1992, but reached a plateau after the period 1989–1992 (Fig. 1). The median serum AFP value at a diagnosis continued decreasing throughout the study period, and the median serum DCP value also continued decreasing after the period 1989–1992 (data not shown).

When the characteristics were compared between two periods (8 years) before the year 2000 (1992–1996 and 1997–2000, $n = 639$) and two periods after the year 2000 (2001–2004 and 2005–2008, $n = 721$) to elucidate the characteristics of patients with HCC after the year 2000, the age of the patients, as well as their serum albumin levels, were significantly higher after the year 2000 (64.7 ± 8.8 years *vs* 68.2 ± 9.3 years and 3.31 ± 0.62 g/dL *vs* 3.59 ± 1.09 g/dL, both $P < 0.0001$). After the year 2000, the percentage of patients with Child–Pugh class A liver function was significantly higher (59.5% *vs* 68.9%, $P = 0.0013$), and the per-

centage of patients with AJCC tumor stage I was significantly higher (41.9% *vs* 51.2%, $P = 0.0011$). Additionally, the percentage of patients with single HCC tumors (44.1% *vs* 54.4%, $P = 0.0002$) and the percentage of patients with HCC that lacked vascular invasion (76.2% *vs* 83.1%, $P = 0.0020$) were significantly higher after the year 2000. The serum AFP value at diagnosis was significantly lower after the year 2000 (38 ng/mL *vs* 24.7 ng/mL, $P = 0.0003$). Also, the serum DCP value at diagnosis was significantly lower after the year 2000 (62 mAU/mL *vs* 38.2 mAU/mL, $P = 0.0027$). In contrast, there was no decrease in maximal tumor size after the year 2000 in comparison to the periods before ($P = 0.4301$). The percentage of patients with hepatitis B virus (HBV) infection who were administered a nucleoside analog against HBV was significantly higher after the year 2000 (7.5% *vs* 58.5%, $P < 0.0001$). The percentage of patients with hepatitis C virus (HCV) infection who had undergone interferon-based antiviral therapy against HCV was significantly higher after the year 2000 (7.6% *vs* 14.3%, $P = 0.0012$), although the rate of patients in whom HCV was eradicated by antiviral therapy (i.e. sustained virological responders) was not significantly different.

An analysis of the initial treatment selected (Table 3) demonstrated that the percentage of patients who underwent surgery as an initial treatment increased from the period 1997–2000, and was approximately 35% in the periods after the year 2000 (1997–2000 *vs* 2001–2004, $P = 0.0002$). In contrast, patients who underwent TACE decreased from over 50% in the period 1985–1988, to approximately 20% in the periods after the year 2000 (1997–2000 *vs* 2001–2004, $P < 0.0001$). LAT treatment increased from the period 1989–1992 (1989–1992 *vs* 1993–1996, $P = 0.0004$), and was almost constant after this period, with some fluctuations. However, when the details of the treatment were analyzed in patients who underwent LAT, the percentage of patients who underwent RFA as LAT markedly increased in the period after the year 2000 (1997–2000 *vs* 2001–2004, $P < 0.0001$), and most patients underwent RFA in this period. In six patients who underwent PEIT as LAT after the year 2000, a HCC tumor was located just beside the bowel (2 patients), gallbladder (1 patient), and main trunk of the intrahepatic bile duct (3 patients), and therefore, RFA should have been avoided. We found no patient who underwent RFA before the period 1993–1996. The percentage of patients who underwent RFA among patients treated by LAT was 21.9% in the period 1997–2000. It was 95.1% and 98.7% in the periods 2001–2004 and 2005–2008, respectively.
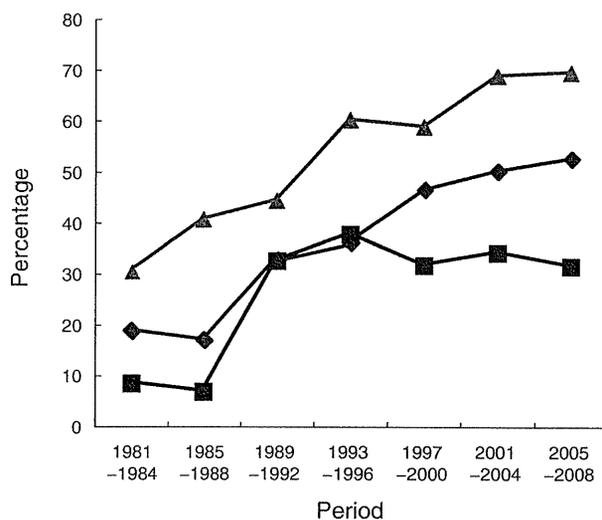

Figure 1 Changes in the percentage of patients with Child–Pugh class A or those without cirrhosis (green line), the percentage of patients with hepatocellular carcinoma (HCC) of maximal tumor size < 2 cm (red line), and the percentage of patients with HCC of American Joint Committee on Cancer (AJCC) tumor stage I (blue line) at the first diagnosis of HCC. Patients with Child–Pugh class A include patients with cirrhosis. ▲, Child–Pugh class A; ■, maximal tumor size < 2 cm; ◆, AJCC tumor stage I.

Table 3 Initial treatment for hepatocellular carcinoma by periods

Periods	Surgery	Locoregional ablative therapies				TACE	Others	None
		Total	PEIT	PMCT	RFA			
1981–1984 ($n = 141$)	19 (13.5)	0	0	0	0	39 (27.7)	38 (26.9)	45 (31.9)
1985–1988 ($n = 220$)	25 (11.4)	6 (2.7)	6 (2.7)	0	0	115 (52.3)	31 (14.1)	43 (19.5)
1989–1992 ($n = 292$)	46 (15.8)	50 (17.1)	50 (17.1)	0	0	114 (39.0)	28 (9.6)	54 (18.5)
1993–1996 ($n = 305$)	42 (13.8)	91 (29.8)	90 (29.5)	1 (0.3)	0	108 (35.4)	23 (7.5)	41 (13.5)
1997–2000 ($n = 334$)	72 (21.5)	64 (19.2)	38 (11.4)	12 (3.6)	14 (4.2)	109 (32.6)	25 (7.5)	64 (19.2)
2001–2004 ($n = 366$)	127 (34.7)	103 (28.2)	5 (1.4)	0	98 (26.8)	59 (16.1)	22 (6.0)	55 (15.0)
2004–2008 ($n = 355$)	128 (36.1)	78 (22.0)	1 (0.3)	0	77 (21.7)	74 (20.8)	21 (5.9)	54 (15.2)

PEIT, percutaneous ethanol injection therapy; PMCT, percutaneous microwave thermocoagulation therapy; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization.

Survival rate for patients with HCC by period

The survival rates were compared according to the periods of initial HCC diagnosis (Fig. 2). The survival rates increased throughout the periods 1981–1984, 1985–1988, and 1989–1992 (1981–1984 *vs* 1985–1988, $P = 0.0003$; 1985–1988 *vs* 1989–1992, $P = 0.0009$; 1989–1992 *vs* 1993–1996, $P = 0.0383$). We found no difference in
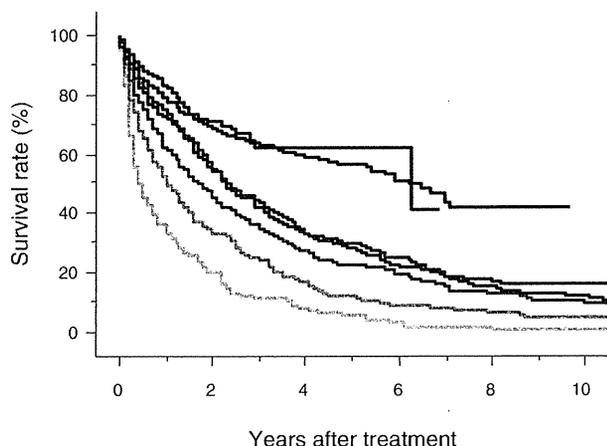

Figure 2 Survival rate of patients with hepatocellular carcinoma (HCC) according to the period of initial HCC diagnosis. ⋯⋯, 1981–1984 (*n* = 141); ▬▬, 1985–1988 (*n* = 220); ▬▬, 1989–1992 (*n* = 292); ▬▬, 1993–1996 (*n* = 305); ▬▬, 1997–2000 (*n* = 334); ▬▬, 2001–2004 (*n* = 366); ▬▬, 2004–2008 (*n* = 355).

the survival rates between the periods 1993–1996 and 1997–2000 ($P = 0.5887$). In contrast, the survival rate markedly increased in the period 2001–2004 in comparison to the period 1997–2000 ($P < 0.0001$). Again, we found no difference in the survival rates between the periods 2001–2004 and 2005–2008 ($P = 0.5151$).

When we compared the survival rates according to treatment, it was highest in patients treated by surgery, followed by patients treated by LAT, by TACE, by other treatment, and patients without treatment in this order (data not shown). In patients who underwent LAT, the survival rate was higher in patients treated by RFA than those treated by PEIT ($P = 0.0073$, Fig. S1).

Multivariate analyses for factors affecting survival rates

We conducted a multivariate analysis to determine factors that were associated with patient survival rate (Table 4). The analysis revealed that Child–Pugh class (B and C), AJCC tumor stage (II and III), treatment (surgery, LAT, TACE, and other treatments), and the period of diagnosis (periods 1997–2000, 2001–2004, and 2005–2008) were independently associated with patient survival rate.

Discussion

In the present study, we analyzed several trends in patients with HCC, including the periods after the year 2000. The incidence of HCC is reportedly increasing in the USA and other Western countries.[11,12] In contrast, a recent study demonstrated that the incidence

Table 4 Multivariate analysis of factors associated with patient survival

Factor		Parameter estimate	Standard error	X	Risk ratio (95% confidence interval)	*P*-value
Age		0.0038	0.0033	1.36	1.0038 (0.9974–1.0102)	0.2428
Sex	Male				1	
	Female	−0.0566	0.0337	2.87	0.9449 (0.8845–1.0095)	0.0904
Child–Pugh class	A†				1	
	B	0.2379	0.0328	52.00	1.2686 (1.1895–1.3529)	< 0.0001
	C	0.4768	0.0481	89.41	1.6109 (1.4658–1.7703)	< 0.0001
AJCC tumor stage	Stage I				1	
	Stage II	0.2559	0.0380	45.36	1.2916 (1.1990–1.3915)	< 0.0001
	Stage III	0.6170	0.0431	206.14	1.8534 (1.7032–2.0168)	< 0.0001
	Stage IV	0.7973	0.0704	97.98	2.2196 (1.9334–2.5481)	< 0.0001
Treatment	No treatment				1	
	Surgery	−0.7011	0.0599	139.24	0.4960 (0.4411–0.5578)	< 0.0001
	LAT	−0.6365	0.0559	129.84	0.5291 (0.4742–0.5904)	< 0.0001
	TACE	−0.4039	0.0451	77.19	0.6677 (0.6113–0.7294)	< 0.0001
	Other	−0.1233	0.0530	5.49	0.8840 (0.7968–0.9808)	0.0192
Period	1981–1984				1	
	1985–1988	−0.0832	0.0569	2.12	0.9202 (0.8231–1.0287)	0.1456
	1989–1992	−0.0818	0.0543	2.23	0.9215 (0.8285–1.0249)	0.1350
	1993–1996	−0.0824	0.0557	2.16	0.9209 (0.8256–1.0272)	0.1419
	1997–2000	−0.1208	0.0553	4.67	0.8862 (0.7952–0.9877)	0.0306
	2001–2004	−0.2904	0.0621	21.52	0.7480 (0.6623–0.8448)	< 0.0001
	2005–2008	−0.2988	0.0707	18.33	0.7417 (0.6456–0.8520)	< 0.0001

†Category of Child–Pugh class A includes patients without cirrhosis. AJCC, American Joint Committee on Cancer; LAT, locoregional ablative therapies including percutaneous ethanol injection therapy, percutaneous microwave thermocoagulation therapy, and radiofrequency ablation; TACE, transcatheter arterial chemoembolization.

of HCC in urban areas of Japan began to decrease by the year 2000.[13] However, we did not find a decrease in the number of patients with HCC during this period. This authors' institution is located in a county area, and the results indicated that the incidence of HCC has not started to decrease and remains constant even after the year 2000 for county regions of Japan.

In the comparisons based on the period of the HCC diagnosis in the present study, patient age continued to increase throughout the study period. One of the most important risk factors for the development of HCC worldwide[14,15] is chronic viral hepatitis, and the majority of patients with HCC in Japan have chronic HCV infection.[16] The age of Japanese individuals with HCV infection is increasing, thus contributing to the higher patient age found in this study.

When we analyzed the characteristics of patients with HCC who were diagnosed after the year 2000, in comparison to those diagnosed in the 1990s, the prevalence of patients with liver function of Child–Pugh class A significantly increased, along with the serum albumin level and patient age. This indicates the trend that patients had better liver function at first diagnosis of HCC after the year 2000. At the same time, it indicates that HCC develops more frequently in livers with less progressed fibrosis, and even in livers without cirrhosis, after the year 2000. The reason for this better liver function at diagnosis is unknown. It could be due to the increase in patient age at diagnosis in Japan, as it has been reported that HCC develops in the liver with less progressed fibrosis in cases of high-aged patients.[17] It could also be due to the increase in the number of patients with a history of several antiviral therapies, including nucleoside analogs for patients with HBV infection and interferon-based therapy for those with HCV infection. Indeed, the percentage of patients who underwent antiviral therapy markedly increased after the year 2000 in both patients with HBV and HCV infection. Further studies will be needed to clarify the reason for the increase of HCC patients with better liver function in the period after the year 2000.

With respect to HCC tumor progression at diagnosis, we did not find an improvement in the size of HCC tumor at diagnosis in the period after the year 2000. The size of maximal HCC tumor at diagnosis reached a plateau after the period 1993–1996. Tremendous effort has been made to increase the detection of small-size HCC tumors, especially during the 1990s. This has led to the development of various scanning techniques and imaging apparatuses,[18,19] identification of highly-sensitive and specific tumor markers,[20,21] and the establishment of surveillance for patients at high-risk of developing HCC.[5,22] It appears that this effort reached a limit during the 1990s, as it currently is difficult to detect smaller HCC tumors. In contrast to the size of maximal HCC tumors, the prevalence of single HCC tumors or HCC without vascular invasion significantly increased after the year 2000. This resulted in an increase in patients with an earlier tumor stage of HCC. Also, serum AFP and DCP values at diagnosis, which are reported to be a biomarker of biological malignant features of HCC, continued decreasing after the year 2000. Advances in techniques for the early detection of HCC continue to effect the improvement not of the size of HCC tumors but of these factors after the year 2000; less advanced HCC continues increasing, even in the same size HCC tumors.

In regards to patient prognosis, the survival rates were almost constant during the period 1993–2000. This is associated with the fact that the detection of small-sized HCC tumors reached a plateau during this period. In contrast, the survival rates significantly increased after the year 2000. The increase in patients with better liver function at diagnosis after the year 2000 resulted in an increase of the percentage of patients who underwent hepatectomy as an initial treatment in this period. This increase in the percentage of patients who underwent hepatectomy for the treatment for HCC might have contributed to the improvement in the survival rate of patients with HCC diagnosed after the year 2000. In addition, the percentage of patients who underwent RFA markedly increased after the year 2000. Considering the higher survival rate in patients who underwent RFA as LAT than that in patients treated by PEIT, the emergence of RFA as a treatment modality for HCC might also have played a role in the increasing survival rate after the year 2000.

In conclusion, the survival rate of patients with HCC continues to increase after the year 2000 in Japan. This is not due to improvements in the detection of small HCC tumors, as observed in 1990s, but is a consequence of the increase in the percentage of patients with better liver function and patients with single HCC tumors or HCC without vascular invasion with low serum tumor marker levels at first diagnosis. These changes resulted in an increase in the number of patients who underwent radical curative treatment and contributed to the continuing improvement of patient prognosis. Further studies will be necessary to elucidate the reasons for these changes in the characteristics of patients with HCC after the year 2000.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Survival rate of patients with hepatocellular carcinoma treated by percutaneous ethanol injection therapy and radiofrequency ablation as a locoregional ablative therapy.

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Clinical utility of highly sensitive *Lens culinaris* agglutinin-reactive alpha-fetoprotein in hepatocellular carcinoma patients with alpha-fetoprotein <20 ng/mL

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The *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) has been used as a diagnostic and prognostic marker of hepatocellular carcinoma (HCC). The analytical sensitivity of a conventional method for AFP-L3% is not sufficient in patients with a low AFP level. This study was performed to determine the clinical utility of a newly developed highly sensitive AFP-L3% (hs-AFP-L3%) assay in patients with an AFP level <20 ng/mL. In the cohort study, serum samples obtained from 270 patients with newly diagnosed HCC before treatment and 396 patients with chronic liver disease at Ogaki Municipal Hospital, in both of which the AFP level was <20 ng/mL, were measured for conventional AFP-L3% (c-AFP-L3%), hs-AFP-L3% and des-gamma-carboxy prothrombin (DCP). Diagnostic sensitivity and specificity of hs-AFP-L3% at a cut-off level of 5% were 41.5% and 85.1%, respectively, significantly increasing the sensitivity from 7.0% for c-AFP-L3%. Multivariate analysis identified hs-AFP-L3% as an independent factor associated with reduced long-term survival. The survival rate of patients with high hs-AFP-L3% (≥5%) before treatment was significantly poorer than that of patients with low hs-AFP-L3% (<5%) (*P* < 0.001). In patients with AFP <20 ng/mL, measurements of AFP-L3% by the highly sensitive method before treatment were more useful for diagnosis and prognosis of HCC than by the conventional method. (*Cancer Sci* 2011; 102: 1025–1031)

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, and the third most common cause of cancer-related death.[1] A diagnosis of HCC is typically marked with a poor prognosis, largely because early HCC is difficult to diagnose. Three tumor markers, alpha-fetoprotein (AFP),[2–5] *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3)[6–8] and des-gamma-carboxy prothrombin (DCP),[9–11] are currently available as serological markers of HCC for surveillance, diagnosis and patient outcome prediction. Alpha-fetoprotein has been widely used as an *in vitro* diagnostic, and different cut-off values have been proposed by previous studies.[12–14] However, total AFP is not always specific for HCC, especially when HCC is in the early stages. In contrast, a fucosylated fraction of AFP (AFP-L3) is highly specific for HCC. A combination assay of AFP-L3% and DCP results in an improvement in diagnostic sensitivity compared with AFP-L3% or DCP alone.[15] Furthermore, higher levels of AFP-L3% prior to treatment are also associated with poorer prognosis.[8,16,17].

The percentage of AFP-L3 is determined by AFP-L3 concentration divided by the total AFP concentration in serum. Therefore, the analytical sensitivity for AFP-L3% can be affected inversely by the total AFP concentration. In addition, the clinical

usefulness of AFP-L3% has been hampered by insufficient analytical sensitivity of the conventional assay system in patients with a low AFP level, which is a liquid-phase binding assay on an auto-analyzer (LiBASys).[18,19]

Recent technical improvements to the highly sensitive analytical methods, by using novel and advanced microfluidics-based separation science, have improved the analytical sensitivity of this assay.[20] This new generation of assays [micro-total analysis system (μTAS)] has enabled the accurate measurement of AFP-L3% at very low AFP concentrations. In patients with an AFP level of ≥20 ng/mL, μTAS AFP-L3% correlated well with LiBASys AFP-L3%.[20] Therefore, we conducted a retrospective cohort study to determine the clinical utility of the new highly sensitive AFP-L3% (hs-AFP-L3%) assay for diagnosis of HCC of our patient population under surveillance who were at risk of HCC and with AFP <20 ng/mL. In addition, we evaluated the clinical usefulness of this new AFP-L3% as a prognostic factor in patients with HCC with AFP <20 ng/mL.

Methods

Patients. Between January 1995 and December 2007 there were 1004 patients diagnosed with primary HCC at Ogaki Municipal Hospital. The AFP levels of all patients had been measured, and 461 patients (46%) had AFP <20 ng/mL. Of these 461 patients, 270 met the eligibility criteria (availability of stored serum samples, patient informed consent and Child–Pugh class A or B). Patients with Child–Pugh class C were not enrolled in the present study, because the influence of poor liver function on patients' survival was too dominant to accurately evaluate HCC-related death in the prognosis part of this study.

Hepatocellular carcinoma was diagnosed by histological examination or the appropriate imaging characteristics using criteria similar to the guidelines accepted by the American Association for the Study of Liver Diseases.[21] Tumor stage on imaging findings was assessed on the basis of the TNM classification of the Liver Cancer Study Group of Japan.[22]

Control samples were obtained from 396 patients with chronic liver disease without HCC, which were collected during routine HCC surveillance in the same period. These patients had AFP <20 ng/mL and met the eligibility criteria of stored samples availability and patient informed consent. There were 98 patients classified by histological confirmation of chronic liver disease, and 298 patients were diagnosed by imaging findings and biochemical tests. To ensure that controls did not have

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HCC, these patients were followed for 3 years after serum sampling by ultrasonography, computed tomography or magnetic resonance imaging to ensure that none had developed HCC.

Individual decisions regarding treatment were made principally on the basis of the treatment guidelines for HCC in Japan.[23] Patients were initially assessed for eligibility for surgical treatment. When patients declined or were deemed ineligible for surgical treatment, they underwent non-surgical treatment. Patients were first offered locoregional ablative therapy (LAT) with percutaneous ethanol injection or, more recently, radiofrequency ablation. Patients who were not eligible for either resection or locoregional ablative therapy were offered transcatheter arterial chemoembolization (TACE). No patient underwent liver transplantation as a treatment.

Each HCC patient's follow-up period began between 1995 and 2007 and continued until death or December 2008, whichever came first. The follow-up period ranged from 0.3 to 101.6 months (a median of 28.2 months).

The study protocol was approved by the Institutional Ethics Review Board and was in compliance with the Declaration of Helsinki.

Assays of AFP, AFP-L3% and DCP. AFP, AFP-L3% and DCP were measured in the same serum sample obtained at the time of HCC diagnosis before any treatment (for HCC group) or from a sample obtained at least 3 years before the examination that confirmed the absence of HCC (for the control group). The measurements of hs-AFP-L3% and DCP were achieved by using a microchip capillary electrophoresis and liquid-phase binding assay on a μTASWako i30 auto analyzer (Wako Pure Chemical Industries, Ltd, Osaka, Japan).[20] The measurements of conventional AFP-L3% (c-AFP-L3%) were performed using a column chromatography and liquid-phase binding assay on a LiBASys auto analyzer (Wako Pure Chemical Industries, Ltd).[18,19] Analytical sensitivity of μTAS is 0.3 ng/mL AFP, and the percentage of AFP-L3 can be measured when AFP-L3 is over 0.3 ng/mL. Analytical sensitivity of LiBASys is 0.8 ng/mL AFP, but AFP-L3% can not be calculated at AFP <10 ng/mL. Therefore the correlation between μTAS and LiBASys was poor at AFP <20 ng/mL.

Statistical analyses. To evaluate the diagnostic value of hs-AFP-L3% and DCP, sensitivity and specificity were calculated. For the evaluation of prognosis, the long-term survival of

Table 1. Demographics of the study population

Characteristics	Patients with HCC (n = 270)	Patients without HCC (n = 396)
Gender (%)		
Male/Female	209 (77)/61 (23)	207 (52)/189 (48)
Age	67.9 ± 8.8	63.5 ± 12.2
Mean ± SD		
Infection of hepatitis virus (%)	38 (14)/193 (71)/7 (3)/32 (12)	82 (21)/217 (55)/12 (3)/85 (21)
HBV/HCV/HBV + HCV/none		
Child–Pugh class (%)	215 (80)/55 (20)/0	323 (82)/73 (18)/0
A/B/C		
Platelet count (×10³/mm³)	12.4 (7.6, 17.1)	11.3 (8.8, 17.6)
Median (25%, 75% quartile)		
ALT (IU/L)	41.5 (27.0, 64.8)	31.0 (20.0, 57.3)
Median (25%, 75% quartile)		
AST (IU/L)	48.0 (34.0, 68.5)	36.0 (26.0, 61.0)
Median (25%, 75% quartile)		
Total bilirubin (mg/dL)	0.8 (0.5, 1.0)	0.7 (0.5, 1.0)
Median (25%, 75% quartile)		
Albumin (g/dL)	3.7 (3.3, 4.1)	3.9 (3.4, 4.2)
Median (25%, 75% quartile)		
AFP (ng/mL)	6.7 (3.6, 10.3)	2.8 (1.8, 4.9)
Median (25%, 75% quartile)		
c-AFP-L3% (%)	0.5 (0.5, 0.5)	0.5 (0.5, 0.5)
Median (25%, 75% quartile)		
hs-AFP-L3% (%)	4.2 (0.5, 7.1)	0.5 (0.5, 0.5)
Median (25%, 75% quartile)		
DCP (mAU/mL)	33 (18, 182)	19 (15, 27)
Median (25%, 75% quartile)		
Tumor stage†		
I	89	NA
II	127	NA
III	47	NA
IV	7	NA
Tumor size		
≤2 cm	123	NA
>2 and ≤3 cm	63	NA
>3 and ≤5 cm	52	NA
>5 cm	32	NA
Tumor number		
Single	189	NA
Multiple	81	NA

†According to TNM staging by the Liver Cancer Study Group of Japan. AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

Table 2. Sensitivity and specificity. (a) Patients with AFP <20 ng/mL (HCC, n = 270; control, n = 396), and (b) patients with AFP <10 ng/mL (HCC, n = 199; control, n = 357)

	Cut-off (%)	Sensitivity (%)	Specificity (%)
(a) AFP <20 ng/mL			
c-AFP-L3%	5	7.0	98.5
	7	5.2	98.7
	10	4.1	99.0
hs-AFP-L3%	5	41.5	85.1
	7	26.7	92.4
	10	14.8	98.2
(b) AFP <10 ng/mL			
c-AFP-L3%	5	0.0	100.0
	7	0.0	100.0
	10	0.0	100.0
hs-AFP-L3%	5	36.2	88.5
	7	23.6	93.8
	10	11.6	98.3

AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma.

patients with HCC was determined by the Kaplan–Meier method, and the log-rank test was used to compare the survival rates. The Cox proportional hazards model was used for multivariate analysis for factors that influenced patient survival. The values were considered significant when the P value was <0.05. All analyses were performed using JMP6 statistical software (SAS Institute, Cary, NC, USA).

Results

Clinical features of patients. The demographics, etiology of liver disease, hepatic functional reserve ranked by Child–Pugh classification, tumor stage, tumor size and tumor number of the study patients are summarized in Table 1. Overall, there were 270 patients with HCC, including 89 in stage I, 127 in stage II, 47 in stage III and seven in stage IV. The majority of HCC (80.0%) were diagnosed stages I and II. The levels of median and quartile for AFP concentration, c-AFP-L3%, hs-AFP-L3%, DCP and other laboratory tests are shown in Table 1. The numbers of HCC patients and non-HCC patients with AFP <10 ng/mL were 199 out of 270 (73.7%) and 357 out of 396 (90.2%), respectively. In addition to evaluating AFP-L3% in patients with AFP <20 ng/mL, we further evaluated the patient group with AFP <10 ng/mL.

Sensitivity and specificity. The receiver-operating characteristic (ROC) curve was constructed to evaluate the area under the curve (AUC) for AFP-L3%. In all patients (HCC, n = 270; control, n = 396), the AUC of μTAS hs-AFP-L3% and prior-generation methodology LiBASys c-AFP-L3% were 0.707 and 0.537 respectively (P < 0.05) (data not shown). In patients with AFP <10 ng/mL (HCC, n = 199; control, n = 357), the AUC of μTAS hs-AFP-L3% was 0.668, but that of LiBASys c-AFP-L3% was not measured because AFP-L3% was out of the reportable range (data not shown). These indicated that the diagnostic accuracy of μTAS hs-AFP-L3% was superior to that of LiBASys c-AFP-L3%.

Overall sensitivity and specificity calculated for patients with AFP <20 ng/mL and for those with AFP <10 ng/mL using three cut-off values (5%, 7% and 10%) are shown in Table 2. In

Table 3. Sensitivity by tumor characteristics. (a) Patients with AFP <20 ng/mL (n = 270), and (b) <10 ng/mL (n = 199)

	Analyte (n)	c-AFP-L3% (%)	hs-AFP-L3% (%)	DCP (%)	hs-AFP-L3% and DCP (%)
	Cut-off	10%	5%	40 mAU/mL	5% + 40 mAU/mL
(a)					
Stage†					
I	89	4.5	34.8	20.2	44.9
II	127	2.4	42.5	57.5	71.7
III	47	6.4	53.2	53.2	74.5
IV	7	14.3	28.6	71.4	85.7
Size					
≤2 cm	123	4.1	36.6	24.4	48.8
>2 and ≤3 cm	63	3.2	46.0	52.4	65.1
>3 and ≤5 cm	52	3.8	44.2	63.5	80.8
>5 cm	32	6.3	46.9	78.1	90.6
Number					
Single	189	3.2	39.2	43.9	60.8
Multiple	81	6.2	46.9	46.9	70.4
(b)					
Stage†					
I	66	0.0	28.8	18.2	40.9
II	97	0.0	37.1	58.8	70.1
III	30	0.0	53.3	56.7	73.3
IV	6	0.0	16.7	83.3	83.3
Size					
≤2 cm	90	0.0	31.1	23.3	45.6
>2 and ≤3 cm	43	0.0	37.2	46.5	55.8
>3 and ≤5 cm	42	0.0	40.5	69.0	81.0
>5 cm	24	0.0	45.8	87.5	95.8
Number					
Single	146	0.0	33.6	43.8	58.2
Multiple	53	0.0	43.4	50.9	69.8

†According to TNM staging by the Liver Cancer Study Group of Japan. AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

patients with AFP <20 ng/mL, when the cut-off value for AFP-L3% was set at 5%, the sensitivity and specificity of c-AFP-L3% were 7.0% and 98.5%, respectively. Those of hs-AFP-L3% were 41.5% and 85.1%, respectively. Sensitivity of hs-AFP-L3% was significantly higher than that of c-AFP-L3% (P < 0.05). Focusing on patients with AFP <10 ng/mL, the sensitivity of hs-AFP-L3% was 36.2%, which was still much higher than that for c-AFP-L3%. A cut-off value of 10% has been reported for diagnosis of HCC using the earlier generation methodology.[8] For this study, to maintain the specificity at 85% or more, we chose a cut-off value of 5% for hs-AFP-L3% and 10% for c-AFP-L3%.

Sensitivity with respect to tumor characteristics. Patients were classified by tumor stage (I, II, III and IV), tumor size (<2, 2–3, 3–5 and >5 cm) and tumor number (single tumor and multiple tumors). In patients with AFP <20 ng/mL, sensitivities by tumor characteristics are shown for c-AFP-L3% (cut-off 10%), hs-AFP-L3% (cut-off 5%), DCP (cut-off 40 mAU/mL) and hs-AFP-L3%-DCP combined in Table 3. Sensitivities of hs-AFP-L3% in stages I and II were 34.8% and 42.5%, respectively, whereas those of c-AFP-L3% were only 4.5% and 2.4%, respectively. Those of DCP in stage I and II were 20.2% and 57.5%, respectively. Combination of hs-AFP-L3% and DCP resulted in an improvement in sensitivity compared with hs-AFP-L3% or DCP alone. Those of the combination in stage I and II were 44.9% and 71.7%, respectively. Focusing on patients with AFP <10 ng/mL, sensitivity using the combination in stages I and II were 40.9% and 70.1%, respectively.

In patients treated by hepatectomy, 13 patients had well-differentiated HCC by postoperative pathological examination. Hs-AFP-L3% was elevated (≥5%) in four patients (30.8%). Hypervascularity of the tumor was not detected with computed tomography during hepatic arteriography, the most sensitive imaging modality to detect hypervascularity, in five patients. Hs-AFP-L3% was elevated in one of these hypovascular HCC (20.0%).

Survival rates of patients with HCC. We evaluated the significance of hs-AFP-L3% on the survival rate of HCC patients (Fig. 1). Statistical significance were not observed between the patients with high c-AFP-L3% (≥10%) and the patients with low c-AFP-L3% (<10%) (P = 0.175). The survival rate of patients with high hs-AFP-L3% (≥5%) was significantly lower than that of patients with low hs-AFP-L3% (<5%) by the log-rank test (P < 0.001). Statistical significance was not observed between the patients with high DCP (≥40 mAU/mL) and the patients with low DCP (<40 mAU/mL) (P = 0.197). Focusing on patients with AFP <10 ng/mL, statistical significance was still observed between the patients with high hs-AFP-L3% (≥5%) and the patients with low hs-AFP-L3% (<5%) (P = 0.035).

Univariate and multivariate analyses for prognostic factors for HCC. Table 4 shows the results of univariate and multivariate analyses of prognostic factors evaluated by Cox proportional hazards model in patients with AFP <20 ng/mL. The factors in the analysis were c-AFP-L3%, hs-AFP-L3%, DCP, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, type of treatment, tumor stage, age and gender. In the univariate analysis, the hazard ratios of hs-AFP-L3%, total bilirubin, albumin, type of treatment and tumor stage were statistically significantly high (P = 0.001, <0.001, 0.001, 0.001 and 0.006, respectively). Those of c-AFP-L3% and DCP were not statistically significant (P = 0.218 and 0.202, respectively). In the multivariate analysis, hs-AFP-L3% and non-resection were independent prognostic factors with significantly high hazard ratios (P = 0.026 and <0.001. respectively). For patients with AFP <10 ng/mL, hs-AFP-L3% was identified as a prognostic factor by univariate analysis (P = 0.045) but not by multivariate analysis (P = 0.457) (data not shown).

Survival rates of patients stratified by the type of treatment. In patients with AFP <20 ng/mL and classified into stages I and II, survival rates evaluated by treatment and by hs-AFP-L3% status are shown in Figure 2. All patients with any treatments (n = 216) are shown in Figure 2a, patients with
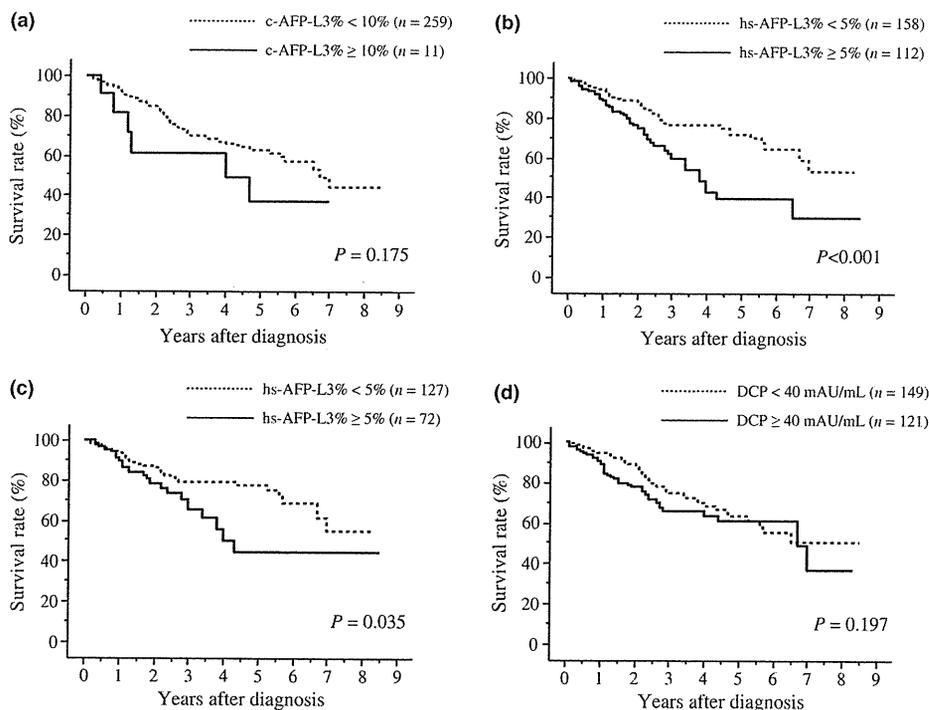

Fig. 1. Survival rates. (a) c-AFP-L3% in patients with AFP <20 ng/mL (n = 270), (b) hs-AFP-L3% in patients with AFP <20 ng/mL (n = 270), (c) hs-AFP-L3% in patients with AFP <10 ng/mL (n = 199), and (d) Des-gamma-carboxy prothrombin (DCP) in patients with AFP <20 ng/mL (n = 270). c-AFP-L3%, conventional AFP-L3%; hs-AFP-L3%, highly sensitive AFP-L3%.

Table 4. (a) Univariate and (b) multivariate analyses for prognostic factors of hepatocellular carcinoma in patients with alpha-fetoprotein <20 ng/mL

	Variables	Hazard ratio (95% confidence interval)	P value
(a) Univariate analyses			
c-AFP-L3%	≥10%	1.765 (0.683–3.739)	0.218
hs-AFP-L3%	≥5%	2.195 (1.401–3.450)	0.001
DCP	≥40 mAU/mL	1.335 (0.855–2.080)	0.202
ALT	≥40 IU/L	1.132 (0.725–1.792)	0.587
AST	≥40 IU/L	1.370 (0.845–2.310)	0.207
Total bilirubin	≥1 mg/dL	2.466 (1.543–3.901)	<0.001
Albumin	<3 g/dL	2.868 (1.567–4.923)	0.001
Treatment	(LAT + TACE/ resection)	4.893 (2.876–8.832)	<0.001
Stage†	III + IV/I + II	2.111 (1.247–3.440)	0.006
Age		1.009 (0.983–1.037)	0.504
Gender	Male/Female	1.185 (0.902–1.616)	0.232
(b) Multivariate analysis			
hs-AFP-L3%	≥5%	1.697 (1.066–2.709)	0.026
Total bilirubin	≥1 mg/dL	1.575 (0.961–2.558)	0.071
Albumin	<3 g/dL	1.650 (0.878–2.930)	0.116
Treatment	(LAT + TACE/ resection)	3.627 (2.066–6.708)	<0.001
Stage†	III + IV/I + II	1.675 (0.982–2.753)	0.058

†According to TNM staging by the Liver Cancer Study Group of Japan. AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DCP, des-gamma-carboxy prothrombin; LAT, locoregional ablative therapies; TACE, transcatheter arterial chemoembolization.

resection ($n = 103$) in Figure 2b, patients with LAT ($n = 56$) in Figure 2c and patients with TACE ($n = 57$) in Figure 2d. The difference in the survival rate of patients with resection was not found patients with high hs-AFP-L3% and with low hs-AFP-L3% ($P = 0.813$). In the case of LAT, the survival rate of patients with high hs-AFP-L3% was significantly lower than that of patients with low hs-AFP-L3% ($P = 0.037$). The survival rate of patients with high hs-AFP-L3% tended to be lower than that of patients with low hs-AFP-L3%, but the difference was not statistically significant in the case of TACE. The survival rate of patients with resection was significantly higher than that of patients with ablation and TACE regardless of the hs-AFP-L3% level ($P = 0.002$) (data not shown).

Discussion

Alpha-fetoprotein, AFP-L3% and DCP are used as markers for HCC, and their utility in the diagnosis of HCC and the evaluation of tumor progression and prognosis has been reported. Alpha-fetoprotein is the most widely used marker for monitoring HCC development. However, elevated AFP is not typically observed in patients with a small tumor or early stage HCC. Recent advances in diagnostic imaging techniques have allowed for the detection of small tumors and early stage HCC,[24–28] and the establishment of surveillance programs for HCC in the high-risk group have also contributed to diagnosis of early stage HCC.[29,30] These trends have resulted in an increase in the number of HCC patients diagnosed by imaging without elevation of AFP. Thus, HCC patients with low AFP represent the appropriate study population in a successful HCC surveillance program. Among the tumor markers, AFP-L3% is highly specific for HCC, and elevated AFP-L3% correlates with tumor progression, poor tumor differentiation and unfavorable prognosis.[8,11,31–33]
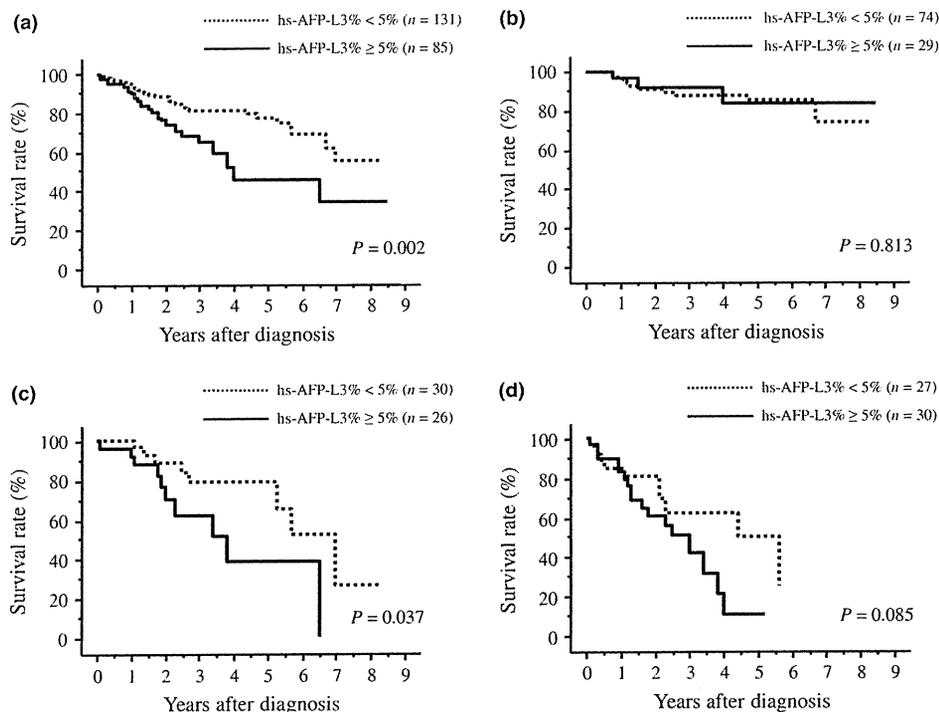

Fig. 2. Survival rates of patients stratified by the type of treatment in patients with alpha-fetoprotein (AFP) <20 ng/mL and classified into stages I and II. (a) All patients with any treatments ($n = 216$), (b) patients treated by surgical resection ($n = 103$), (c) patients treated by locoregional ablative therapies ($n = 56$), and (d) patients treated by transcatheter arterial chemoembolization ($n = 57$).

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However, measurement of AFP-L3% by the conventional assay system has not always been reliable in patients with AFP <20 ng/mL due to low analytical sensitivity. Therefore, the clinical utility of conventional AFP-L3% has limited use in the diagnosis and prediction of outcome of this subpopulation. The present study focused on patients with AFP <20 ng/mL, and further, the subgroup with AFP <10 ng/mL, and revealed that the hs-AFP-L3% assay could diagnose earlier stage HCC than the c-AFP-L3 assay (cut-off 5%). The combination assay with DCP resulted in a significant improvement in diagnostic sensitivity. Parallel measurement of hs-AFP-L3% and DCP will identify additional HCC patients in the early stage because the markers are complementary for different subgroups of HCC.

Regarding prognosis, in patients with AFP <20 ng/mL, the survival rate of patients with elevated hs-AFP-L3% (≥5%) was significantly lower than that of patients with low hs-AFP-L3% (<5%). Univariate and multivariate analysis identified hs-AFP-L3% as an independent factor associated with long-term survival. Furthermore, high hs-AFP-L3% (≥5%) in the present study suggested an unfavorable prognosis, even when focusing on patients with stages I and II. In patients with stages I and II HCC treated by surgical resection, there was no statistically significant difference in survival between patients with high hs-AFP-L3% (≥5%) and those with low hs-AFP-L3% (<5%). The survival rate of patients treated by hepatic resection was much higher than that of patients with LAT or TACE. Thus, hepatic resection demonstrated favorable effects on survival compared with the other treatments, which might confound the clinical utilities of hs-AFP-L3%. In patients with AFP <10 ng/mL,

hs-AFP-L3% was not identified as a prognostic factor by multivariate analysis, probably for the same reason. Although in our previous study using conventional AFP-L3% there was no difference in survival between patients with high AFP-L3% and those with low AFP-L3% in the patients treated surgically,[34] postoperative AFP-L3% has been reported as a predictive marker for recurrence and long-term survival.[35] To evaluate the prognosis of patients with resection, measurements of hs-AFP-L3% using samples after treatment should be performed.

The lower survival rate of patients with elevated hs-AFP-L3% and high rate of elevation in early stage HCC indicated that hs-AFP-L3 will be useful in identifying early stage HCC but with poorer prognosis, for which early diagnosis and treatment would be important. It may be advisable that hs-AFP-L3% should be included as a routine screening tool for HCC in the surveillance of patients at high risk of the development of HCC, together with imaging modalities.

In conclusion, the present study shows that hs-AFP-L3% was a useful marker for the diagnosis of early stage HCC in patients with AFP <20 ng/mL, and parallel measurement with DCP improved sensitivity. In addition, measurement of hs-AFP-L3% before treatment could help predict patient prognosis.

Disclosure Statement

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Amino Acid Substitutions in the Hepatitis C Virus Core Region Are Associated With Postoperative Recurrence and Survival of Patients With HCV Genotype 1b-Associated Hepatocellular Carcinoma

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Objective: We researched the molecular marker for prognosis of postoperative patients with hepatocellular carcinoma (HCC).

Background: The association of amino acid substitutions in the hepatitis C virus (HCV) core region and hepatocarcinogenesis has recently been explored. We investigated if these amino acid substitutions are associated with recurrence or survival in patients with HCC after attempted curative treatment by hepatectomy.

Methods: A total of 163 patients infected with HCV genotype 1b who previously underwent hepatectomy for primary, not recurrent HCC were analyzed. Amino acid substitutions in the HCV core region were measured by direct sequencing. Postoperative recurrence or survival rates were compared according to tumor characteristics, tumor markers, and amino acid substitutions in the core region.

Results: Recurrence rates after hepatectomy were higher in patients bearing a methionine at residue 91 of the HCV core region than in patients with leucine (*P* = 0.0002). Survival was also decreased in patients with methionine at this residue from that seen in patients with leucine at this position (*P* = 0.0061). The associations between amino acid substitutions at residue 91 of the HCV core region and either recurrence or survival rates were independent of liver function, progression of HCC, or tumor marker levels.

Conclusions: Amino acid substitutions at residue 91 of the HCV core region are associated with postoperative recurrence or survival in patients infected with HCV genotype 1b who developed HCC and treated by hepatectomy. This factor should be taken into consideration for the postoperative management of patients with HCC.

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H epatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third most common cause of cancer-related death.[1,2] In Japan, HCC is the third and fifth most common causes of death from cancer in men and women, respectively.[3] One of the most important risk factors for the development of HCC[4,5] is chronic viral hepatitis. Hepatitis C virus (HCV) infection, one of main causes of chronic viral hepatitis, can result in liver cirrhosis and HCC.[6] The majority of patients in Japan with HCC exhibit chronic HCV infection.[7]

Amino acid substitutions in the HCV core region, especially at residues 70 and/or 91 have been associated with poor responses to antiviral therapy with peginterferon and ribavirin as curative therapy for HCV (eradication of HCV) in patients infected with HCV genotype 1b.[8,9] More recently, an association between substitutions in this region and aspects of hepatocarcinogenesis, including the incidence of HCC and patient prognosis, has been reported.[10–13] In previous reports, researchers demonstrated a difference in the incidence of HCC according to amino acid differences in residues 70 and/or 91 of the HCV core region.[10–12] In addition, Ogura et al[13] reported that the mortality of patients with HCC was affected by amino acid substitutions at residue 91 in the HCV core region as well as the type of initial treatment or preservation of liver function. This study, however, included patients who underwent a variety of treatments, both surgical and nonsurgical, and only survival rate was analyzed. It remains unclear if amino acid substitutions in the HCV core region affect the prognosis of patients with HCC who have been treated with curative intent.

In this study, we evaluated the impact of amino acid substitutions in the HCV core region (residues 70 and 91) on the survival and recurrence rates in patients with HCC after hepatectomy with curative intent.

METHODS

Patients

A total of 969 patients were diagnosed with primary, not recurrent HCC between January 1999 and December 2008 at Ogaki Municipal Hospital. Of these patients, 331 patients were treated with hepatectomy. Decisions regarding individual treatments were made based on the treatment guidelines for HCC in Japan.[14] In all patients, HCC tumors were resected with ample margins; enucleation of tumors without margins was not performed. HCV infection was confirmed in 229 of the 331 patients by positive serum HCV RNA at the time of HCC diagnosis using PCR-based detection. HCV genotype was assessed in 209 patients with PCR amplifying the core gene sequences using genotype-specific primers,[15] determining that 166 patients had infections with HCV genotype 1b. We excluded 3 patients who had coinfection with hepatitis B virus to avoid a possible impact of HBV on outcomes, resulting in a final study population of 163 patients (Supplemental Digital Content 1, available at: http://links.lww.com/SLA/A144). A diagnosis of HCC was confirmed by pathologic diagnosis of resected specimens.

After hepatectomy, all patients were followed for 1.65 to 128.9 months (median follow-up period, 48.3 months) at our institution with US and CT or MRI performed every 3 to 6 months. Regular monitoring of serum tumor markers (alpha-fetoprotein [AFP], *lens culinaris* agglutinin-reactive AFP [AFP-L3], and des-gamma-carboxy prothrombin [DCP]) was performed every 3 months. When an elevation of tumor markers was detected, additional imaging examination (usually by CT or MRI) was performed to check for a

recurrence of HCC. If the presence of a recurrence was confirmed, patients underwent treatment for recurrent HCC based on the treatment guidlines. Recurrent HCC was categorized into intrahepatic metastasis and multicentric recurrence according to the previous study.[16] Intrahepatic metastasis was defined as recurrent tumors consisting of moderately or poorly differentiated HCC with the same or lower degree of differentiation compared with the differentiation of the primary tumors. Multicentric recurrence was defined according to previously reported criteria with some modifications[17] as follows: (1) The recurrent tumors consisted of well-differentiated HCC occurring in a different hepatic segment, than even moderately or poorly differentiated preexisting HCCs; (2) Both the primary and recurrent tumors were well-differentiated HCCs; and (3) The recurrent tumor contained regions of dysplastic nodule in peripheral areas.

The entire protocol was approved by the hospital institutional review board and carried out in compliance with the Helsinki Declaration.

Measurements of Amino Acid Substitutions of HCV Core Region

Amino acid substitutions in the core region of HCV were analyzed by direct sequencing of amino acids 1–191 of genotype 1b[8] using stored serum samples. HCV RNA was extracted from serum samples and were PCR amplified using the following primer pairs:

5'-GCCATAGTGGTCTGCGGAAC-3' (CC11: outer, sense primer)

5'-GGAGCAGTCCTTCGTGACATG-3' (e14: outer, antisense primer),

5'-GCTAGCCGAGTAGTGTT-3' (CC9: inner, sense primer), and

5'-GGAGCAGTCCTTCGTGACATG-3' (e14: inner, antisense primer).

Amplified PCR products were purified and used for direct sequencing. Sequencing results were used to detect substitutions of arginine or glutamine at amino acid 70 and leucine or methionine at amino acid 91.

Measurement of the Tumor Markers for HCC

Three tumor markers for HCC, AFP, AFP-L3, and DCP, were measured in serum samples taken at the time of HCC diagnosis. Serum AFP levels were determined by enzyme-linked immunosorbent assay using a commercially available kit (ELISA-AFP, International Reagents, Kobe, Japan). A cut-off value of 20 ng/mL AFP was used to define AFP positivity, as proposed by Oka et al.[18] Serum AFP-L3, expressed as the percentage of total AFP (AFP-L3 level/total AFP level × 100), was measured by lectin-affinity electrophoresis followed by antibody-affinity blotting (AFP Differentiation Kit L, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cut-off value used to establish AFP-L3 positivity was 10%, as proposed by Shimizu et al.[19] The serum DCP level was determined by a specific enzyme immunoassay (Eitest PIVKA-II kit, Eisai Laboratory, Tokyo, Japan) according to the manufacturer's instructions. The cut-off value used to establish DCP positivity was 40 mAU/mL, as proposed by Okuda et al.[20]

Statistical Analyses

Differences in percentages between groups were analyzed by the χ^2 test. Differences in mean quantitative values were analyzed by the Mann–Whitney U test. The date of treatment (hepatectomy) was defined as time zero for calculations of patient recurrence and survival rates. In the analysis of recurrence rate, patients in whom

HCC recurred were noncensored, and those in whom HCC did not recur were censored. In the analysis of cancer specific survival rates, patients who died from HCC-related cause were noncensored and the other patients were censored. In the analysis of overall survival rate, all patients who died were not censored and surviving patients were censored. The Kaplan–Meier method[21] was used to calculate survival rates, whereas the log-rank test[22] was used to analyze differences in survival.

The Cox proportional hazards model[23] was used for univariate and multivariate analyses of factors related to recurrence and survival. Variables analyzed included patient age and gender, Child-Pugh class (A/B), tumor size (≤ 2 cm / >2 cm and ≤ 5 cm / >5 cm), number of tumors (single/multiple), macroscopic portal vein invasion (absent/present), amino acid substitutions of the HCV core region (at residue 70: arginine/glutamine, at residue 91: leucine/methionine), pretreatment serum AFP level (<20 ng/mL/≥ 20 ng/mL), pretreatment AFP-L3 proportion ($<10\%$/$\geq 10\%$), and pretreatment serum DCP level (<40 mAU/mL/≥ 40 mAU/mL). Data analyses were performed using the JMP statistical software, version 6.0 (Macintosh version; SAS Institute, Cary, NC, USA). All P values were derived from 2-tailed tests, with a $P < 0.05$ accepted as statistically significant.

RESULTS

Patients Characteristics

Table 1 summarizes the pretreatment characteristics of the study patients. This population was composed of 121 males and

TABLE 1. Characteristics of Study Patients (n = 163)

Age (mean ± SD, years) (range)	67.4 ± 7.1 (47–83)
Sex ratio (female/male)	42 (25.8)/121 (74.2)
Surveillance state at diagnosis (our institution/ others/ none)*	114 (69.9)/ 45 (27.6)/ 4 (2.4)
HCV infection	163 (100)
Child-Pugh class (A/B)†	152 (93.3)/ 11 (6.7)
Albumin (mean ± SD, g/dL)	3.85 ± 0.43
Total bilirubin (mean ± SD, mg/dL)	0.74 ± 0.35
15-minute retention rate of ICG (%)	15.5 ± 7.3
Prothrombin (%)	90.4 ± 14.3
Platelet (×1000/μL)	124 ± 52
Tumor size (mean ± SD, cm) (range)	2.93 ± 2.02 (0.6-7.6)
≤ 2 cm / > 2 cm and ≤ 5 cm / > 5 cm	63 (38.6)/ 80 (49.1)/ 20 (12.3)
Tumor number (single/multiple)	137 (84.0)/ 26 (16.0)
Macroscopic-portal vein invasion (absent/present)	143 (87.7)/ 20 (12.3)
Microscopic-portal vein invasion (absent/present)	133 (81.6)/ 30 (18.4)
AFP (median, ng/mL) (range)‡	18.0 (0.8–5280)
≥ 20 ng/mL / <20 ng/mL	84 (52.2)/ 77 (47.8)
AFP-L3 (median, %) (range)‡	0.5 (0–87.2)
$\geq 10\%$ / <10%	125 (82.8) /26 (17.2)
DCP (median, mAU/mL) (range)‡	42.0 (10–36,164)
≥ 40 mAU/mL / <40 mAU/mL	78 (49.4)/ 80 (50.6)
Interferon therapy after hepatectomy (no/yes)	139 (85.3)/ 24 (14.7)

Percentages were in parentheses.

HCV indicates hepatitis C virus; ICG, indocyanine green test; AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin.

*Our institution, patients had been under surveillance at our institution before the detection of HCC; others, patients had been under surveillance at family physician before the detection of HCC; none, patients had not beem under surveillance and had admitted to our institution with symptoms.

†Category of Child-Pugh class A includes patients without cirrhosis.

‡AFP, AFP-L3, and DCP were not measured in 2, 12, and 5 patients, respectively.

42 females with a mean age of 67.4 ± 7.1 years. HCC was detected under surveillance at our institution in 69.9% of patients, and 93.3% of patients exhibited Child-Pugh class[24] A liver function. Multiple tumors were present in 16.0% of patients. Macroscopic portal vein invasion was observed in 11.7% of patients, whereas microscopic portal vein invasion was observed in 18.4% of patients. Pretreatment AFP, AFP-L3, and DCP were above the specified cut-off levels in 47.8%, 17.2%, and 50.6% of patients, respectively. Antiviral therapy with interferon was performed in 24 (14.7%) patients after hepatectomy.

Recurrence Rate after Hepatectomy Based on Amino Acid Substitutions of the HCV Core Region and Pretreatment Serum Tumor Markers

Sequencing of the HCV core region failed in 6 patients, preventing detection of amino acid substitutions at residues 70 and 91. At residue 70, 87 of 157 patients (55.4%) possessed arginine, whereas 70 patients (44.6%) had glutamine at that position. At residue 91, 97 of 157 patients (61.8%) had leucine and 60 patients (38.2%) had methionine.

We determined the rates of recurrence for patients after attentive curative treatment with hepatectomy based on the amino acid residue at position 91 of the HCV core region (Fig. 1). The recurrence rate for patients with methionine at this position was significantly higher than that of patients bearing a leucine ($P = 0.0002$). In contrast, we found no difference in recurrence according to amino acid substitutions at residue 70 (Supplemental Digital Content 2, available at: http://links.lww.com/SLA/A145). When we analyzed recurrence rates according to pretreatment tumor markers, there was no difference in recurrence rate according to pretreatment serum AFP, AFP-L3, or DCP levels (Supplemental Digital Content 3, available at: http://links.lww.com/SLA/A146). Univariate analysis identified tumor size (>5 cm), macroscopic portal vein invasion, and amino acid substitution at residue 91 of the HCV core region as factors that significantly associated with recurrence rate after hepatectomy. By multivariate analysis, these 3 factors were also selected as independent factors associated with increased recurrence rates (Table 2). Recurrent HCC was categorized into multicentric recurrence in 25 of 55 patients (45.5%) with recurrence bearing a leucine and in 33 of
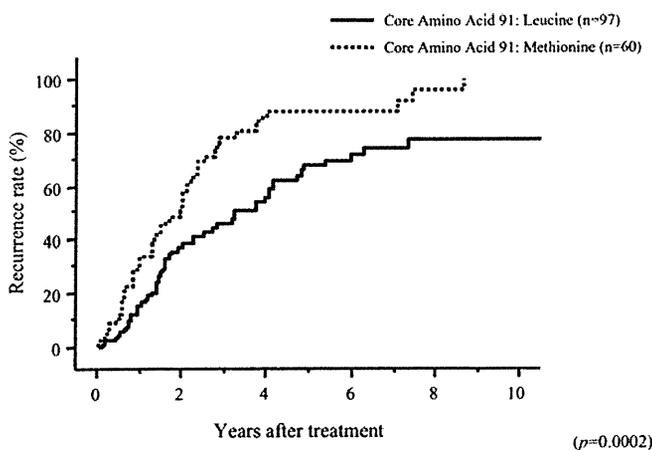

FIGURE 1. Recurrence rates after hepatectomy in patients bearing leucine (solid line) or methionine (dotted line) at residue 91 of the HCV core region. Recurrence rates were significantly higher in patients with methionine than in patients with leucine at residue 91 of the HCV core region ($P = 0.0002$).

48 patients (68.8%) with methionine, the prevalence being higher in patients with methionin ($P = 0.0292$).

Comparison of the characteristics of patients according to amino acid substitution at residue 91 of the HCV core region did not reveal differences in patient age, gender, liver function, the progression of HCC, or pretreatment AFP, AFP-L3, and DCP levels between patients with leucine and methionine at residue 91 (Table 3). The rate of patients who underwent postoperative interferon therapy and the rate of patients who achieved the eradication of HCV by interferon therapy were not different between these 2 groups.

Cancer Specific and Overall Survival Rates after Hepatectomy According to Amino Acid Substitutions of the HCV Core Region and Pretreatment Serum Tumor Markers

We determined the cancer specific and overall survival rates of patients after hepatectomy as a function of amino acid substitution at residue 91 of the HCV core region. The cancer specific survival of patients bearing methionine at residue 91 was significantly lower than that of patients with leucine at residue 91 ($P = 0.0010$, Supplemental Digital Content 4, available at: http://links.lww.com/SLA/A147) and consequently, overall survival of patients with methionine was significantly lower than that of patients with leucine ($P = 0.0061$, Fig. 2). In contrast, we did not find a difference in survival correlating with the amino acid substitution at residue 70 (Supplemental Digital Content 5, available at: http://links.lww.com/SLA/A148). When we analyzed patient survival rates according to pretreatment tumor markers, there was no difference in patient survival according to pretreatment serum AFP or DCP levels. We did identify a significant difference in survival associated with pretreatment AFP-L3 proportions ($P = 0.0473$) (Supplemental Digital Content 6, available at: http://links.lww.com/SLA/A150). We found significantly higher survival rate of patients who underwent postoperative antiviral therapy with interferon than that of patients who did not ($P = 0.0065$, Supplemental Digital Content 7, available at: http://links.lww.com/SLA/A151). Univariate analysis identified patient age, tumor number, macroscopic portal vein invasion, amino acid substitution at residue 91 of the HCV core region, and postoperative interferon therapy as factors significantly associated with survival after hepatectomy. By multivariate analysis, tumor number, portal vein invasion, amino acid substitution at residue 91 of the HCV core region, and postoperative interferon therapy were selected as independent factors associated with patient survival (Table 4).

DISCUSSION

The results of this study demonstrated that amino acid substitutions at residue 91 in the HCV core region were associated with recurrence and survival rates for patients with HCC bearing HCV genotype 1b infection who underwent hepatectomy with curative intent. Comparison of patient background characteristics, including liver function at diagnosis and HCC tumor progression (size, number, and portal vein invasion), did not reveal any difference between HCV genotype 1b-positive patients with leucine at residue 91 and those with methionine at that position, indicating that the differences in recurrence and survival seen for different amino acid substitutions at residue 91 were not due to the differences in liver function or tumor progression before treatment. Multivariate analyses identified that methionine substitution at this position was an independent factor associated with increased recurrence and decreased survival after hepatectomy. In previous reports, Akuta et al[10] and Nakamoto et al[12] studied the association between the incidence of primary, not recurrent HCC and amino acid substitutions in the HCV core region, combining substitutions at residues 70 and 91. They

TABLE 2. Univariate and Multivariate Analyses for Factors Associated with Postoperative Recurrence in HCC Patients Infected with HCV Genotype 1b (n = 157)

Factor		Univariate analysis	Multivariate analysis	Risk ratio (95% confidence interval)
Age		0.0731	–	
Sex	Male			
	Female	0.0892	–	
Child-Pugh class	A			
	B	0.7315	–	
Tumor size	≤2 cm			1
	>2 cm and ≤ 5 cm	0.1061	–	
	>5 cm	0.0018	0.0165	1.5254 (1.0869–2.0542)
Tumor number	Single			
	Multiple	0.5379	–	
Macroscopic-PV invasion	Absent			1
	Present	0.0011	0.0011	1.8240 (1.2972–2.4585)
Core-70 amino acid	Arginine			
	Glutamine	0.1130	–	
Core-91 amino acid	Leucine			1
	Methionine	0.0023	0.0207	1.2878 (1.0399–1.5895)
Pretreatment AFP	<20 ng/mL			
	≥20 ng/mL	0.6394	–	
Pretreatment AFP-L3	<10%			
	≥10%	0.0763	–	
Pretreatment DCP	<40 mAU/mL			
	≥40 mAU/mL	0.0643	–	
IFN therapy after hepatectomy	No			
	Yes	0.1859	–	

PV indicates portal vein; IFN, interferon; AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin.
*Category of Child-Pugh class A includes patients without cirrhosis.

TABLE 3. Comparison of Clinical Characteristics of Study Patients Based on the Amino Acid Substitutions at Residue 91 of the HCV Core Region (n = 157)

	Core Amino Acid 91: Leucine (n = 97)	Core Amino Acid 91: Methionine (n = 60)	P value
Age (mean ± SD, years) (range)	66.8 ± 6.9 (47-79)	68.7 ± 7.3 (49-83)	0.0737
Sex ratio (female/male)	28 (28.9)/ 69 (71.1)	14 (23.3)/ 46 (76.7)	0.5642
Child-Pugh class (A/B)*	93 (95.9)/ 4 (4.1)	54 (90.0)/ 6 (10.0)	0.2581
Albumin (mean ± SD, g/dL)	3.85 ± 0.42	3.83 ± 0.45	0.5523
Total bilirubin (mean ± SD, mg/dL)	0.73 ± 0.33	0.76 ± 0.37	0.5546
15-minute retention rate of ICG (%)	15.7 ± 6.7	15.4 ± 8.4	0.8114
Prothrombin (%)	90.4 ± 14.2	90.1 ± 14.6	0.6752
Platelet (× 1000/μL)	127 ± 55	117 ± 46	0.1679
Tumor size (mean ± SD, cm) (range)	2.86 ± 2.25 (0.6-17.6)	2.93 ± 1.64 (0.7-11.0)	0.2020
≤2 cm/>2 cm and ≤5 cm/>5 cm	37 (38.2)/ 46 (47.4)/ 14 (14.4)	25 (41.7)/ 30 (50.0)/ 5 (8.3)	0.5047
Tumor number (single/multiple)	82 (84.5)/ 15 (15.5)	50 (83.3)/ 10 (16.7)	0.8414
Macroscopic-portal vein invasion (absent/present)	84 (86.6)/ 13 (13.4)	54 (90.0)/ 6 (10.0)	0.7003
Microscopic-portal vein invasion (absent/present)	77 (79.4)/ 20 (20.6)	51 (85.0)/ 9 (15.0)	0.5021
AFP (median, ng/mL) (range)†	16.0 (0.8-5280)	22.6 (0.8-3480)	0.1780
≥ 20 ng/mL / <20 ng/mL	53 (55.2)/ 43 (44.8)	27 (45.8)/ 32 (54.2)	0.3280
AFP-L3 (median, %) (range)†	0.5 (0-87.2)	0.5 (0-65.7)	0.0893
≥ 10% / <10%	77 (87.5)/11 (12.5)	43 (75.4)/ 14 (24.6)	0.0980
DCP (median, mAU/mL) (range)†	45.5 (10-36164)	40.0 (10-11638)	0.7514
≥ 40 mAU/mL / <40 mAU/mL	45 (47.9)/ 49 (52.1)	30 (51.7)/ 28 (48.3)	0.7684
Interferon therapy after hepatectomy (no/yes)	85 (87.6)/ 12 (12.4)	53 (88.3)/ 7 (11.7)	0.8954
Eradication of HCV by interferon therapy (no/yes)	94 (96.9)/ 3 (3.1)	57 (95.0)/ 3 (5.0)	0.8571

Percentages were in parentheses.
ICG indicates indocyanine green test; AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin; HCV, hepatitis C virus.
*Category of Child-Pugh class A includes patients without cirrhosis.
†AFP, AFP-L3, and DCP were not measured in 2, 12, and 5 patients, respectively.
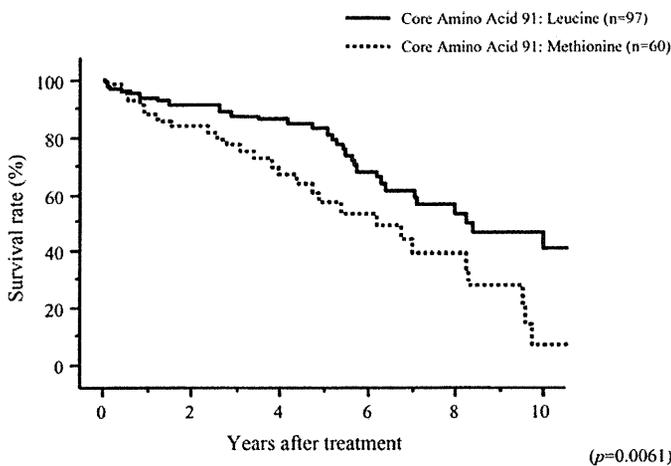
FIGURE 2. Overall survival rates after hepatectomy in patients bearing leucine (solid line) or methionine (dotted line) at residue 91 of the HCV core region. Survival rates were significantly lower in patients with methionine at residue 91 of the HCV core region than those with leucine at residue 91 ($P = 0.0061$).

TABLE 4. Univariate and Multivariate Analyses for Factors Associated with Postoperative Survival in HCC Patients Infected with HCV Genotype 1b (n = 157)

Factor		Univariate analysis	Multivariate analysis	Risk ratio (95% confidence interval)
Age		0.0198	0.1698	
Sex	Male			
	Female	0.0934	–	
Child-Pugh class	A			
	B	0.3398	–	
Tumor size	≤2 cm			
	>2 cm and ≤5 cm	0.3474	–	
	>5 cm	0.0898	–	
Tumor number	Single			1
	Multiple	0.0190	0.0434	1.3841 (1.0102–1.8466)
Macroscopic-PV invasion	Absent			1
	Present	0.0022	0.0031	1.8280 (1.2460–2.5621)
Core-70 amino acid	Arginine			
	Glutamine	0.1483	–	
Core-91 amino acid	Leucine			1
	Methionine	0.0063	0.0076	1.4517 (1.1063–1.8994)
Pretreatment AFP	<20 ng/mL			
	≥20 ng/mL	0.3632	–	
Pretreatment AFP-L3	<10%			
	≥10%	0.0617	–	
Pretreatment DCP	<40 mAU/mL			
	≥40 mAU/mL	0.5713	–	
IFN therapy after hepatectomy	No			1
	Yes	0.0013	0.0203	0.5052 (0.2036–0.9141)

PV indicates portal vein; IFN, interferon; AFP, alpha-fetoprotein; AFP-L3, *lens culinaris* agglutinin-reactive AFP; DCP, des-gamma-carboxy prothrombin.
*Category of Child-Pugh class A includes patients without cirrhosis.

reported that lower incidence of the development of HCC in patients bearing an arginine at residue 70 and a leucine at residue 91 (double-wild type) than in patients with other substitutions at these positions (nondouble-wild type). We also found significant difference in both recurrence and survival rates after hepatectomy between these 2 groups ($P = 0.0219$ and 0.0384, Supplemental Digital Content 8 and 9, available at: http://links.lww.com/SLA/A152 and http://links.lww.com/SLA/A153). However, the differences in recurrence and survival rates after hepatectomy were more marked when stratifying patients according to amino acid substitutions at residue 91 in the HCV core region, indicating different effects of the amino acid substitutions of the HCV core region between de novo HCC cases and recurrences after curative resection.

Pretreatment elevations of tumor markers for HCC, especially AFP-L3 and DCP, have been reported to indicate malignant potential of HCC tumor and be associated with higher recurrence rates and lower survival rates.[25,26] In this study, however, we were unable to identify differences in recurrence or survival according to pretreatment elevations of tumor markers, except for a mild association of AFP-L3 elevation and decreased survival rates. This may be due to a focus in our study on patients who underwent hepatectomy as a curative radical treatment and our exclusion of patients who underwent nonsurgical treatment or no treatment. Hepatectomy may overcome the malignant potential of HCC tumor associated with a pretreatment elevation of tumor markers.

Postoperative interferon therapy has been reported to decrease recurrence rate[27] and increase survival rate[28] after hepatectomy, especially in patients who achieved the eradication of HCV by the therapy. We found higher survival rates in patients who underwent postoperative interferon therapy than in those who did not. The effect

of interferon therapy to improve liver function might have contributed to the increased survival[28] in our study patients. In contrast, we failed to find the effect of interferon on the suppression of recurrence of HCC after hepatectomy. Recent studies revealed that the efficacy of interferon therapy is strongly associated with amino acid substitutions in the HCV core region,[8] mainly with amino acid substitutions at residue 70.[9,29] In contrast, our results showed the association between amino acid substitutions at residue 91 and postoperative recurrence and survival rates of patients with HCC after hepatectomy. Together with previous reports and our results, it seems that postoperative interferon therapy did not play a role in the association between postoperative recurrence and survival of patients with HCC and amino acid substitutions in the HCV core region observed in this study.

Previous studies examining the patterns of HCC recurrence in patients with HCV-related HCC reported that intrahepatic metastases are predominant within 2 to 3 years of treatment; multicentric recurrence of HCC becomes predominant after that period.[16] A comparison of the recurrence curve for patients with leucine at residue 91 of the HCV core region with that for patients bearing methionine at that position (Fig. 1) revealed that the difference in recurrence rates became marked 2 years after hepatectomy and suggests that amino acid substitution at residue 91 is associated with multicentric recurrence of HCC in patients with HCV genotype 1b infection. Indeed, the prevalence of multicentric recurrence was significantly higher in patients bearing a methionine at residue 91 in the HCV core region than in patients with leucine.

There are several limitations to this study. The study population included only patients infected with HCV genotype 1b; our study did not examine the association between amino acid substitutions at residue 91 and recurrence or survival rates in patients with other HCV genotypes. All patients were Mongoloid Japanese; these results, therefore, should be evaluated in other ethnicities to demonstrate the generalization of our findings. In addition, the percentage of macroscopic and microscopic portal vein invasion in the study patients was lower in comparison to HCC patients from Japanese general population.[30] This will be because of the high rate of patients in whom HCC was detected under surveillance at our liver center in this study; HCC was diagnosed in early stage in most of these patients.[31] Furthermore, as none of our patients were treated with liver transplantation, we have no data on the effect of amino acid substitutions at residue 91 on recurrence or survival in this subpopulation. Finally, the mechanism underlying the effect of this amino acid substitution on patient recurrence and survival remains unknown, which we hope will be investigated in the future to shed light on potential treatment approaches.

In conclusion, our examination of 163 patients treated by hepatectomy with curative intent revealed that amino acid substitution at residue 91 of the HCV core region influenced recurrence and survival after hepatectomy; patients bearing methionine at this position demonstrated higher recurrence and lower survival rates. Further studies will be needed to confirm this association in other population and elucidate the mechanism underlying this effect.

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mentioned in the recent editorial by McColl and Gillen (4). Howden and Kahrilas state that, "in summary, there is no clear clinical- or clinical trial-evidence of undue difficulty in reducing or discontinuing PPI treatment in GERD patients, apart from those with erosive esophagitis" (2). This is not correct as a placebo-controlled trial of discontinuation of PPIs in patients on long-term therapy was performed a few years ago (5). Most patients participating had gastroesophageal reflux disease (GERD) as the indication for the PPI and GERD patients had significantly more difficulties discontinuing PPI therapy as compared with patients with other indications (5). Exclusion criterion for participation was erosive esophagitis (5). In the articles mentioned above (2,3) there is no disagreement with the last paragraph of the editorial, that "PPI treatment remains an important, valuable and safe intervention for a multitude of patients with appropriate indications" (1). Finally, it is somewhat surprising that authors that are chosen to write the editorial of a study showing that PPI therapy can induce dyspeptic symptoms have strong and multiple conflicts of interest with the pharmaceutical companies producing PPIs (1).

CONFLICT OF INTEREST

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Withdrawing PPI Treatment

Colin W. Howden, MD, FACG¹ and Peter J. Kahrilas, MD, FACG¹

To the Editor: Our editorial (1) sought to highlight both the strengths and limitations of the study by Niklasson et al. (2), of which Dr Bjornsson was a co-author. We presume that Dr Bjornsson would agree that the results of controlled studies should not be overinterpreted, which is precisely what we had observed with the previously reported study of Reimer et al. (3) and what we caution of here. We do not dispute that some (although by no means all) investigators have shown rebound acid hypersecretion following PPI withdrawal and that such an effect is biologically plausible. Rather, our concern regards the clinical relevance of this phenomenon. Furthermore, although the observations of Reimer et al. (3) and Niklasson et al. (2) might be explained on the basis of rebound acid hypersecretion, readers should understand that neither study actually measured this phenomenon.

Regarding our stated conflicts of interest, readers are free to make of them what they choose. We welcome and adhere to the Journal's policy of making a declaration of all relevant financial relationships mandatory ("strong" and otherwise); this helps to ensure transparency and objectivity. However, a thoughtful reading of our editorial would conclude that we advocate minimizing and withdrawing PPI treatment whenever appropriate, hardly a viewpoint steeped in bias. To re-state our main argument, this is generally easily accomplished in clinical practice.

CONFLICT OF INTEREST

Dr Howden has been a consultant for Takeda Pharmaceuticals North America, Takeda Global Research and Development, Santarus, XenoPort, Schering-Plough Healthcare, Novartis Consumer Health, Novartis Oncology, Procter & Gamble, Eisai, Otsuka, and Boehringer Ingelheim. He has received speaking honoraria from Takeda Pharmaceuticals North America, Otsuka, and Novartis. He has received research support for an investigator-initiated project from AstraZeneca. Dr Kahrilas has been a consultant for AstraZeneca, Xenoport, ARYx Therapeutics, Eisai, EndoGastric Solutions, Novartis, Movetis, and Revalesio. He has received research support for investigator-initiated studies from the National Institutes of Health and Reckitt Benckiser Group plc.

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Double-Contrast Ultrasound: A Novel Surveillance Tool for Hepatocellular Carcinoma

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This letter underwent AJG editorial review.

To the Editor: Hepatocellular carcinoma (HCC) is the third most common cause of

Table 1. Results of surveillance by double contrast US: B-mode US vs. double contras US

No.	Sex	Age	Virus	Location	Size (mm)	B-mode US	CEUS Kupffer phase	Double-contrast US	Pathological diagnosis
1	M	64	HCV	S6	6×6	Not detected	Defect	Positive	HCC
2	M	53	HCV	S8	7×7	Not detected	Defect	Positive	HCC
3	M	76	HCV	S6	8×8	Not detected	Defect	Positive	HCC
4	F	72	HCV	S7	8×7	Not detected	Defect	Positive	HCC
5	M	68	HBV	S5	8×8	Not detected	Defect	Positive	HCC
6	M	72	HCV	S2	9×8	Not detected	Defect	Positive	HCC
7	M	71	HCV	S3	10×9	Not detected	Defect	Positive	HCC
8	M	70	HBV	S8	10×10	Not detected	Defect	Positive	HCC
9	M	68	HCV	S2	10×7	Not detected	Defect	Positive	HCC
10	F	75	HCV	S6	11×11	Not detected	Defect	Positive	HCC
11	M	67	HCV	S6	11×10	Not detected	Defect	Positive	HCC
12	M	73	HBV	S7	12×11	Not detected	Defect	Positive	HCC
13	M	74	HCV	S5	12×11	Not detected	Defect	Positive	HCC
14	F	69	HCV	S2	12×10	Not detected	Defect	Positive	HCC
15	M	70	HCV	S6	12×11	Not detected	Defect	Positive	HCC
16	M	76	HCV	S8	13×12	Not detected	Defect	Positive	HCC

CEUS, contrast-enhanced US; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; US, ultrasound.

cancer death worldwide. Practice guidelines in the West (1) and East (2) recommend ultrasound (US) surveillance as a first-line test. However, despite the performance of periodic surveillance, some HCCs are still detected at advanced stages because of the coarse liver parenchyma. Furthermore, even HCCs detected at early stages, such as single nodular HCCs smaller than 3 cm, still show high annual recurrence rates (15–20%) after resection or ablation (3). These phenomena are attributed to the tumor biology of HCCs, which frequently metastasize via the portal vein even when they are less than 2 cm (4). Detection of much smaller HCC nodules that do not yet have microsatellites or vascular invasion is an urgent clinical need.

In 2007, Sonazoid, a second-generation US contrast agent, was approved for routine clinical use in Japan. The most important property of this agent is that it allows very stable Kupffer phase imaging for at least 60 min, which is tolerable for multiple scanning in addition to real-time imaging. From December 2007 to November 2009, Kupffer phase surveillance was performed for 292 consecutive patients with hepatitis B- or C-related cirrhosis, who are at very

high risk for HCC. At the outpatient clinic, 0.01 ml/kg of Sonazoid was injected, followed by entire liver scanning at the Kupffer phase. Among the 292 patients, 27 Kupffer defects that were not detected by B-mode US were detected by Kupffer phase surveillance. Of these defects, 16 hypervascular nodules (5.5%) were confirmed as HCC by re-injecting Sonazoid at the Kupffer phase (double-contrast US) (5). All 16 nodules were proven to be HCC histologically, with a size range of 6–13 mm (Table 1). After resection ($n = 2$) or radiofrequency ablation ($n = 14$), none of these nodules showed local recurrence or intrahepatic recurrence during a median follow-up period of 2.3 years. Only one HCC nodule located at the subphrenic region was missed during detection by double-contrast US. The sensitivity of detecting B-mode US-undetectable hypervascular HCC was 94% using double-contrast US.

In conclusion, Kupffer phase surveillance of the cirrhotic liver followed by re-injection of Sonazoid (double-contrast US) is a novel technique in the surveillance program for detecting small hypervascular HCCs that are in a completely curable state. Based on these findings, a prospective

randomized phase III multicenter controlled trial comparing B-mode and double-contrast US surveillance for virus-related cirrhotic patients is now ongoing (http://www.clinicaltrials.com; NCT 00822991).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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