCC.

Methods: Nine serum cytokines (angiopoietin-2 [Ang-2], follistatin, granulocyte colony-stimulating factor [G-CSF], hepatocyte growth factor [HGF], interleukin-8 [IL-8], leptin, platelet-derived growth factor-BB, platelet endothelial cell adhesion molecule-1, and vascular endothelial growth factor) were measured in 30 HCC patients treated with sorafenib, and the effects of treatment were compared using modified Response Evaluation Criteria in Solid Tumors.

Results: All but IL-8 were significantly higher at baseline in patients with progressive disease. Progression-free survival was significantly shorter in patients with high levels of Ang-2, G-CSF, HGF, and leptin, and the hazard ratios were 2.51, 6.89, 2.55, and 4.14, respectively. As the number of cytokines at a high level increased, the treatment response deteriorated. Disease progression was seen in three of 12 (25.0%) patients with zero to two high biomarkers, two of six (33.3%) patients with 3–5 high biomarkers, and 10 of 12 (83.3%) patients with six to eight high biomarkers ($P = 0.008$). The prognosis of all patients with eight high biomarkers was progressive disease.

Conclusion: High levels of serum cytokines at baseline were correlated with poor effects of sorafenib treatment in patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with more than half a million new cases each year, and it is the third highest cause of cancer-related death globally, behind only lung and stomach cancers.[1,2] Although potentially curative treatments are available for patients diagnosed at early stages, such as radiofrequency ablation, resection, and liver transplantation, disease that is diagnosed at an advanced stage or progresses after locoregional therapy has a dismal prognosis, owing to the underlying liver disease and lack of effective treatment options.[3–5] However, the recent development of molecular-targeted therapies is changing the landscape. The efficacy and safety of sorafenib in advanced HCC patients were assessed in the Sorafenib HCC Assessment Randomized Protocol study, which demonstrated that monotherapy with sorafenib prolonged overall survival (OS) and delayed the time to progression (TTP) in patients with advanced HCC[6]. The efficacy in prolonging OS and TTP in patients from the Asia–Pacific region was confirmed in a phase III randomized, double-blind, placebo-controlled study (Asia–Pacific study).[7]

Sorafenib is an orally-active multikinase inhibitor affecting angiogenesis and tumor proliferation. It inhibits the activity of targets, such as raf, the vascular endothelial growth factor (VEGF) receptor, and the platelet-derived growth factor (PDGF) receptor.[8] Some components of these proliferative and pro-angiogenic signaling pathways, or molecules related to other angiogenesis pathways, can be detected in serum or plasma, and might provide sensitive indicators of tumor activity, response to treatment with sorafenib, and drug-related adverse events.

In the present study, we simultaneously analyzed nine cytokines related to angiogenesis or cell proliferation in the serum of HCC patients before sorafenib treatment, and attempted to determine their utility as biomarkers of the treatment effect.

Methods

Patients and diagnosis of HCC

Between July 2009 and October 2010, 30 of 35 patients with advanced HCC who were treated with sorafenib in our institute and affiliated hospitals, and who could be evaluated for treatment response, were enrolled in this study (Table 1). All of the patients were treated by sorafenib as a first-line therapy. The patients enrolled in this study consisted of 24 males and six females, with a median age of 71.5 years. Hepatitis B surface antigen and anti-hepatitis C virus antibody were positive in eight and 18 patients, respectively. In accordance with the American Association for the Study of Liver Disease guidelines, we confirmed the diagnosis of HCC by at least two dynamic imaging modalities.[4] Written, informed consent was obtained from all patients. This study was approved by the ethical committee of the institutes (approval no. 850).

Treatment regimen, evaluation of response, and adverse events

All patients received continuous oral treatment with sorafenib. Twenty patients received 400 mg twice daily, and 10 patients received 200 mg twice daily. The reasons why some patients received a reduced dose of sorafenib were for due to their high age, high Child–Pugh score, high aminotransferases, or low white blood cell count. Patients were followed every month for routine surveillance, including serological and radiological examinations, such as dynamic computed tomography or magnetic resonance imaging. Patients had at least one untreated target lesion that could be measured in one dimension, and patients were evaluated for radiographic response in the primary tumor and metastatic lesions before and 33.6 ± 9.6 (mean ± standard deviation [SD]) days after starting therapy, in accordance with the conventional and modified Response Evaluation Criteria in solid Tumors (RECIST).[9,10] Adverse events were estimated with the use of version 4.0 of the National Cancer Institute's Common Terminology Criteria for Adverse Events.

Measurement of cytokines

Baseline serum of all patients was collected at the time of admission, just before starting the therapy. Serum was also collected at 7.0 ± 1.1 (mean ± SD) days after starting therapy in 21 patients. Serum samples were centrifuged for 10 min at 1000 × *g* prior to the analysis, and the supernatants were used for the assay.

Table 1 Clinicopathological characteristics of the patients with hepatocellular carcinoma

Variables	Total (*n* = 30)	PD (*n* = 15)	Non-PD (*n* = 15)	*P*-value
Age (years)	71.5 (36–84)	73 (36–84)	66 (63–84)	0.950†
Sex (%)				1.000
Male	24 (80.0)	12 (80.0)	12 (80.0)	
Female	6 (20.0)	3 (20.0)	3 (20.0)	
Viral infection (%)				
HBsAg (positive)	8 (26.7)	3 (20.0)	5 (33.3)	0.682
HCVAb (positive)	18 (60.0)	10 (66.7)	8 (53.3)	0.710
ECOG performance status (%)				1.000
0–1	29 (96.7)	14 (93.3)	15 (100)	
2	1 (3.3)	1 (6.7)	0 (0.0)	
Child–Pugh grade (%)				1.000
A	24 (80.0)	12 (80.0)	12 (80.0)	
B	6 (20.0)	3 (20.0)	3 (20.0)	
T category (%)				1.000
T1–T2	23 (76.7)	11 (73.3)	12 (80.0)	
T3–T4	7 (23.3)	4 (26.7)	3 (20.0)	
N category (%)				1.000
N0	19 (63.3)	10 (66.7)	9 (60.0)	
N1	11 (36.7)	5 (33.3)	6 (40.0)	
M category (%)				0.139
M0	13 (43.3)	4 (26.7)	9 (60.0)	
M1	17 (56.7)	11 (73.3)	6 (40.0)	
TNM stage (%)				0.651
II–III	6 (20.0)	2 (13.3)	4 (26.7)	
IV	24 (80.0)	13 (86.7)	11 (73.3)	
Tumor markers				
AFP (ng/mL)	50 (1.3–8 074)	45 (1.3–8 074)	57.5 (2.2–4 840)	0.852†
AFP-L3 (%)	21.6 (0–88.1)	26.7 (0–84.1)	20.4 (0–88.1)	0.867†
DCP (mAU/mL)	284.5 (15–226 930)	688 (15–11 577)	256 (15–226 930)	0.740†

Values are indicated as median (range) unless otherwise noted. †*P*-values from Wilcoxon rank sum test; all other *P*-values are from Fisher's exact test. AFP, α-fetoprotein; AFP-L3, Lens culinaris-reactive AFP; DCP, des-γ-carboxy prothrombin; ECOG, Eastern Cooperative Oncology Group; HBsAg, hepatitis B surface antigen; HCVAb, anti-hepatitis C virus antibody; PD, progressive disease.

Concentrations of nine molecules (angiopoietin-2 [Ang-2], follistatin [FST], granulocyte colony-stimulating factor [G-CSF], hepatocyte growth factor [HGF], interleukin-8 [IL-8], leptin, PDGF-BB, platelet endothelial cell adhesion molecule-1 [PECAM-1/CD31], and VEGF) were quantified using the BioPlex 200 System (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's protocol. Samples were tested in duplicate, and the mean value was used for the analysis. When out-of-range values were included in the analysis, the highest value across all samples was substituted for values above the upper limit of detection. For values below the lower limit of detection, half the lowest value was substituted.

Statistical analysis

Progression-free survival (PFS) and OS were calculated from the first day of therapy. The Wilcoxon rank sum test was used to compare the continuous data, and Fisher's exact test was used to compare categorical data. The survival of the patients was compared by the Kaplan–Meier method, and the differences were evaluated by the log–rank test. Cox's proportional hazards model was used to analyze hazard ratios (HR).

For these exploratory analyses, $P < 0.05$ was considered significant. All statistical analyses were carried out using JMP statistical software, version 8 (SAS Institute, Cary, NC, USA).

Results

Characteristics of patients

Treatment interruptions and dose reductions of sorafenib were allowed for drug-related toxicity in five and eight patients before radiographic evaluation, and in four and seven patients after the evaluation, respectively. There were no patients who needed treatment interruptions or dose reductions before serum collections at 1 week after starting sorafenib. Of the 30 patients, one had a complete response (CR), six had a partial response (PR), eight had a stable disease (SD), and 15 had a progressive disease (PD), an increase of at least 20% in the sum of the diameters of viable target lesions, according to modified RECIST. One patient with a CR and two patients with PR, by modified RECIST, were classified as having a SD by conventional RECIST, but there was no difference of PD classification between conventional and modified versions of RECIST. Patients who had a CR, PR, or SD in classical or modified RECIST were classified into the non-PD group. The distribution of the TNM stage was five, one, and 24 for TNM II, III, and IV respectively. There were no significant differences in demographics (age, sex), baseline clinical characteristics (viral infection, Eastern Cooperative Oncology Group [ECOG] performance status, Child–Pugh class, T category, N category, M category, TNM stage), and serum tumor markers (α-fetoprotein [AFP], Lens culinaris-reactive AFP, AFP-L3, and des-γ-carboxy prothrombin) between patients with PD and non-PD (Table 1).

Cytokine expression and treatment response

The expression of each cytokine at baseline was classified into two groups: high biomarker and low biomarker (the cut-off values were set at the median of each marker), and was compared with other clinical parameters. There were no significant differences in demographics (age, sex) or baseline clinical characteristics (viral infection, ECOG performance status, Child–Pugh class, T category, N category, M category, TNM stage) between patients with high and low biomarkers for all nine markers examined.

Baseline cytokine levels were compared between patients with PD ($n = 15$) and non-PD ($n = 15$) by Wilcoxon rank sum test, and eight of nine biomarkers were significantly higher in patients with PD (Fig. 1). These were Ang-2, FST, G-CSF, HGF, leptin, PDGF-BB, PECAM-1/CD31, and VEGF. PFS was significantly shorter in the group with high biomarkers of Ang-2, G-CSF, HGF, and leptin (Fig. 2). HR of the markers were 2.51 (95% confidence interval [CI]: 1.01–6.57; $P = 0.048$) for Ang-2, 6.89 (95% CI: 2.29–25.9, $P < 0.001$) for G-CSF, 2.55 (95% CI: 1.03–6.61, $P = 0.042$) for HGF, and 4.14 (95% CI: 1.52–13.3, $P = 0.005$) for leptin.

Subsequently, we examined whether simultaneous elevation of these molecules at baseline could be used as a predictive marker of disease progression after sorafenib treatment. For each patient, the number of biomarkers at high levels (above median) was counted and compared to the treatment response (Fig. 3). There was a tendency for the number to be higher with a worse treatment response. The prognosis of all patients with eight high biomarkers was PD. When the patients were classified into groups with zero to two, three to five, and six to eight high biomarkers, disease progression was seen in three of 12 (25.0%) patients with zero to two high biomarkers, two of six (33.3%) patients with three to five high biomarkers, and 10 of 12 (83.3%) patients with six to eight high biomarkers ($P = 0.008$). PFS was significantly different between these three groups ($P = 0.009$) (Fig. 4). HR of the group with six to eight high biomarkers compared to the other groups was 4.65 (95% CI: 1.59–15.5; $P = 0.005$).

We also measured the changes of nine biomarkers from baseline to 1 week after starting the therapy. Fold changes of cytokine levels were compared between patients with PD ($n = 9$) and non-PD ($n = 12$) by Wilcoxon rank sum test, and only the increase of PDGF-BB was significantly higher in patients with non-PD. The median fold change of PD and non-PD patients were 0.9 and 1.5, respectively ($P = 0.011$).

In addition, no correlation was observed between the cytokine levels and OS.

Baseline cytokine expression and adverse events

We also analyzed the correlations between cytokine levels and the known adverse events of sorafenib, such as hand–foot syndrome, hypertension, and diarrhea; however, no relationship was observed.

Discussion

To the best of our knowledge, this is the first report on the simultaneous measurement of cytokines in advanced HCC treated with sorafenib with a comparison of the response evaluated by conventional and modified RECIST. Modified RECIST is a new, proposed set of criteria measuring arterial enhanced lesions for the evaluation of "viable" tumors, such as HCC, especially when applied to molecular-targeted therapies or other therapeutic interventions, rather than to cytotoxic agents.[10] The decision about treatment response was different in three cases between conven-

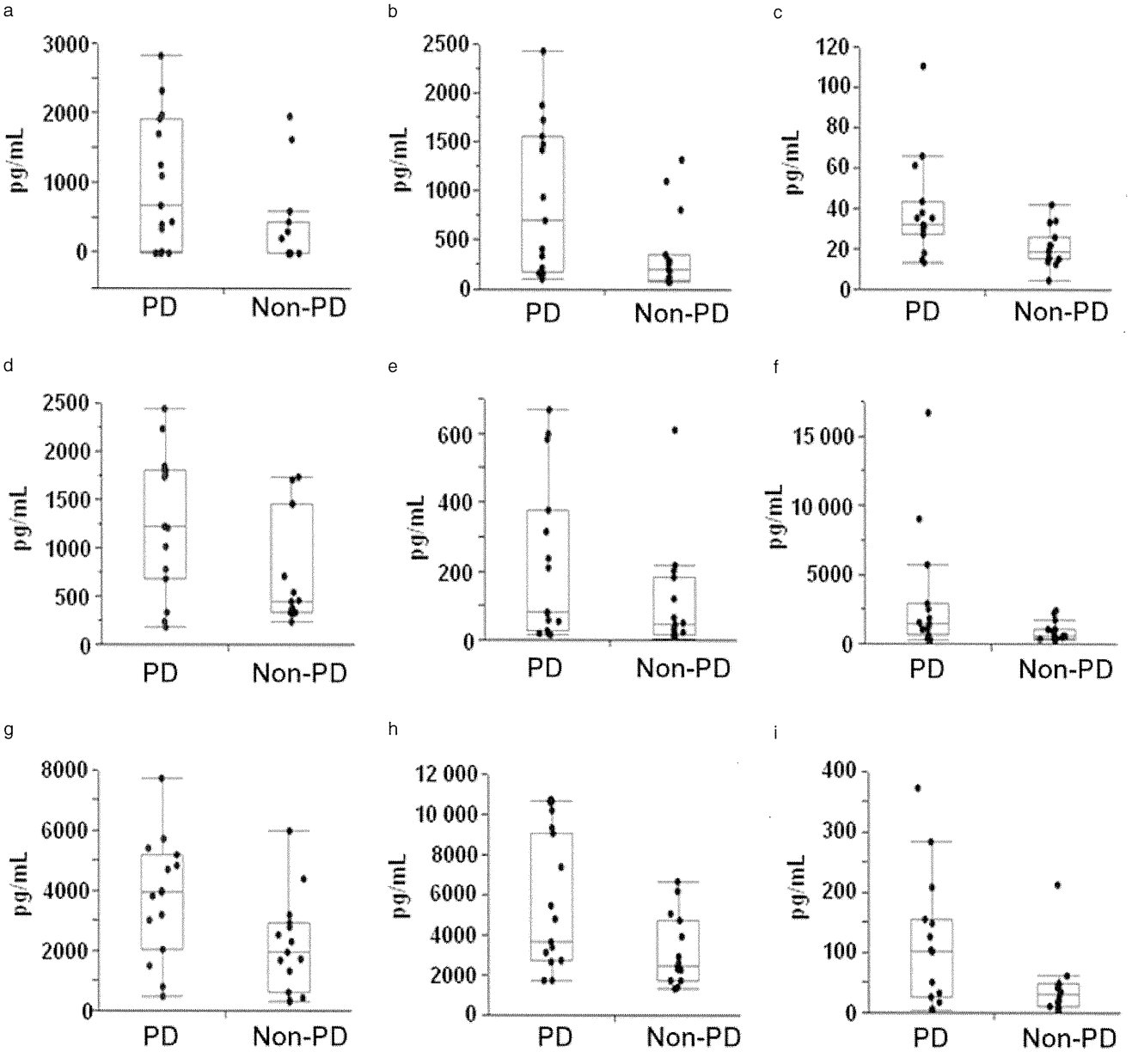

Figure 1 Box plot of baseline biomarker levels in patients with progressive disease (PD) and non-PD. All cytokines but interleukin-8 (IL-8) were higher in PD than in non-PD. (a) angiopoietin-2; (b) follistatin; (c) granulocyte colony-stimulating factor; (d) hepatocyte growth factor; (e) IL-8; (f) leptin; (g) platelet-derived growth factor-BB; (h) platelet endothelial cell adhesion molecule-1; (i) vascular endothelial growth factor. Horizontal bars in the boxes indicate median.

tional and modified RECIST. The differences were observed within the non-PD group and did not influence the disease control rate, which referred to the status of PD or non-PD.

We measured the expressions of Ang-2, FST, G-CSF, HGF, IL-8, leptin, PDGF-BB, PECAM-1/CD31, and VEGF, which are molecules related to angiogenesis. The reason why we chose these molecules is that sorafenib is a representative anti-angiogenic drug, and angiogenesis plays an important role in the aggressive biological behavior of HCC, which is one of the most hypervascular human cancers.[11] Our biomarker study identified eight serum markers at baseline, namely, Ang-2, FST, G-CSF, HGF, leptin, PDGF-BB, PECAM-1/CD31, and VEGF, which were correlated with the initial response, and also four markers, Ang-2, G-CSF, HGF, and leptin, which were correlated with PFS after sorafenib treatment. In addition, a large number of biomarkers at high levels were correlated with a worse response.

A few reports have been published demonstrating relationships between biomarkers and outcomes in anti-angiogenic therapy. Low HGF levels and high c-kit levels in serum at baseline were reported to be associated with longer survival in HCC patients

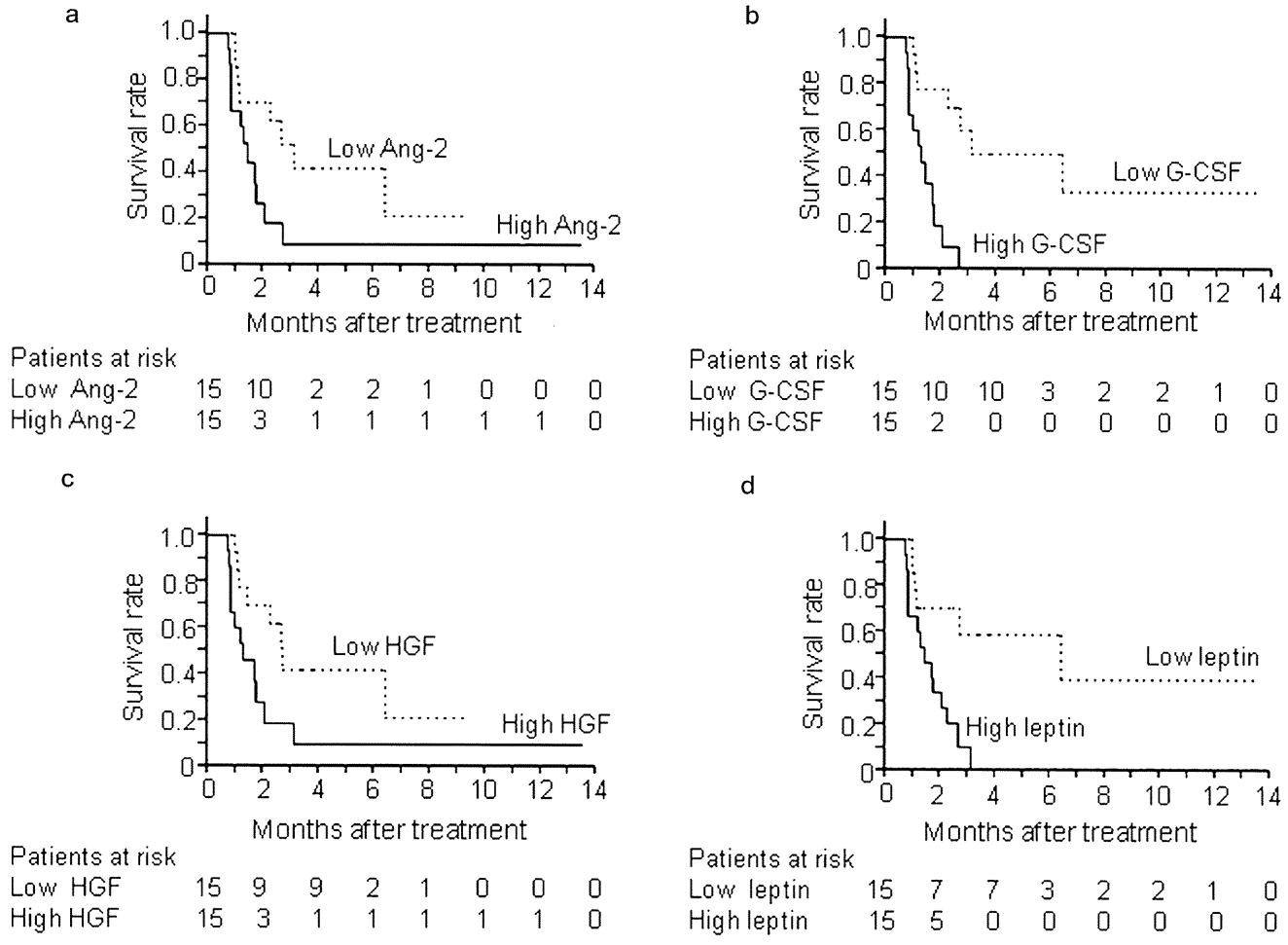

Figure 2 Progression-free survival of high and low biomarker groups. All high cytokine groups showed lower progression-free survival than low cytokine groups. (a) angiopoietin-2 (Ang-2); (b) granulocyte colony-stimulating factor (G-CSF); (c) hepatocyte growth factor (HGF); (d) leptin. (a) $p = 0.043$; (b) $p < 0.001$; (c) $p = 0.037$; (d) $p = 0.005$.

treated with sorafenib.[12] In addition, lower baseline VEGF levels were found to be correlated with increased PFS in HCC patients treated with sunitinib[13] and in metastatic breast cancer patients treated with bevacizumab combination therapy.[14] Several studies reported that no correlation was observed between VEGF levels and clinical outcome in metastatic colorectal cancer[15] and non-small cell lung cancer.[16] In fact, an inverse correlation was reported between high VEGF levels and PFS in advanced renal cell carcinoma.[17] One possible explanation for these differences is the pathological differences of tumors, or physiological differences associated with ethnicity, as we observed in the adverse events with sorafenib.[6,7]

Of the eight cytokines, serum VEGF and HGF were reported as molecules associated with tumor progression.[18–22] In addition, PDGF-B,[23,24] Ang-2,[25–28] leptin,[29] and G-CSF receptor[30–32] were overexpressed in at least some of the HCC tissues and were associated with tumor progression, angiogenesis, or dedifferentiation, among others. Therefore, a large number of cytokines at high levels might be representative of tumor aggressiveness as an additive effect of multiple cytokines. The elevation of the cytokines might also be correlated with the resistance to anti-angiogenic therapies in terms of the regulation of multiple pro-angiogenic mechanisms. For example, G-CSF was reported to induce tumor refractoriness to anti-VEGF therapy by recruiting myeloid cells to tumors and mediating tumor angiogenesis.[33] Molecules, such as vascular endothelial-cadherin and matrix metalloproteinases, play a role in another mechanism called vasculogenic mimicry,[34] although they were not measured in the present study. From this point of view, it is possible that monotherapies targeting limited numbers of molecules were not so effective when multiple pro-angiogenic molecules were activated, and it is important to investigate multiple molecules simultaneously as a reflection of the activation of multiple pro-angiogenic mechanisms.

We demonstrated that cytokines related to angiogenesis could be biomarkers predicting the effect of sorafenib treatment in patients with HCC, but there are several weak points in this study. We detected candidate markers related to tumor response and PFS; however, we could not strictly evaluate OS in this study. This study population was not adequate to analyze OS, because various treatments were performed after PD, such as transcatheter arterial

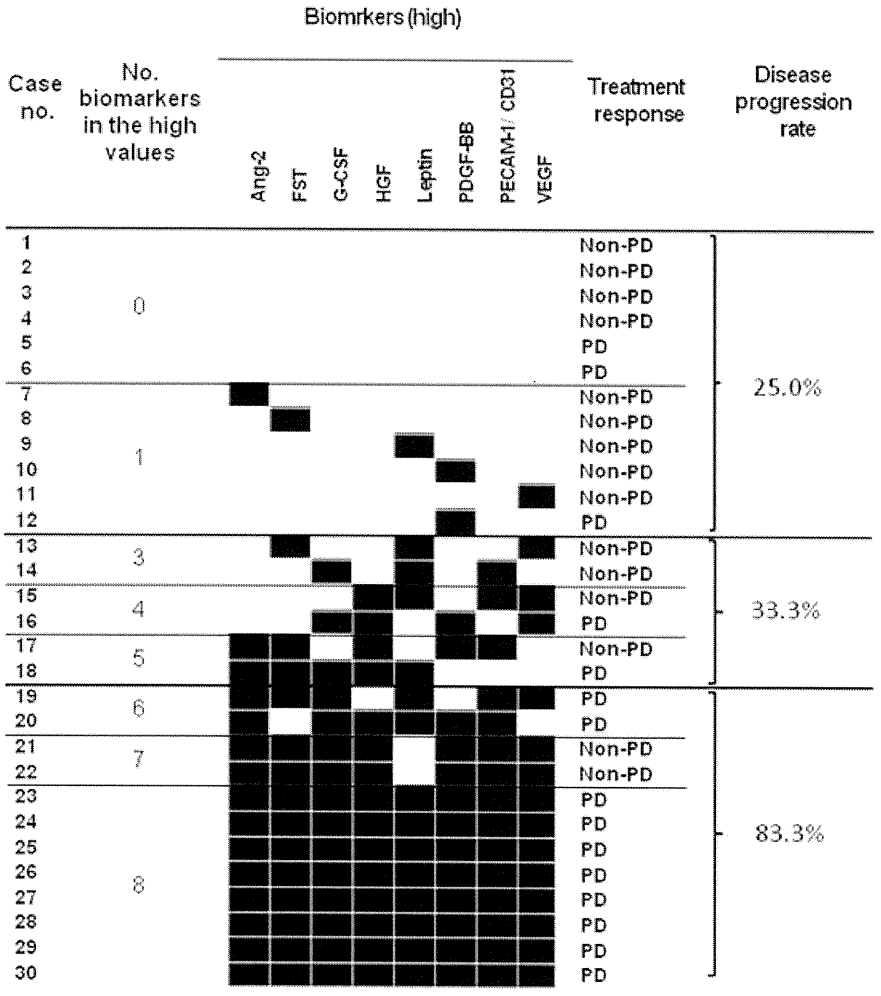

Figure 3 Combinations of biomarkers at high levels (cut-off values are the median). Closed square indicates that the expression was above the median. Progressive disease (PD) was seen in three of 12 (25%) patients with 0–2 high biomarkers, two of six (33.3%) patients with 3–5 high biomarkers, and 10 of 12 (83.3%) patients with 6–8 high biomarkers ($P = 0.008$). Ang-2, angiopoietin-2; FST, follistatin; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; PDGF-BB, platelet-derived growth factor-BB; PECAM-1/CD31, platelet endothelial cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

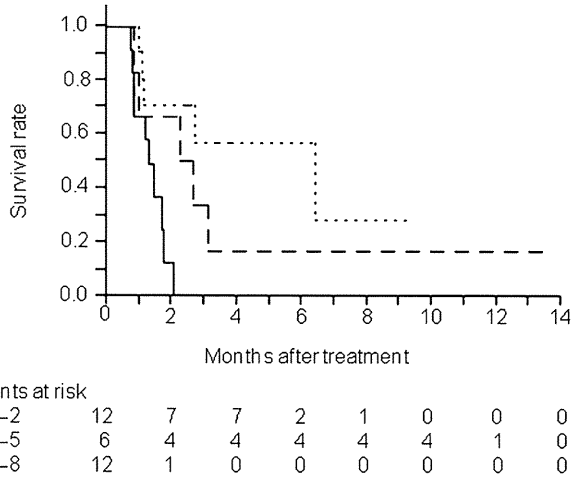

Figure 4 Progression-free survival of groups with 0–2, 3–5, and 6–8 high biomarkers. These refer to the number of biomarkers above the median at baseline. $P = 0.009$ (log–rank test). ·····, 0–2; ---, 3–5; —, 6–8.

embolization and chemoembolization. Another weak point is that this study is an exploratory analysis with a small sample size and a short observation period. There might be an effective combination of a few cytokines to predict the treatment effect; however, the number of patients in this study was too small to reach the conclusion. In addition, the difficulty in setting the standard values of these cytokines is another limitation. The values of serum cytokines in healthy controls differ between reports, and the difference extends from over 10-fold in VEGF to over 100-fold in PDGF-BB.[35–38] Therefore, it is necessary to measure them among a specific population with one method for obtaining a consistent result.

We demonstrated the expressions of angiogenesis-related factors in HCC patients who received sorafenib in this study, and we proposed a new concept of simultaneous measurement of serum markers of angiogenesis for the prediction of the treatment effect. Further examination is necessary to validate our findings by increasing the sample size and extending the observation period.

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HEPATOLOGY

Prognostic importance of fucosylated alpha-fetoprotein in hepatocellular carcinoma patients with low alpha-fetoprotein

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alpha-fetoprotein, fucosylation, hepatocellular carcinoma.

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Abstract

Background and Aim: Fucosylated alpha-fetoprotein (AFP-L3) is known to be a marker of poor prognosis in patients with hepatocellular carcinoma (HCC). However, it has been difficult to measure AFP-L3 under low AFP (≤ 20 ng/mL). The aim of this study was to elucidate the role of AFP-L3 in HCC patients with low AFP conditions.

Methods: One hundred and ninety six consecutive newly developed HCC patients with low AFP (≤ 20 ng/mL) were examined for serum AFP-L3 expression by a newly-developed micro-total analysis system that could stably measure AFP-L3 in low AFP circumstances, and its clinical importance was analyzed.

Results: Positivity of AFP-L3 in HCC patients was 13.3% at a cut-off level of 10%. Five-year survivals of HCC patients with AFP-L3 (< 10%) and AFP-L3 (≥ 10%) were 69.4% and 41.1%, respectively ($P = 0.001$). Among 18 clinical parameters, low alanine aminotransferase, large tumor size, presence of portal vein tumor thrombus, high AFP and high des-gamma carboxy prothrombin were observed in the high AFP-L3 (≥ 10%) group. Multivariate analysis revealed that high aspartate aminotransferase (AST) (risk ratio [RR] = 3.24, 95% confidence interval [CI] = 1.27–8.26), the presence of ascites (RR = 3.44, 95% CI = 1.22–9.34), multiple tumor number (RR = 3.06, 95% CI = 1.33–7.17), and high AFP-L3 (RR = 8.36, 95% CI = 2.79–25.5) were risk factors for survival. High AFP-L3 was also a risk factor for survival in HCC patients who received radiofrequency ablation ($P = 0.048$).

Conclusions: AFP-L3 is a strong prognostic factor for survival even in HCC patients with low AFP (≤ 20 ng/mL).

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer death in the world and represents a major health problem.[1] There are many reports about the risk factors for survival of the patients with HCC. They include tumor factors such as tumor size and tumor numbers, and background liver factors such as serum albumin and total bilirubin.[2–5] Alpha-fetoprotein (AFP), fucosylated AFP (AFP-L3), and des-gamma carboxy prothrombin (DCP) are three major tumor markers of HCC (tumor factors). All of these markers, especially AFP-L3, are closely correlated with the prognosis of patients with HCC.[6] Although the clinical importance of AFP-L3 has been reported, the level obtained by conventional measurement methods is unstable or even undetectable when the serum AFP level is less than 20 ng/mL.

Recently, a new method that can stably measure AFP-L3 in patients with low levels of AFP was developed.[7] The method uses the electrokinetic analyte transport assay technique that enables the on-tip integration of all assay steps, and can detect minute amounts of AFP-L3 by Laser-induced-fluorescence following lectin affinity electrophoresis.

In this report, we measured the AFP-L3 in HCC patients with low AFP levels (≤ 20 ng/mL) and analyzed the clinical importance of AFP-L3 in this patient population.

Methods

Patients

Among 776 consecutive newly diagnosed HCC patients who were admitted to Okayama University Hospital between 2002 and 2009,

196 patients with AFP less than or equal to 20 ng/mL were enrolled. The mean age of the patients was 70.3 years, 127 patients (64.8%) were male, and the median tumor diameter was 17 mm. The percentage of tumor < 2 cm, 2–5 cm and > 5 cm were 65%, 23%, and 12%, respectively. One hundred and sixty-six patients, 29, and one patient were Child–Pugh grade A, B, and grade C, respectively. Radiofrequency ablation (RFA), transcatheter arterial chemoembolization (TACE), surgical resection, chemotherapy, and percutaneous ethanol injection therapy were performed in 139, 29, 21, five and two patients, respectively. As a control, 87 patients with liver cirrhosis (LC) with low AFP (less than or equal to 20 ng/mL) who also visited our hospital during the same period were also examined for AFP-L3. All LC patients were confirmed not to have HCC with ultrasonography or contrast enhanced computed tomography (CT), and no patients developed HCC during a 6-month follow up period. Seven out of the 10 patients received liver transplantation and no clear evidence of HCC was found in the patients by the pathological examination of the explanted livers. Informed consent was obtained from all patients for the use of their clinical data. The study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by our institutional review board.

Diagnosis

Hepatocellular carcinoma was diagnosed by imaging modalities such as angiography, CT, and magnetic resonance imaging (MRI) ($n = 141$) or by tumor biopsy ($n = 55$). The criteria for HCC via imaging was based on previous reports of hyperattenuation at the arterial phase, hypoattenuation at the portal phase in dynamic CT or magnetic resonance imaging (MRI), and tumor staining on angiography. According to the American Association for the Study of Liver Disease guidelines, we confirmed the diagnosis at least by two dynamic imaging modalities.[8] The nodules without these findings were histologically confirmed as HCC via US-guided, fine-needle biopsy.

Treatment and follow up

The patients were treated basically according to the evidence-based clinical practice guidelines for HCC in Japan.[9] The rate of observance of the guidelines was 83.1%. After the initial treatments, patients were followed up with ultrasonography, dynamic CT, or MRI as well as by biochemical liver function tests at least every 3 months. Two patients received interferon therapy after the treatment. Recurrence was diagnosed with the same criteria used for the initial diagnosis, and re-treatment was performed based on the same decision criteria. The recurrence rates of RFA treated patients were 57.5% and 70.7% at 3 years and 5 years after treatment, respectively.

Measurement of AFP-L3

Serum AFP concentrations were determined using a commercially available EIA kit. The percentage of AFP-L3 was determined by a micro-total analysis system developed by Wako Pure Chemical Industries, Ltd. (Osaka, Japan).[7]

Statistical analysis

The Wilcoxon rank sum test was used to compare the continuous data and the χ^2 test was used to compare categorical data. The survival of the patients was compared by the Kaplan–Meier method and the differences were evaluated by the log-rank test. Cox proportional hazards regression analysis was used to examine prognostic factors including AFP-L3. Factors exhibiting significant values in univariate analysis were further analyzed by multivariate analysis. The reason for using 3 cm as a break point is that this tumor size is frequently used in many algorithms and studies including ours.[3] JMP (version 8.02) software packages (SAS Institute, Cary, NC, USA) were used for the analyses, and $P < 0.05$ was considered significant. Bonferroni correction was used for multiple-comparisons of three groups and $P < 0.05/3$ was considered significant.

Results

Positivity of AFP-L3 at different cut-off

Positivity of AFP-L3 in patients with HCC was 51.5%, 13.3%, and 8.7% at cut-off levels of 5%, 10%, and 15%, respectively (Table 1). The positivity in patients with LC was lower than that for those with HCC; however, no significant difference was observed between HCC and LC. The area under the receiver operating characteristic curve (AUROC) was 0.534.

Relationship between AFP and AFP-L3

A very weak correlation was observed between AFP and AFP-L3 ($P < 0.001$, $R^2 = 0.064$, Fig. 1). High AFP-L3 (≤ 10%) was not observed in patients with AFP below 3.2 ng/mL.

Survival of the patients with HCC

Patients were divided into two groups according to the percentage of AFP-L3 and the survival rates of each group were compared. The 3-year (5-year) survival rates of patients with AFP-L3 (< 10%) and AFP-L3 (≥ 10%) were 90.1% (69.4%), and 68.4% (41.1%), respectively ($P = 0.001$, Fig. 2). Because the cut-off value of AFP-L3 was 10% in our institute for AFP above 20 ng/mL, we adopted the cut-off value of 10% in subsequent studies.

Characteristics of HCC with high AFP-L3

Eighteen clinical parameters were analyzed in patients with different AFP-L3 (Table 2). In the high AFP-L3 (≥ 10%) group, alanine

Table 1 Positivity of fucosylated alpha-fetoprotein (AFP-L3) in patients with low AFP (≤ 20 ng/mL)

Cut-off	Positivity of AFP-L3		
	HCC	LC	*P*-value
5%	101/196 (51.5%)	40/87 (46.0%)	0.440
10%	26/196 (13.3%)	10/87 (11.5%)	0.846
15%	17/196 (8.7%)	3/87 (3.5%)	0.136

HCC, hepatocellular carcinoma; LC, liver cirrhosis.

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aminotransferase (ALT) was low ($P = 0.003$), tumor size was large ($P < 0.001$), the presence of portal vein tumor thrombus (PVTT) was high ($P = 0.026$) and AFP and DCP were high ($P = 0.049$ and 0.002, respectively). No differences were observed in total bilirubin, albumin, prothrombin time or presence of ascites, which represented liver function and factors in the Child–Pugh score.

Risk factors for survival

Among 18 parameters, Child–Pugh grade B/C, high T. Bil (≥ 1.5 mg/dL), low albumin (< 3.5 g/dL), high AST (≥ 80 IU/mL), low platelet count (< $10 \times 10^4/mm^3$), low prothrombin time (PT) (< 80%), the presence of ascites, multiple tumor number, and high AFP-L3 (≥ 10%) were risk factors for survival according to univariate analysis (Table 3). In multivariate analysis, high AST, the presence of ascites, multiple tumor number, and high AFP-L3 were risk factors for survival. The risk ratio of AFP-L3 (RR = 8.36, 95% confidence interval [CI] = 2.79–25.5) was the highest among the factors examined. Child–Pugh grade was not included in multivariate analysis to avoid multicollinearity.

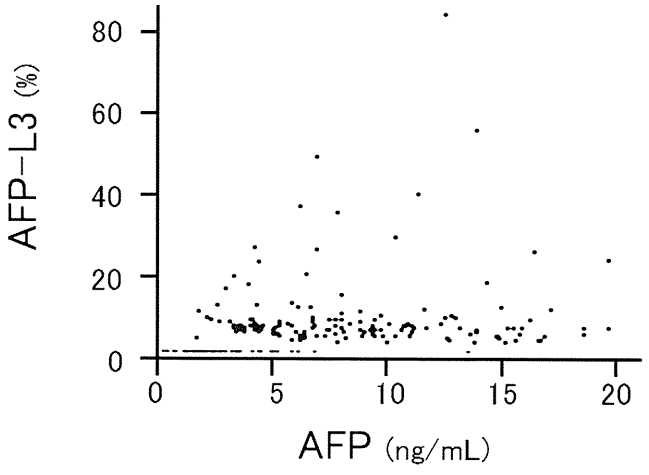

Figure 1 Relationship between fucosylated alpha-fetoprotein (AFP-L3) and alpha-fetoprotein (AFP) under 20 ng/mL in patients with hepatocellular carcinoma (HCC). A weak correlation was observed between AFP and AFP-L3 ($P < 0.001$, $R^2 = 0.064$). High AFP-L3 (10% ≤) was not observed in patients with AFP below 3.2 ng/mL. AFP-L3 under the lowest detection limit was considered as zero.

Survival and recurrence of RFA-treated patients

In this study, 139 patients were treated by RFA. Curative ablation could be performed in 129 of them, and these 129 were enrolled in the subsequent examination. The same 18 variables were analyzed. In the high AFP-L3 (≥ 10%) group, tumor size was larger (18 mm *vs* 15 mm, median), DCP was higher (68 mAU/mL *vs* 24 mAU/mL), AST was lower (33 IU/L *vs* 40 IU/L), and PT was lower (91% *vs* 98%) than in the low AFP-L3 (< 10%) group. Multivariate analysis revealed that high AST (≥ 80 IU/L, RR = 11.6, 95% CI = 3.14–47.6), low prothrombin time (< 80%, 3.50, 1.14–11.1), presence of ascites (RR = 5.37, 95% CI = 1.14–20.3), and high AFP-L3 (≥ 10%, 13.5, 2.99–69.1) were risk factors for survival.

The 3-year (5-year) recurrence free survival rates of patients with AFP-L3 (< 10%) and AFP-L3 (≥ 10%) were 42.7% (30.7%), and 43.8% (21.9%), respectively. The 3-year (5-year) survival rates of patients with AFP-L3 (< 10%) and AFP-L3 (≥ 10%) were 88.4% (66.3%), and 72.9% (43.8%), respectively ($P = 0.048$, Fig. 3).

The recurrence free survival curve tended to be higher in the low AFP-L3 group in the first 3 years ($P = 0.031$, Wilcoxon); however, no significant difference was observed between the two groups ($P = 0.123$, Log-rank test, Fig. 4). RFA and TACE were, respectively, performed in 62.1% and 12.1% for the treatment of recurrent HCC; however, no significant difference of the treatment was observed between high AFP-L3 (≥ 10%) group and low AFP-L3 (< 10%) group.

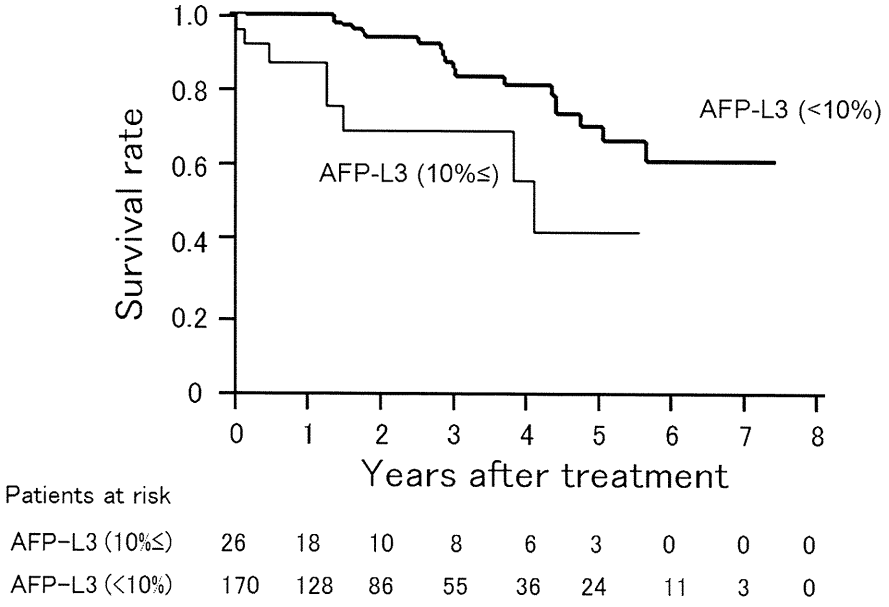

Figure 2 Survival of hepatocellular carcinoma (HCC) patients with different fucosylated alpha-fetoprotein (AFP-L3) level. The 3-year (5-year) survivals of patients with AFP-L3 (< 10%, thick solid line), and AFP-L3 (≥ 10%, thin solid line) were 90.1% (69.4%), and 68.4% (41.1%), respectively ($P = 0.001$).

Table 2 Characteristics of patients with hepatocellular carcinoma (HCC) at different fucosylated alpha-fetoprotein (AFP-L3) levels

Variables	AFP-L3		*P*-value
	< 10%	10% ≤	
Patient number	170 (86.7%)	26 (13.3%)	
Age (years)	70.2 (38.1–85.4)	71.4 (46.3–87.7)	0.988
Sex (male)	109 (64.1%)	18 (69.2%)	0.611
HCVAb (positive)	124 (72.9%)	17 (65.4%)	0.665
HBsAg (positive)	16 (9.4%)	4 (15.4%)	0.312
Child–Pugh A	145 (85.3%)	19 (76.0%)	0.116
Total bilirubin (mg/dL)	0.78 (0.36–2.88)	0.78 (0.33–4.44)	0.611
Albumin (g/dL)	3.8 (2.5–4.9)	3.6 (2.5–4.4)	0.278
AST (IU/L)	48 (19–198)	48 (16–243)	0.406
ALT (IU/L)	44 (13–235)	32 (14–115)	0.003
Platelet ($\times 10^4/mm^3$)	12.2 (3.4–74.0)	12.2 (3.5–29.8)	0.630
Prothrombin time (%)	98 (10–146)	92 (62–126)	0.166
Ascites (present)	22 (12.9%)	6 (23.1%)	0.169
Alcohol (≥ 90 g/day)	16 (9.6%)	4 (15.4%)	0.372
Tumor size (mm)	16 (7–87)	26 (9–170)	< 0.001
Tumor number (multiple)	61 (35.9%)	14 (53.8%)	0.079
PVTT (present)	11 (6.5%)	5 (19.2%)	0.026
Alpha-fetoprotein (ng/mL)	6.4 (0.9–20)	7.5 (3.2–20)	0.049
Des-gamma carboxy prothrombin (mAU/mL)	26 (10–363 350)	86 (11–75 000)	0.002

All numbers are medians (inter-quartile range) unless otherwise noted.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBsAg, hepatitis B virus surface antigen; HCVAb, hepatitis C virus antibody; PVTT, portal vein tumor thrombus.

Table 3 Risk factors for survival in patients with hepatocellular carcinoma (HCC) with low alpha-fetoprotein (AFP)

	Univariate			Multivariate		
	RR	95%CI	*P*-value	RR	95%CI	*P*-value
Age (≥ 65 years)	1.39	0.60–3.79	0.455			
Sex (male)	1.80	0.77–4.89	0.179			
HCVAb (positive)	1.55	0.60–5.33	0.385			
HBsAg (positive)	0.47	0.02–2.25	0.415			
Child–Pugh B/C	2.74	1.18–5.90	0.020			
Total bilirubin (≥ 1.5 mg/dL)	3.94	1.68–8.54	0.002	1.24	0.43–3.36	0.804
Albumin (< 3.5 g/dL)	2.17	1.02–4.62	0.042	0.87	0.31–2.35	0.796
AST (≥ 80 IU/L)	3.22	1.48–6.80	0.003	3.24	1.27–8.26	0.014
ALT (≥ 80 IU/L)	2.05	0.85–4.51	0.104			
Platelet (< $10 \times 10^4/mm^3$)	2.15	1.02–4.67	0.044	2.07	0.70–6.08	0.180
Prothrombin time (< 80%)	2.35	1.06–5.02	0.035	1.51	0.54–4.00	0.417
Ascites (present)	4.39	1.87–9.53	0.001	3.44	1.22–9.34	0.019
Alcohol (≥ 90 g/day)	0.55	0.08–1.85	0.377			
Tumor size (≥ 3 cm)	1.38	0.46–3.39	0.524			
Tumor (multiple)	2.20	1.03–4.68	0.040	3.06	1.33–7.17	0.008
PVTT (present)	3.40	0.99–8.92	0.051			
AFP L3 (≥ 10%)	3.50	1.44–7.75	0.007	8.36	2.79–25.5	< 0.001
Des-gamma carboxy prothrombin (≥ 40 mAU/mL)	1.18	0.52–2.51	0.674			

95% CI, 95% confidence interval; RR, risk ratio; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBsAg, hepatitis B virus surface antigen; HCVAb, hepatitis C virus antibody; PVTT, portal vein tumor thrombus.

Discussion

AFP-L3 is known to be a predictor of poor prognosis; however, it has been difficult to evaluate its value in cases with low AFP under 20 ng/mL, because conventional measuring methods yield unstable or even undetectable AFP-L3 levels under these circumstances. Methodological development enabled us to overcome this difficulty and revealed that AFP-L3 was closely related to poor prognosis even in HCC patients with low AFP. The risk ratio for survival was 3.50 and was comparable with the ratio (2.43) in

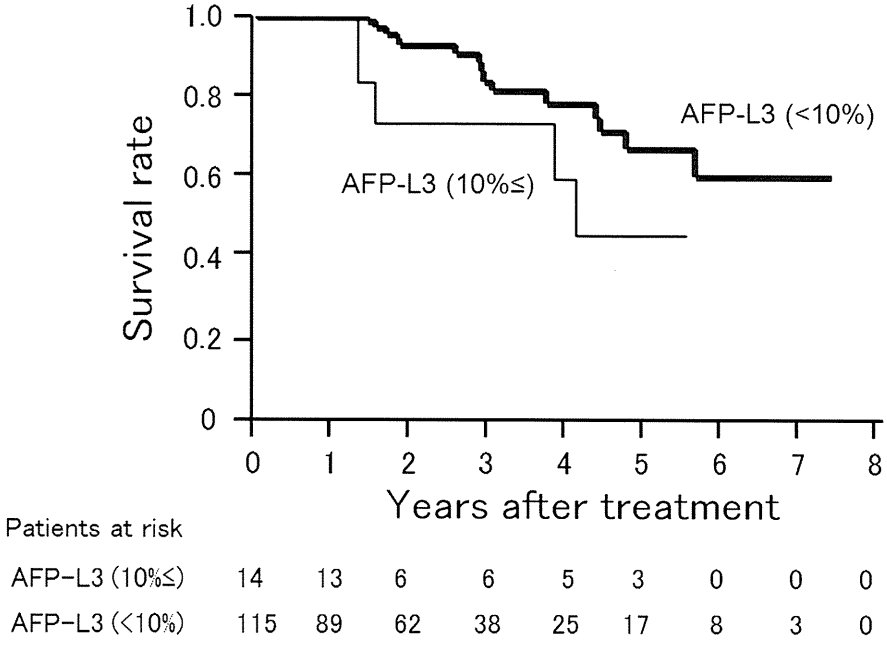

Figure 3 Survival of radiofrequency ablation (RFA)-treated patients. The 3-year (5-year) survivals of RFA-treated patients with fucosylated alpha-fetoprotein (AFP-L3) (< 10%, thick solid line), and AFP-L3 (≥ 10%, thin solid line) were 88.4% (66.3%), and 72.9% (43.8%), respectively ($P = 0.048$).

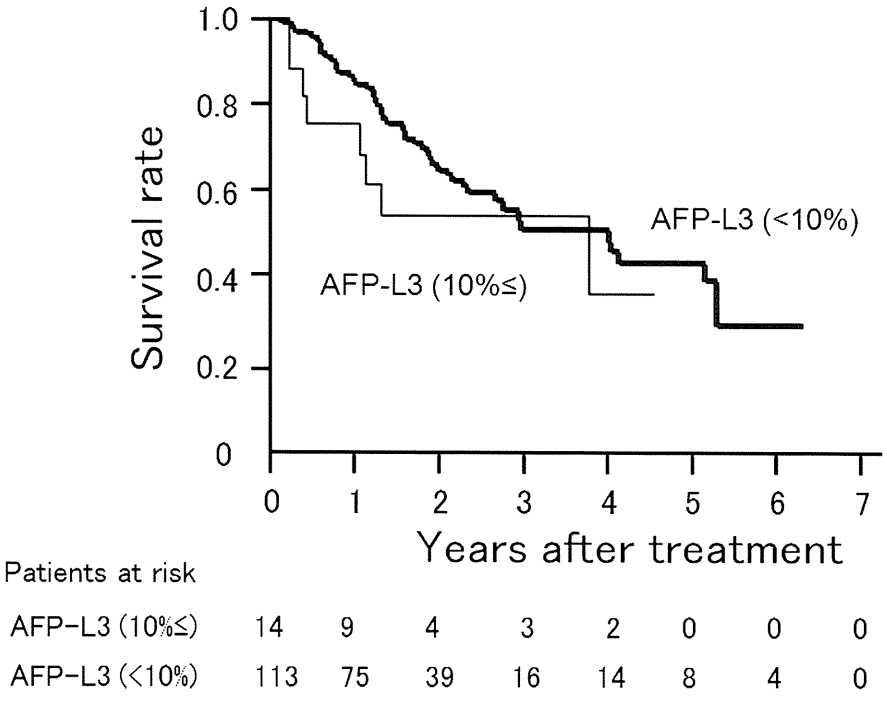

Figure 4 Recurrence free survival of radiofrequency ablation (RFA)-treated patients. The 3-year (5-year) recurrence free survivals of RFA-treated patients with AFP-L3 (< 10%, thick solid line), and AFP-L3 (≥ 10%, thin solid line) were 42.7% (30.7%), and 43.8% (21.9%), respectively.

HCC patients with high AFP in our institute (Nouso, unpubl. data, 2010). There were other differences in patients' backgrounds at different AFP-L3 levels. Total AFP level and DCP were high and tumor size was large in the high AFP-L3 group. The factors were known to correlate with the prognosis of patients with HCC.

Recently, many fucosylated glycoproteins other than AFP-L3 have been reported to be useful for the diagnosis of HCC. They include fucosylated hemopexin and fucosylated kininogen;[10,11] however, the mechanism of the increase of fucosylated glycoproteins is not fully understood. Fut 8 is known to be a key enzyme of fucosylation.[12] This enzyme might act to produce AFP-L3 but an activation of the enzyme is not the only reason for increased AFP-L3 in HCC, because Fut8 was also reported to exist in the non-cancerous cirrhotic liver.[13]

The sensitivity of AFP-L3 was quite low (13.3%) in this study, meaning that the diagnostic role of AFP-L3 is limited when AFP is below 20 ng/mL. This low specificity of AFP-L3 was completely different from the results of previous reports that demonstrated a high specificity of AFP-L3.[14–19] The reports analyzed AFP-L3 in patients with HCC with high AFP. We analyzed only patients with

low AFP (≤ 20 ng/mL), meaning that a small amount of AFP-L3 production could have easily raised the percentage of AFP-L3. Small constitutive expression of AFP-L3 by FUT8 activation in cirrhotic liver as well as the possibility of occult HCC in some patients might have led to the low specificity in cases with low AFP.

Despite the low sensitivity and specificity of AFP-L3, its increase was closely related to the prognosis of HCC patients; the risk ratio was 8.36 and was the highest among the factors examined in this study. The sensitivities of AFP at 20 ng/mL cut-off were reported to be approximately 25–55% and 53–70% when the tumor size was less than or equal to 2 cm and 5 cm, respectively.[14,15,20–23] The sensitivity of AFP in our department is 62%,[14] meaning that approximately 40% of our patients have previously undergone unreliable AFP-L3 measurements. Measuring AFP-L3 with the new method provides a good prediction of prognosis in large patient populations with normal AFP (≤ 20 ng/mL).

In the present study, we demonstrated the prognostic value of AFP-L3 in patients with low AFP (≤ 20 ng/mL). The outcome of LC patients with high AFP-L3 is a future issue that must be solved.

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Original Article

Long-term outcome and hepatocellular carcinoma development in chronic hepatitis B or cirrhosis patients after nucleoside analog treatment with entecavir or lamivudine

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Aim: We conducted this prospective study to elucidate the long-term outcome and incidence of hepatocellular carcinoma (HCC) development after nucleos(t)ide analog (NA) treatment in patients with chronic hepatitis B (CHB) or cirrhosis.

Methods: CHB or cirrhosis patients without past NA treatment or HCC were started on entecavir (ETV) or lamivudine (LVD), and prospectively followed up with monthly blood tests, and with abdominal imaging every 6 months in CHB and every 3 months in cirrhosis patients.

Results: A total of 256 subjects with CHB ($n = 194$) or cirrhosis ($n = 62$) received ETV ($n = 129$) or LVD ($n = 127$) for 4.25 years (range: 0.41–10.0). After NA treatment, serum HBV DNA, alanine aminotransferase and α-fetoprotein (AFP) dropped significantly, along with significant increases in serum albumin and prothrombin time. Drug-resistance developed in 60 cases in the LVD group and in only one case in the ETV group. HCC developed in 35 patients, and the incidence at years 1, 3, 5, 7 and 10 was significantly higher in patients with cirrhosis (8.1%, 17.5%, 43.2%, 46.7% and 53.4%, respectively) than chronic hepatitis (1.6%, 3.5%, 3.5%, 7.1% and 29.6%, respectively), with no difference between ETV and LVD. After NA treatment, the sensitivity/specificity for HCC of AFP and des-γ-carboxy prothrombin (DCP) was 45.7%/97.3% and 33.3%/96.2%, respectively, with the specificity of AFP being higher than at baseline (64.4%), at the cut-off of 10 ng/mL.

Conclusion: NA exerted a long-term efficacy and improved hepatic reservation in CHB and cirrhosis. After NA treatment, AFP dropped to lower than 10 ng/mL with marked elevation of specificity, leading to an earlier detection of HCC.

Key words: α-fetoprotein, chronic hepatitis B, entecavir, hepatitis B virus, hepatocellular carcinoma, lamivudine

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a serious health problem because of its potential to induce a variety of liver diseases, namely, acute hepatitis, chronic hepatitis (CH), liver cirrhosis, hepatocellular carcinoma (HCC) and fulminant hepatic failure.

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The prevalence of HBV carriage is reported to be 350–400 million people worldwide, and the prevalence rate of HBV infection in Japan is estimated to be 0.8%.[1,2] It has been reported that 15–20% of chronic hepatitis B (CHB) patients progress to cirrhosis within 5 years and that the annual incidence of HCC is 2.8%.[3] Nucleos(t)ide analogs (NA) suppress HBV DNA replication by inhibiting HBV DNA polymerase activity in the reverse transcription process from pregenomic RNA derived from a HBV closely covalent circular (ccc)DNA template.[4] Three NA anti-HBV agents, namely lamivudine (LVD), adefovir-dipivoxil (ADV) and entecavir (ETV), have been approved for coverage by the health

insurance program in Japan. They have been shown to be effective in decreasing serum HBV DNA and alanine aminotransferase (ALT) levels, improving hepatic reservation and liver histology.[5–11] It has also been reported that LVD decreases the risk of developing liver failure and hepatocarcinogenesis.[12,13]

A high serum HBV DNA load has been shown to be the most critical risk factor for HCC in HBV carriers.[14] Therefore, current treatment guidelines for patients with CHB or cirrhosis stress the importance of suppression of the serum HBV DNA load by antiviral treatment including NA administration in order to minimize the risk of liver disease progression and hepatocarcinogenesis.[15–18] However, the long-term effect of NA treatment on prognosis, especially on development of HCC in patients with CHB or cirrhosis, has not been fully elucidated.

Therefore, we designed this prospective cohort study to elucidate the virological and biochemical treatment effects and the long-term prognosis – particularly with respect to development of HCC – of patients with CHB or cirrhosis, who were started on LVD treatment, with or without co-administration with ADV, and ETV treatment.

METHODS

Study design

THIS STUDY WAS a prospective, non-randomized open-label cohort study on NA treatment for patients with CHB or cirrhosis without complications or past history of HCC. It was started as a study on LVD treatment for CHB or cirrhosis in November 2000, and then later modified to include ETV treatment when ETV was approved for coverage by the health insurance program and became commercially available in Japan in September 2006. This study was designed and performed in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines. The study was approved by the Institutional Committee for Human Rights. This study was also registered for the University Hospital Medical Information Network CTR system (ID: UMIN 000000594).

Subject population

The eligible patients were men and women aged 20 years or older, diagnosed with CHB or cirrhosis, without previous NA administration, and without complications or past history of HCC. The diagnosis of CHB and cirrhosis was made by positive hepatitis Bs antigen and positive serum HBV DNA with elevated serum ALT. The indication criteria for NA included serum HBV DNA greater than 5 log copies/mL with an elevated ALT level over twice the upper normal limit (ULN) or with complications of hepatic insufficiency, such as jaundice, ascites or encephalopathy.

The diagnosis of cirrhosis was confirmed by liver histology, clinical signs (encephalopathy or ascites), evidence of esophagogastric varices by endoscopy, or imaging modalities such as ultrasonography (US), computed tomography (CT) or magnetic resonance imaging (MRI).

All patients were deemed to be without complications of HCC by US, CT or MRI performed within 3 months before enrollment. Patients with acute hepatitis, fulminant hepatitis, alcoholic liver injury, co-infection with hepatitis C virus, autoimmune hepatitis, primary biliary cirrhosis, non-alcoholic steatohepatitis or hereditary liver diseases were excluded. All patients were informed of the aim and methodology of the study, received a written synopsis and gave their written consent to participate. The patients were started on LVD 100 mg/day or ETV 0.5 mg/day p.o. Some patients who had been started on ETV as participants of ETV phase II clinical study in Japan were also enrolled in this study.[9] In this paper, the study population consisted of the subjects enrolled between November 2000 and December 2009, and the patients' data between enrollment and November 2010 were used.

Patient follow up

The patients were followed up every month with a medical consultation and the following blood examinations: serum albumin, total bilirubin, aspartate AST, ALT, prothrombin time (PT%), platelet count (Plt), hepatitis B e-antigen (HBeAg), anti-HBe antibody by chemiluminescence immunoassay (Chemi-luminescent Immunoassay; Abbott Japan, Tokyo), and serum HBV DNA by polymerase chain reaction (PCR) assay (Roche Amplicor PCR assay; Roche Diagnostics, Tokyo, Japan), real-time PCR assay (Cobas TaqMan HBV Auto; Roche Diagnostics), or transcription-mediated amplification (TMA) assay (Chugai Diagnostics, Tokyo, USA). The detection ranges for the HBV DNA assay of PCR, real-time PCR and TMA were from 2.6–7.6 log copies/mL, from 1.8–8.8 log copies/mL and from 3.7–8.7 log genome equivalent/mL, respectively, permitting close determination with nearly equal quantitative accuracies. Levels of HBV DNA under the lower limit of normal (LLN) or over the ULN were assigned the value of the LLN or the ULN, respectively, in statistical analysis.

Drug resistance was confirmed by virological breakthrough and defined as an increase in serum HBV DNA by more than 1 log copy/mL greater than nadir. If virological breakthrough developed in patients receiving LVD, co-administration of ADV 10 mg/day or change to ETV 1.0 mg/day was selected. If virological breakthrough developed in patients receiving ETV, ETV was stopped and changed to co-administration of LVD 100 mg/day and ADV 10 mg/day.

Surveillance and diagnosis of HCC

As surveillance for HCC, α-fetoprotein (AFP) by electrochemiluminescent immunoassay (Roche Diagnostics) and des-g-carboxy prothrombin (DCP) by electrochemiluminescent immunoassay (Sanko, Tokyo, Japan) were measured alternately every month. Image diagnosis by US, dynamic enhanced CT or dynamic enhanced MRI was performed every 6 months in CH patients and every 3 months in cirrhosis patients. If elevation of AFP or DCP was observed, imaging diagnosis was performed within a month. Diagnosis of HCC was confirmed by the finding of enhanced arterial contrast uptake followed by washout in the portal venous phase and equivalent phase by dynamic enhanced CT or dynamic enhanced MRI, or histologically by fine-needle tumor biopsy. The methods used for the confirmation of diagnosis and staging of HCC conformed to the standards by Liver Cancer Study Group of Japan.[19]

End-points

The primary end-point was the development of HCC. Secondary end-points included changes in serum HBV DNA, HBeAg, AST, ALT, serum albumin, PT%, AFP, DCP, Plt and Child–Turcotte–Pugh class between baseline and the latest visit. Seroconversion was defined as loss of HBeAg and development of anti-HBe. In the case of patients who developed HCC during the study period, the last data gathered before the first detection of HCC by imaging were adopted as the latest data. In the patients who developed HCC, the size, number and stage of tumors were also estimated. The sensitivity and specificity of AFP and DCP were estimated using the values at diagnosis of HCC for the patients who developed HCC, and those at the latest visit for patients without HCC. Only the specificity of AFP and DCP, without sensitivity, was estimated at baseline using the values of AFP and DCP at baseline, when no patients had yet developed HCC.

Statistical analysis

Parameters represented by continuous variables were expressed as the median and range (minimum and maximum). Parameters at baseline were compared between ETV patients and LVD patients by Mann–Whitney *U*-test for continuous variables and by χ^2-test for categorical data. Each parameter at the latest visit was compared with the corresponding one at baseline by Mann–Whitney *U*-test for continuous variables and by χ^2-test for categorical data. The incidence of HCC development was estimated by Kaplan–Meier analysis, and compared between the patients with CH and cirrhosis, and between the patients started on ETV and those administrated LVD, using a log–rank test. Receiver–operator curves (ROC) for serum AFP and DCP were estimated to search for the optimal cut-off value to distinguish between the patients with and without HCC. A *P*-value of less than 0.05 was considered statistically significant. Statistical analysis was performed with JMP software ver. 5.01J.

RESULTS

Study populations and baseline characteristics (Table 1)

A TOTAL OF 256 patients were enrolled in this study. ETV and LVD were administrated to 129 and 127 patients, respectively, as the first-line NA. The baseline characteristics and demographics of all patients, the ETV group and the LVD group, are shown in Table 1. All patients were Japanese, and consisted of 179 men and 77 women, with a median age of 50 years. The median follow-up period was 4.25 years in all patients, 2.96 years in ETV patients and 5.97 years in LVD patients.

As for viral markers, the median serum HBV DNA level was 7.0 log copies/mL and HBeAg was positive in 132 patients of the total patient group, with no significant difference between the ETV and LVD groups.

The clinical diagnosis in all patients was CH in 194 and cirrhosis in 62 patients and there was no significant difference in distribution between the ETV and LVD groups. In the 62 patients with cirrhosis, the Child–Turcotte–Pugh class was A, B and C in 43, 16 and three patients, respectively. The serum albumin concentration, PT% and Plt were significantly lower in the LVD group than the ETV group, indicating that hepatic reservation was somewhat lower in the LVD group.

Liver biopsy was performed and assessed using the New Inuyama Classification system in 166 patients in

Table 1 Baseline characteristics of the subjects

	All ($n = 256$)	ETV ($n = 129$)	LVD ($n = 127$)	*P*-value
Sex (male/female)†	179/77	86/43	93/34	0.2964
Age (years)‡	50 (22–88)	51 (26–88)	50 (22–81)	0.4369
Follow-up period (years)‡	4.25 (0.41–10.0)	2.86 (0.41–7.47)	5.97 (0.51–10.0)	<0.0001*
Fibrosis stage (1/2/3/4)†	42/57/50/17	23/31/21/6	19/26/29/11	0.2665
Activity grade (1/2/3)†	49/80/37	29/34/18	20/46/19	0.2276
Clinical diagnosis (CH/LC)†	194/62	101/28	93/34	0.2934
CTP class A/B/C (in LC patients)†	43/16/3	23/4/1	20/12/2	0.1253
HBV DNA (log copies/mL)‡	7.0 (2.6–8.8)	6.8 (2.6–8.8)	7.3 (3.0–8.7)	0.1763
HBeAg (positive/negative)†	132/124	67/62	65/62	0.952
Total bilirubin (mg/dL)‡	0.87 (0.2–22.67)	0.86 (0.2–5.19)	0.875 (0.38–22.67)	0.0617
Albumin (g/dL)‡	4.0 (2.07–5.0)	4.1 (2.3–5.0)	3.96 (2.0–5.0)	0.0090*
AST (IU/L)‡	66 (12–1216)	61 (12–811)	73 (19–1216)	0.0427*
ALT (IU/L)‡	87 (13–1660)	82 (13–1250)	96 (14–1660)	0.0569
Platelet count (×104/μL)‡	14.0 (3.9–47.2)	17.5 (3.5–32.8)	15.7 (3.6–52.2)	<0.001*
Prothrombin time (%)‡	88.0 (27.8–132.0)	93.0 (58.0–132.0)	83.0 (27.8–122.6)	<0.001*
AFP (ng/mL)‡	5.8 (1.4–1057.1)	5.7 (1.4–820.6)	5.8 (1.4–1057.1)	0.839
DCP (mAU/mL)‡	20.0 (6.0–145.0)	19.0 (6.0–145.0)	20.0 (10.0–89.0)	0.2282

*Different between ETV and LVD groups with statistical significance.
†Values are numerical and analyzed by χ^2-test.
‡Values are median (range) and analyzed by Mann–Whitney *U*-test.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; CTP, Child–Turcotte–Pugh; DCP, des-γ-carboxy prothrombin; ETV, entecavir; HBeAg, hepatitis B e-antigen; LC, liver cirrhosis; LVD, lamivudine.

total. Fibrosis stages of 1, 2, 3 and 4 were observed in 42, 57, 50 and 17 patients, and activity grades of 1, 2 and 3 were observed in 49, 80 and 37 patients in total, both with no significant difference between the ETV and LVD groups.

As for tumor markers, the median AFP was 5.8 ng/mL and the median DCP was 20.0 mAU/mL, and neither marker was significantly different between the ETV and LVD groups. The complication of HCC was neglected in all patients by the inclusion criteria.

Changes in virological and biochemical markers (Fig. 1)

Median serum HBV DNA dropped significantly from 7.0 log copies/mL at baseline to 2.1 log copies/mL at the latest visit ($P < 0.0001$) (Fig. 1a). Serum HBV DNA was under the LLN in 220 out of 256 cases at the latest visit. There was no difference in the latest serum HBV DNA or the rate of decline between the ETV and LVD groups. Among the 132 patients with positive HBeAg at baseline, HBeAg seroconversion was observed in 48 patients (36.4%) after a median period of 4.25 years.

Median serum albumin was significantly elevated from 4.0 g/dL at baseline to 4.4 g/dL at the latest visit ($P < 0.0001$) (Fig. 1b). The median AST dropped from 66 to 22 IU/L ($P < 0.0001$), and the median ALT dropped from 87 to 19 IU/L ($P < 0.0001$) (Fig. 1c,d). The median PT% was elevated from 88% to 100% ($P < 0.0001$), and the median Plt increased from 14.0 to $16.7 \times 10^4/\mu L$ ($P < 0.0001$) (Fig. 1e,f). By subgroup

Figure 1 Comparison of the measured parameters between baseline and the latest visit (a–f) by Mann–Whitney *U*-test for continuous valuables and (g) by χ^2-test for Child–Turcotte–Pugh class distribution. (a) Median serum hepatitis B virus (HBV) DNA dropped significantly from 7.0 log copies/mL at baseline to 2.1 log copies/mL at the latest visit ($P < 0.0001$). (b) Median serum albumin was significantly elevated from 4.0 g/dL at baseline to 4.4 g/dL at the latest visit ($P < 0.0001$). (c) Median aspartate aminotransferase (AST) dropped significantly from 66 IU/L at baseline to 22 IU/L at the latest visit ($P < 0.0001$). (d) Median alanine aminotransferase (ALT) dropped significantly from 87 IU/L at baseline to 19 IU/L at the latest visit ($P < 0.0001$). (e) Median prothrombin time (PT) was significantly elevated from 88% at baseline to 100% at the latest visit ($P < 0.0001$). (f) Median platelet count (PLT) increased significantly from 14.0 at baseline to $16.7 \times 10^4/\mu L$ at the latest visit ($P < 0.0001$). (g) In the 62 patients with cirrhosis, the distribution of Child–Turcotte–Pugh class A, B and C changed from 43, 16 and three at baseline to 56, six and zero at the latest visit, with significant difference by χ^2-test ($P = 0.0173$).