DCP (r^2 = 0.235, P = 0.001). There was no association between serum albumin concentration and either AFP or DCP. The results of multivariate analysis of risk factors for the dropout due to death without tumor progression are shown in Table 2. In univariate analysis, lower serum albumin (<3.5 g/dL), prolonged prothrombin time (international normalized ratio, continuous), Child-Turcotte-Pugh class B or C, presence of ascites, higher serum DCP (>100 mAU/mL), PEI had a P value < 0.1. Multivariate analysis with stepwise selection of factors showed that lower serum albumin concentration (<3.5 g/dL) (P = 0.002, RR: 3.968, 95% CI: 1.668-9.438) and presence of ascites (P = 0.033, RR: 2.391, 95% CI: 1.074- 5.324) were significant. Alternatively, Child-Turcotte-Pugh class B/C was the only significant factor (P < 0.001, RR: 3.761, 95% CI: 1.892-7.475) when associated factors (serum albumin, bilirubin, ascites, and prothrombin time) were removed from the multivariate analysis.

Liver Function-Stratified Analysis

Since liver function, as represented by serum albumin, ascites or Child-Turcotte-Pugh class, significantly affected failures due to both tumor progression and death, failure-free survival was analyzed again as stratified by liver function, Child-Turcotte-Pugh class A vs. class B/C. Figure 3A compares overall failure-free survival between Child-Turcotte-Pugh class A and class B/C patients (P = 0.005). When failures due to tumor progression and death were separately analyzed (Fig. 3B), there was significant difference between class A and class B/C with regard to death without tumor progression (P = 0.0001) but no difference with regard to tumor progression (P = 0.853).

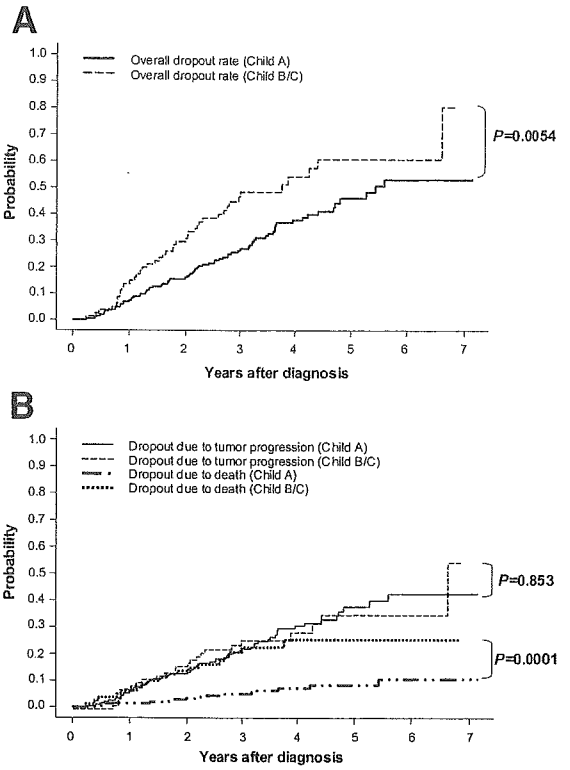

Figure 3. (A) Overall failure-free survival between Child-Turcotte-Pugh class A (solid line) and class B/C patients (dashed line). (B) Failures due to tumor progression and death were separately analyzed between Child-Turcotte-Pugh class A and class B/C patients. There was a significant difference between class A (dash-dot line) and class B/C (dotted line) with regard to deaths without tumor progression, whereas there was no significant difference between class A (solid line) and class B/C (dashed line) with regard to tumor progression.

Discussion

In this study we focused on the probability of losing transplantability after primary local ablation therapy for HCC. We assumed that 288 HCC patients who underwent percutaneous ablation therapy were put on a waiting list for liver transplantation. The dropout from the list was judged based on the Milan criteria. Ablation therapy was repeated for HCC recurrence whenever possible, and the number of treatments was not considered in judging transplantability as long as the extent of HCC met the Milan criteria. The results showed a dropout rate of 9% at 1 year and 33% at 3 years. Reported dropout rates varied largely depending on the transplant program and the availability of donor organs, and also possibly on the mode of adjuvant bridging therapy. Llovet et al. reported a 1-year dropout rate of 38%, where 84 of 87 patients received no adjuvant therapy for HCC.[10] At the University of California, San Francisco, 41% of transplant candidates with HCC received bridge therapy, including PEI, RFA, and transcatheter arterial chemoembolization, and showed a 1-year dropout rate of 37.8 %.[11] At the University of Miami in Florida, 24% of transplant candidates with HCC underwent microwave coagulation therapy and the 1-year dropout rate was 11.8%. The dropout rate calculated in the current study was substantially lower than these reported values.[15]

The patients on our virtual waiting list may have differed from the actual ones in liver function. Indeed, while 72% of the former were classified as Child-Turcotte-Pugh class A, in actual settings Child-Turcotte-Pugh class A patients constituted 25% to 45% of transplant candidates, and class C patients constituted 11% to 32%.[2,3,10,11,14,15] Thus, it was important to check whether the dropout due to Milan criteria violation was dependent on liver function among our subjects. We found that the dropout due to

tumor progression was similar between patients in Child-Turcotte-Pugh class A and those in class B/C but that mortality without tumor progression was higher in the latter group. It is difficult to compare our current results directly with reported ones controlling for liver function. Nevertheless, the overall dropout rate in the class B/C group in this study was 14% at 1 year, which was comparable to or even better than the overall dropout rates reported for the actual waiting lists.

A recent report showed the effectiveness of RFA as an adjuvant treatment for liver transplant candidates, with histological complete necrosis achieved in 55% after 1 session of the RFA procedure.[24] There was no dropout due to accelerated tumor progression during the waiting time, where the mean interval from RFA to liver transplantation was 9.5 months. While we tried to achieve complete ablation whenever possible, it may not be necessary if RFA is performed as a "bridge" to transplantation. Such a scenario may be taken into account, provided that transplantation is assuredly feasible in a reasonable period. Nevertheless, complete ablation, which leads to lower dropout rates due to tumor progression, will be preferable where a longer waiting time should be expected, even if transplantation eventually fails to be performed. Survival after PEI is now considered to be comparable to that after surgical resection in selected cases.[33] RFA would provide comparable or better prognosis with local tumor progression rate of 2%, lower than that after PEI.[34] Among this study population, local tumor progression occurred in only 5 cases (1.7%), and 4 of them were subsequently ablated successfully. This high efficacy of local ablation therapy may be partially due to a selection bias by enrolling patients who had small HCC and well-preserved liver function, which may have led to the low dropout rate in this simulative study.

Liver transplantation for HCC patients who have indication also for surgical resection may be controversial. Surgical resection has shown a lower risk of perioperative death and comparable short-term survival as compared to transplantation. However, HCC recurrence is frequent in the remaining liver, with 5-year cumulative recurrence rates as high as 80%.[18,35] Some researchers consequently encourage primary transplantation for such patients, while others consider surgical resection preferable and transplantation as the salvage therapy in setting of tumor recurrence or deterioration of liver function. However, advanced tumor stage may contraindicate transplantation at recurrence. If the indication for salvage transplantation is assessed based on pathological evaluation of the resected specimen, and transplantation is subsequently performed only when the risk of recurrence is high, donor organs can be optimally allocated.[36]

Similarly to this strategy, local ablation may be performed in varying manners according to prognostic factors. If a patient eligible for transplantation is at a high risk for dropout due to tumor progression, ablation therapy should be considered as an emergent bridge to transplantation and performed as soon as possible. In the current study, we found that some characteristics of the primary tumor—namely, elevated tumor markers before the initial therapy (AFP >100 ng/mL or DCP >100 mAU/mL) and a tumor size larger than 3 cm—were associated with future dropout due to tumor progression despite a high rate of complete ablation. Larger tumor sizes may be associated with a high risk of microvascular invasion and intrahepatic metastasis and thus correlate with tumor recurrence. Elevated tumor markers may reflect a higher malignant potential of the primary tumor and possibly be associated with a higher risk of invasion and metastasis.

On the other hand, patients with a low risk for dropout may as well be followed after initial local ablation therapy until recurrence is detected or liver preservation worsens. Furthermore, we have shown previously that interferon therapy after tumor ablation can improve survival in patients with hepatitis C virus-associated HCC, leading to survival rates of 78% at 5 years and 68% at 7 years among sustained virologic responders.[37] These outcomes were indeed comparable to the prognosis after liver transplantation. Complete ablation or surgical resection of HCC followed by antiviral therapy with high efficacy may be as effective as, and far less expensive than, liver transplantation.

In conclusion, local ablation for HCC may be useful as bridging therapy to liver transplantation by suppressing dropout from the waiting list due to tumor progression. However, its indication may be limited to patients with relatively good liver function, since it cannot suppress dropout due to liver failure.

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LIVER

Proposal of a new prognostic model for hepatocellular carcinoma: an analysis of 403 patients

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Background: The prognosis of hepatocellular carcinoma (HCC) is highly dependent on tumour extension and liver function. Recently, two new prognostic scoring systems—the CLIP score, developed by Italian investigators and the BCLC score, developed in Barcelona—have been widely used to assess prognosis in patients presenting with hepatocellular carcinoma. Each system has its own relative limitations.
Aims: To create a new prognostic scoring system which is simple, easy to calculate, and suitable for estimating prognosis during radical treatment of early HCC.
Methods: A total of 403 consecutive patients with HCC treated by percutaneous ablation at the Department of Gastroenterology, University of Tokyo Hospital, between 1990 and 1997 were used as the training sample to identify prognostic factors for our patients and used to develop the Tokyo score. As a testing sample, 203 independent patients who underwent hepatectomy at the Department of Hepato-Biliary-Pancreatic Surgery were studied. Prognostic factors were analysed by univariate and multivariate Cox proportional hazard regression.
Results: The Tokyo score consists of four factors: serum albumin, bilirubin, and size and number of tumours. Five year survival was 78.7%, 62.1%, 40.0%, 27.7%, and 14.3% for Tokyo scores 0, 1, 2, 3, and 4–6, respectively. The discriminatory ability of the Tokyo score was internally validated by bootstrap methods. The Tokyo score, CLIP score, and BCLC staging were compared by Akaike information criterion and Harrell's c index among training and testing samples. In the testing sample, the predictive ability of the Tokyo score was equal to CLIP and better than BCLC staging.
Conclusions: The Tokyo score is a simple system which provides good prediction of prognosis for Japanese patients with HCC requiring radical therapy.

The prognosis of hepatocellular carcinoma (HCC) depends on tumour extension as well as liver function. Worldwide, most patients with hepatocellular carcinoma have cirrhosis caused by chronic viral hepatitis (hepatitis C (HCV), hepatitis B virus).[1] Assessment of tumour related factors in isolation, such as the tumour node metastasis (TNM) staging,[2] does not accurately predict the prognosis of patients who have HCC and cirrhosis.[3] The Child-Pugh classification has been widely used to evaluate liver function in cirrhotic patients, and has a relatively good correlation with prognosis,[4] but cannot be used to predict survival in patients with HCC.

Okuda staging of HCC, established in 1985, is based on tumour size and liver function, as assessed by three of the four factors used in the Child Pugh score—namely, serum albumin, bilirubin, and the presence of ascites—and for some time has been used as the gold standard for prognostic assessment of HCC patients.[5] However, this prognostic system was established by analysing patients mostly at an advanced stage of HCC, with a median survival of 4.1 months. With current advances in clinical practice, survival of HCC patients is now much longer, and the Okuda staging is unable to accurately predict prognosis in these patients.

Recently, several groups from Italy,[6] Spain,[7] France,[8] Austria,[9] and China[10] proposed new prognostic systems for HCC. While the latter three were developed using a sample of patients with advanced disease (median survival 4–8 months), the CLIP and BCLC systems developed scores based on patients with early disease. The CLIP (Cancer of the Liver Italian Program) score showed a good correlation with the prognosis of HCC patients receiving various treatments, including surgery, percutaneous ablation, transarterial chemoembolisation, and liver transplantation.[6 11 12] Along with liver function, as assessed by Child-Pugh stage, three tumour factors were included. Tumour morphology was divided into three categories: a single tumour ⩽50% of the size of the liver (score 0); multinodular HCC ⩽50% of the size of the liver (score 1); and massive HCC or tumour >50% of the size of the liver (score 2). As in Okuda staging, tumour size was broadly divided at 50% of liver volume. Seropositivity for α fetoprotein (AFP) and the presence of portal vein thrombosis were also included. However, due to advances in liver imaging techniques, especially ultrasound and computed tomography, HCC can now be detected at a much smaller size, usually smaller than 5 cm in diameter, and tumours smaller than 2 cm are frequently diagnosed. Tumour size is associated with the pathological grade of HCC, the probability of vascular invasion, and also with the prognosis of HCC patients after potentially curative treatments such as surgical resection and medical ablation.[13 14] However, it is not known whether a HCC of 2 cm is a determinant of prognosis as previous models have not discriminated between large and small tumours.

Abbreviations: HCC, hepatocellular carcinoma; TNM, tumour node metastasis; CLIP, Cancer of the Liver Italian Program; BCLC, Barcelona Clinic Liver Cancer; AIC, Akaike information criterion; PEIT, percutaneous ethanol injection therapy; PMCT, percutaneous microwave coagulation therapy; TAE, transcatheter arterial embolisation; AST, aspartate aminotransferase; AFP, α fetoprotein; HCV, hepatitis C virus; HVPG, hepatic venous pressure gradient

Table 1 Definition of the Okuda staging system for hepatocellular carcinoma

	Points	
	0	1
Tumour size	<50% of liver	>50% of liver
Ascites	No	Yes
Albumin (g/dl)	≥3	<3
Bilirubin (mg/dl)	<3	≥3

Okuda stage I, 0 points; Okuda stage II, 1 or 2 points; Okuda stage III, 3 or 4 points.

More recently, another staging system, the BCLC (Barcelona Clinic Liver Cancer), was developed and based on both advanced and early HCC, dividing HCC into four early stages (A1–A4) and three more advanced ones (B–D). It contains elements of both the Okuda and Child-Pugh classifications. Subclassification of early stages requires formal measurement of hepatic venous pressure gradient (HVPG), which is not applicable in all patients, although clinical parameters (splenomegaly, etc) are now frequently applied.[7] In the present study, we sought to establish a new scoring system that provides a more precise prediction of prognosis in patients with early stage HCC.

PATIENTS AND METHODS

Training sample

Between January 1990 and December 1997, 403 patients with naïve HCC received medical ablation, either percutaneous ethanol injection therapy (PEIT) or percutaneous microwave coagulation therapy (PMCT), at the Department of Gastroenterology, University of Tokyo Hospital. Their prognosis was followed up until August 2001, and survival data were used as the training samples in this study. HCC was detected with ultrasound and/or computed tomography, and confirmed histopathologically by percutaneous tumour biopsy. Inclusion criteria for ablation were as follows: total bilirubin <3 mg/dl; platelet count $>4\times10^5/mm^3$; prothrombin activity >35%; and no intractable ascites. Although most investigators performed PEIT in early stage HCC, such as for a single nodule of ≤5 cm in diameter or less than three nodules ≤3 cm in diameter,[15 16] we did not limit the indication for ablation to tumour size alone. Patients received ablation therapy because surgery was not an option in terms of impairment in liver function, or they voluntarily chose ablation after informed consent although surgery was also possible. The ablation procedures have been described previously.[17 18]

The following variables obtained at initial ablation therapy were used: age; sex; treatment modality (PEIT, PMCT, with or without transcatheter arterial embolisation (TAE)); tumour factors, including size, number of nodules, lobar distribution, and presence of extrahepatic metastasis; clinical manifestations, including ascites and hepatic encephalopathy; laboratory data, including albumin, bilirubin, prothrombin activity, aspartate aminotransferase (AST), alanine aminotransferase, platelet count, and AFP; positivity for viral markers (hepatitis B surface antigen and anti-hepatitis C antibody); and alcohol consumption. Okuda stage, CLIP score, and BCLC staging were also calculated using these variables (tables 1–3). We substituted the presence of either oesophageal varices or splenomegaly with platelet count less than 100 000/mm^3 for HVPG ≥10 mm Hg, as described by Llovet and colleagues.[7]

All patients were placed under strict observation for recurrence of HCC, with regularly repeated ultrasound, computed tomography, and determination of serum tumour markers. If recurrence of HCC was detected, patients received additional treatments whenever possible—tumour ablation, TAE, or systemic chemotherapy. For survival analysis, the end point was death, and survival was censored on 31 August 2001.

Table 2 Definition of the Cancer of the Liver Italian Program (CLIP) scoring system for hepatocellular carcinoma

	Score		
	0	1	2
Child-Pugh stage	A	B	C
Tumour morphology	Uninodular and extension ≤50%	Multinodular and extension ≤50%	Massive or extension >50%
AFP (ng/ml)	<400	≥400	
Portal vein thrombosis	No	Yes	

AFP, α fetoprotein.

Table 3 Definition of the Barcelona Clinic Liver Cancer (BCLC) staging for hepatocellular carcinoma

		Tumour status		
BCLC stage	PST	Tumour stage	Okuda stage	Liver function status
Stage A: early HCC	0			
A1	0	Single	I	No portal hypertension and normal bilirubin
A2	0	Single	I	Portal hypertension and normal bilirubin
A3	0	Single	I	Portal hypertension and abnormal bilirubin
A4	0	3 tumours <3 cm	I–II	Child-Pugh A–B
Stage B: intermediate HCC	0	Large multinodular	I–II	Child-Pugh A–B
Stage C: advanced HCC	1–2*	Vascular invasion or extrahepatic spread*	I–II	Child-Pugh A–B
Stage D: end stage HCC	3–4†	Any		Child-Pugh C

Stages A and B: all criteria should be fulfilled.
Stage C: at least one criterion; *PST 1–2 or vascular invasion/extrahepatic spread.
Stage D: at least one criterion; †PST 3–4 or Okuda stage III/Child-Pugh C.

Testing sample

Between October 1994 and December 1999, 203 patients with naïve HCC underwent hepatectomy at the Department of Hepato-Biliary-Pancreatic Surgery, University of Tokyo Hospital, and their survival data served as the testing sample. Indications for hepatectomy and selection of the area for resection were previously published.[19] Briefly, the surgical procedure was determined according to residual liver function, as determined by the severity of ascites, serum level of bilirubin, and indocyanine green retention rate at 15 minutes. Patients with a bilirubin level >2 mg/dl or with intractable ascites were contraindicated for hepatectomy. Patients were followed up as described above, and survival was censored on 31 August 2001.

Establishing a new prognostic score

We sought to construct a new prognostic model based on the following principles.

(1) It is preferable to have two break points for continuous variables such as tumour size or serum albumin concentration because their distribution is wide and a single break point may not be optimal.
(2) Variables must be those commonly assessed in clinical practice to enable comparison between different institutions.
(3) The model should not include established classifications because they may be modified in the future, as was the case for TNM staging, and different versions may be confused.

We used survival time as the only end point in this analysis. Firstly, we performed univariate Cox proportional hazard regression to assess the statistical significance of each candidate potential factor, and the factor was retained if a significance level of $p<0.05$ was attained. Polychotomous categorical data were represented by corresponding binary dummy variables. Continuous variables, such as serum concentration of albumin and size (diameter) of the tumour, were transformed into categorical variables. We divided each of these continuous variables into two or three levelled categorical data by setting one or two break point(s), respectively, which were then represented by one or two binary variable(s); p values were calculated for each set of break points with univariate or multivariate Cox proportional hazard regression, and the set of break points showing the lowest p value was retained if the value reached significance.

Factors showing statistical significance as a predictor were further analysed using a multivariate Cox proportional hazard regression model with stepwise selection of variables based on the Akaike information criterion (AIC). AIC is a measure of the goodness of fit (log likelihood) with a "penalty score" for the complexity of the model (number of variables included), defined as

AIC = −2 × (maximum log likelihood) + 2 × (total number of parameters),

and the optimum (that is, simplest effective) model gives the lowest AIC value.[20]

A new prognostic score, designated the Tokyo score, was established, assigning ordinal scores (0, 1 and 2) to each of the selected factors according to the estimated regression coefficient in the final model.

Internal validation

We used the bootstrap method for internal validation of the Tokyo score system.[21] Bootstrap validation is a method of random re-sampling from a given set of samples to simulate the effect of drawing samples from the same population. A re-sampled data set of the same size as the original (training) data set was obtained by random sampling with replacement—in other words, each sample can be drawn more than once or not at all. Differences in three and five year survival rates were calculated between each pair of contiguous stages (for example, between Tokyo scores 1 and 2) using Kaplan-Meier estimation. Mean and 95% confidential interval of the difference in three and five year survival rates between the stages were determined by 2000 times iteration of such re-sampling.

External validation

We validated the Tokyo score in the testing sample as well as in the training sample with AIC and Harrell's c index.[22] Firstly, AIC was calculated in a Cox proportional regression model containing Tokyo, CLIP, and BCLC stages. Then, AIC was recalculated after removing each one of the scores, and the changes in AIC were compared. The c index is equivalent to the area under the receiver operator characteristic curve, and ranges from 0.0 to 1.0. A c index of 1 indicates perfect

Table 4 Baseline characteristics of the training sample (n = 403)

Variable	n (%)
Age (y) (median (range))	64 (35–87)
Male sex	293 (72.7%)
Viral infection	
HBsAg positive	39 (9.6%)
Anti-HCVAb positive	336 (83.4%)
Both positive	6 (1.5%)
Both negative	34 (8.4%)
Child-Pugh classification	
Class A	222 (55.1%)
Class B	160 (39.7%)
Class C	21 (5.2%)
Tumour characteristics	
Size of tumour (cm)	
<2	134 (33.3%)
2–5	234 (58.1%)
>5	35 (8.7%)
No of nodules	
Single	215 (53.3%)
2–3	135 (33.5%)
>3	53 (13.2%)
Portal invasion, present	4 (1.0%)
AFP (ng/ml)	
<100	308 (76.4%)
100–400	58 (14.3%)
>400	37 (9.2%)
Okuda stage	
I	296 (73.4%)
II	107 (26.6%)
III	0 (0%)
CLIP score	
0	118 (29.3%)
1	165 (40.9%)
2	98 (24.3%)
3	18 (4.5%)
4≤	4 (1.0%)
BCLC staging	
A1	70 (17.4%)
A2	58 (14.4%)
A3	32 (7.9%)
A4	100 (24.8%)
B	115 (28.5%)
C	7 (1.7%)
D	21 (5.2%)
Treatment modalities	
PEIT	363 (90.0%)
PMCT	26 (6.5%)
PEIT + PMCT	14 (3.5%)
Combined with TAE	54 (13.4%)

CLIP, Cancer of the Liver Italian Program; BCLC, Barcelona Clinic Liver Cancer; PEIT, percutaneous ethanol injection therapy; PMCT, percutaneous microwave coagulation therapy; TAE, transcatheter arterial embolisation; AFP, α fetoprotein; HBsAg, hepatitis B surface antigen; HCVAb, anti-hepatitis C virus antibody.

Table 5 Univariate analysis

Variable (n)	Hazard ratio (95% CI)	p Value
Age (y) ≥65	1.34 (1.04–1.73)	0.026
Male sex (293)	1.02 (0.769–1.36)	0.87
HBsAg positive (39)	1.04 (0.685–1.57)	0.87
Anti-HCVAb positive (336)	0.682 (0.494–0.943)	0.02
Drinking >80 g/day (90)	1.09 (0.807–1.47)	0.58
Ascites present (86)*	2.39 (1.82–3.15)	<0.0001
Encephalopathy present (27)	1.42 (1.14–1.76)	0.0015
Albumin g/(dl)		
>3.5 (192)	1	
2.8–3.5 (183)	1.99 (1.52–2.59)	<0.0001
<2.8 (28)	3.13 (2.01–4.88)	<0.0001
Bilirubin (mg/dl)		
<1 (271)	1	
1–2 (110)	1.19 (1.03–1.36)	0.016
>2 (22)	1.48 (1.26–1.72)	<0.0001
Prothrombin time		
>70% (225)	1	
40–70% (172)	1.36 (0.1.05–1.77)	0.021
<40% (6)	4.43 (1.79–10.99)	0.0013
Platelet count $\geq 10\times10^3/mm^3$ (173)	0.640 (0.494–0.829)	0.00063
AST >80 IU/l (178)	0.798 (0.620–1.03)	0.079
ALT >80 IU/l (166)	0.771 (0.598–0.994)	0.045
Size of tumour (cm)		
<2.0 (134)	1	
2.0–5.0 (234)	2.31 (1.70–3.13)	<0.0001
>5.0 (35)	3.73 (2.37–5.86)	<0.0001
No of nodules		
Single (215)	1	
2–3 (135)	1.18 (0.896–1.57)	0.24
>3 (53)	2.13 (1.499–3.03)	<0.0001
Lobar distribution		
Unilobar (292)	1	
Bilobar (111)	1.27 (1.11–1.45)	<0.0001
Extrahepatic metastasis present (3)	20.1 (6.12–66.3)	<0.0001
Portal vein invasion present (4)	14.6 (4.5–47.6)	<0.0001
AFP (ng/ml)		
<100 (308)	1	
100–400 (58)	1.99 (1.43–2.76)	<0.0001
>400 (37)	3.57 (2.46–5.18)	<0.0001

*Diuretic controllable ascites were included.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; AFP, α fetoprotein; HBsAg, hepatitis B surface antigen; HCVAb, anti-hepatitis C virus antibody.

concordance between the two variables (that is, the order of survival time and magnitude of prognostic score in the current study) while an index of 0.5 indicates a chance association.

Statistics

Data are expressed as mean (SD) unless otherwise specified. All statistical analyses were performed with S-plus 2000 (MathSoft Inc., Seattle, Washington, USA). Statistical significance was set at $p<0.05$.

Table 6 Multivariate analysis

Variable	Hazard ratio (95%CI)	p Value
Albumin (g/dl)		
>3.5	1	
2.8–3.5	1.74 (1.31–2.30)	0.00014
<2.8	2.45 (1.55–3.88)	0.00013
Bilirubin (mg/dl)		
<1	1	
1–2	1.40 (1.06–1.85)	0.02
>2	2.40 (1.47–3.92)	0.00049
Size of tumour (cm)		
<2.0	1	
2.0–5.0	2.21 (1.63–3.01)	<0.0001
>5.0	3.46 (2.20–5.45)	<0.0001
No of nodules		
≤3	1	
>3	1.88 (1.34–2.64)	<0.001

Table 7 Tokyo score

	Score		
	0	1	2
Albumin (g/dl)	>3.5	2.8–3.5	<2.8
Bilirubin (mg/dl)	<1	1–2	>2
Tumour size (cm)	<2	2–5	>5
Tumour No	≤3		>3

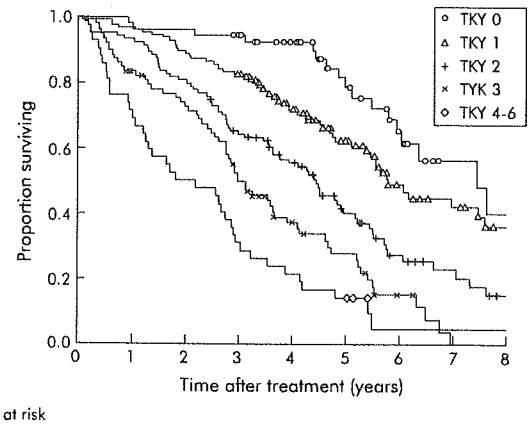
RESULTS

Patient profiles in the training sample

The training sample contained data from 293 male and 110 female patients. Baseline characteristics of the patients are shown in table 4. Median age was 64 years, with 25% and 75% percentiles at 59 and 69 years, respectively. The majority (83.4%) were HCV positive. The observation period was 3.9 (2.1) years, during which 250 patients died. Estimated 50% survival time was 4.75 years. Only eight patients (2%) were lost to follow up.

Selection of predictive factors

Univariate Cox proportional hazard analysis of the training data set revealed that 15 factors were significantly associated with prognosis of HCC patients (table 5). We included AST, which showed marginal significance ($p = 0.079$), and performed multivariate analysis on a total of 16 factors with stepwise selection of variables using the AIC. There were four variables which retained significance as independent predictors—namely, serum concentration of albumin, as ranked by 3.5 g/dl and 2.8 g/dl, bilirubin concentration (1 mg/dl and 2 mg/dl), size of the tumour (diameters of 2 cm and 5 cm), and number of tumour nodules (1–3 *v* >3) (table 6). Scores were assigned to each of the four factors according to the estimated regression coefficient in the final model (table 7) and the Tokyo score was defined as the sum of each score.



No at risk

TKY 0	53	53	52	51	47	28	19	12	6
TKY 1	126	123	114	105	73	47	27	18	13
TKY 2	104	98	85	66	49	29	17	10	7
TYK 3	78	66	55	37	23	15	8	0	
TKY 4-6	42	31	22	14	10	7	2	2	2

Figure 1 Kaplan-Meier estimated survival curves by Tokyo score (TKY).

Table 8 Pairwise comparisons of three and five year survival rates (with 95% confidence interval (CI)) between each stage of the Tokyo score

	3 year survival (%)		5 year survival (%)	
Stage	Difference	95% CI	Difference	95% CI
Tokyo 0 and 1	11.6	2.02–20.3	16.4	3.80–32.1
Tokyo 1 and 2	18.4	6.86–29.5	22.0	8.75–36.8
Tokyo 2 and 3	14.7	0.204–28.7	12.5	2.53–26.8
Tokyo 3 and 4–5	18.8	0.08–35.9	13.2	2.38–28.3

Internal validation

Among the training sample, 55, 126, 104, 78, 30, 9, and 3 patients were classified as Tokyo scores 0, 1, 2, 3, 4, 5, and 6, respectively. Observed cumulative survival of patients grouped by Tokyo score was calculated using the Kaplan-Meier method (fig 1). Prognosis was well distributed among the groups based on the Tokyo score. Five year survival rates for Tokyo scores 0, 1, 2, 3, and 4–6 were 78.7%, 62.1%, 40.0%, 27.7%, and 14.3%, respectively. This was confirmed by internal validation where differences in three and five year survival rates were calculated, along with 95% confidence interval, between each pair of two contiguous stages using the bootstrap method. The lower confidence limit of difference between each pair of two contiguous stages was greater than zero, indicating that all differences were statistically significant (table 8). The Tokyo score was therefore shown to be highly robust in estimating prognosis in distinct groups.

External validation

Baseline characteristics of the patients in the testing sample, who underwent surgical resection, are shown in table 9. Median age and sex proportions were similar to those in the training sample while these patients had better liver function reserve and the average tumour size was larger. Sixty five patients died during the observation period and median survival time was 5.7 years. Tokyo, CLIP, and BCLC stages were calculated according to the variables obtained from each patient.

The Tokyo Score, CLIP score, and BCLC staging were compared in both the training and testing samples by evaluating the AIC on Cox proportional hazard regression models. Goodness of fit of the model estimated by AIC was improved by removing BCLC from the model containing Tokyo, CLIP, and BCLC. AIC was greater in the model with either the Tokyo score or CLIP score alone than in the model containing both (table 10). These results indicate that the Tokyo and CLIP scores complement each other whereas addition of BCLC resulted in no improvement to the model. However, the increment was smaller when the CLIP score was removed, indicating that the model with the Tokyo score was more informative than that with the CLIP score. The c indices for the Tokyo score, CLIP score, and BCLC staging were 0.733, 0.707, and 0.657 in the testing sample and 0.737, 0.758, and 0.710 in the training sample, indicating that the Tokyo score was steadily effective in patients from different backgrounds.

DISCUSSION

The aim of this study was to create a novel, simple, prognostic scoring system that would provide a precise prediction of prognosis for patients who were candidates for radical therapy, such as percutaneous ablation or surgical resection. In addition, this scoring system would be used to stratify patients to enable comparison of the efficacy of distinct

Table 9 Baseline characteristics of the testing sample (n = 203)

Variable	n (%)
Age (y) (median (range))	64 (13–83)
Male sex	160 (78.8%)
Viral infection	
HBsAg positive	30 (14.8%)
Anti-HCVAb positive	138 (68.0%)
Both positive	2 (1.0%)
Both negative	37 (18.2%)
Child-Pugh classification	
Class A	155 (76.4%)
Class B	48 (23.6%)
Class C	0 (0%)
Tumour characteristics	
Size of tumour (cm)	
<2	28 (13.8%)
2–5	118 (58.1%)
>5	57 (28.1%)
No of nodules	
Single	146 (71.9%)
2–3	46 (22.7%)
>3	11 (5.4%)
Portal invasion present*	14 (6.9%)
AFP (ng/ml)	
<100	132 (65.0%)
100–400	29 (14.3%)
>400	42 (20.7%)
Okuda stage	
I	184 (90.6%)
II	19 (9.4%)
III	0 (0%)
CLIP score	
0	86 (42.4%)
1	67 (33.0%)
2	43 (21.2%)
3	4 (2.0%)
4⩽	3 (1.5%)
BCLC staging	
A1	84 (41.4%)
A2	31 (15.3%)
A3	20 (9.9%)
A4	21 (10.3%
B	33 (16.3%)
C	14 (6.9%)
D	0 (0%)
Tokyo score	
0	11 (5.4%)
1	59 (29.0%)
2	73 (36.0%)
3	45 (22.2%)
4⩽	15 (7.4%)

*Based on imaging diagnosis.
CLIP, Cancer of the Liver Italian Program; BCLC, Barcelona Clinic Liver Cancer; AFP, α fetoprotein; HBsAg, hepatitis B surface antigen; HCVAb, anti-hepatitis C virus antibody.

Table 10 Comparison of the Tokyo score with the Cancer of the Liver Italian Program (CLIP) score and Barcelona Clinic Liver Cancer (BCLC) staging in the training and testing samples

Model	AIC	ΔAIC
Training sample (n = 403)		
Tokyo+CLIP+BCLC	2521.560	
Tokyo+CLIP	2519.722	−1.838
Removing CLIP	2523.301	+3.579*
Removing Tokyo	2552.765	+33.043†
Testing sample (n = 203)		
Tokyo+CLIP+BCLC	710.4624	
Tokyo+CLIP	709.4629	−0.9995
Removing CLIP	714.6591	+4.1967*
Removing Tokyo	715.2137	+4.7513†

*Difference in AIC (Akaike information criterion) between a model containing Tokyo and CLIP and that containing only Tokyo.
†Difference in AIC between a model containing Tokyo and CLIP and that containing only CLIP.

treatments or among different institutions. Hence the ideal staging system should provide maximal discrimination of outcomes between different stages of disease while keeping the variability of outcomes within each stage to a minimum. The Okuda staging system is not applicable to HCC patients at an early stage of disease as these patients are now eligible for potentially curative treatments, such as medical ablation or surgical resection. As such, while it was useful when first devised, the Okuda scoring or staging system is now generally considered obsolete. Thus 75% of the patients in the training sample and 90% in the testing sample were classified as Okuda stage I. Further stratification of the group is clearly needed. Recently proposed systems from France,[8] Austria,[9] and China[10] were developed in patients with advanced disease with a median survival of only 4–8 months. We calculated the scores using the French, Austrian, and Chinese criteria in the training sample but all of these systems classified patients into only two stages, and most patients belonged to stage I (data not shown).

One of the outstanding merits of Okuda staging is the fact that it consists of four simple parameters—namely, tumour size, ascites, serum albumin, and bilirubin. In constructing the Tokyo score, we followed this simplistic approach. We examined only those parameters that are easily obtainable and avoided criteria that are not generally available. Moreover, we used the AIC in the selection of parameters to obtain a simple model without too many independent parameters or complicated computation. We believe this quality to be very important, especially in projecting retrospective analysis without omitting any patient because of missing values.

We adopted two tumour factors, size and number, in the final model. Tumour size was divided into three groups with break points of 2 cm and 5 cm in diameter. Several studies have identified tumour size in this range as significant,[7 13 14 23 24] and a strong correlation with microvascular invasion and pathological grade of malignancy has been demonstrated.[23 25] However, previously proposed scoring or staging systems, except for BCLC staging, did not include tumour size,[8] or else divided the tumour broadly into massive and other, usually using the break point of half the volume of the liver.[5 6 9] Thus differences in the present and previous scoring systems are primarily seen in the characteristics of the target populations, as reflected by our objective of defining a prognostic scoring system which would discriminate between the relatively early stages of HCC. By the same token, we did not adopt tumour related symptoms, which were included in the BCLC and French systems,[7 8] as almost all of our patients were asymptomatic.

The number of tumour nodules is also known to be associated with intrahepatic spread of malignant cells. Some authors reported a major difference in prognosis between solitary and multinodular HCC after surgical resection.[13 26 27] However, we found that the number of nodules was best divided dichotomously between 1–3 and ≥4. The suggestion is that the presence of two or three nodules might often be the result of simultaneous independent carcinogenesis rather than intrahepatic metastasis in patients with advanced cirrhosis.

Evident vascular invasion such as portal vein tumour thrombus is an absolute predictor of ominous prognosis.[3 13 26] Overt metastases to extrahepatic organs or lymph nodes are also associated with poor prognosis. As the testing sample contained few patients with these two manifestations, they were not selected after stepwise variable selection. It should be noted that the Tokyo score may not be predictive for advanced disease.

We selected two factors, albumin and bilirubin, as indicators of liver function. Both are included in the Child-Pugh classification, together with prothrombin time, ascites, and encephalopathy, and thus were also included in the CLIP score as a factor in the Child-Pugh classification. However, the latter three were not selected after stepwise variable selection because they were strongly correlated with the former two. Thus liver function is represented by two parameters, which we believe is preferable for the sake of model simplicity.

Portal hypertension is accepted as a strong predictor of poor prognosis. Among our candidate factors, ascites, encephalopathy, and platelet count were considered as related to portal hypertension and all were significant in the univariate analysis. Bruix *et al* reported that HVPG was a significant predictor of decompensation after hepatic resection[28] and it is included in the BCLC staging system. However, HVPG is a special examination which is not routinely carried out in daily practice. We substituted the presence of oesophageal varices and platelet count less than 100 000/mm^3 for HVPG ≥10 mm Hg, as described by the author, but the substitution may have impaired the prognostic power of the BCLC staging in the samples.

Prognostic scores can be divided into two groups: those based on expert opinion, such as TNM staging, and those developed through regression analysis of actual data, such as the CLIP and Tokyo scores. We applied bootstrap methods to avoid possible overfitting bias that often accompanies regression analysis. Nevertheless, the fact that the Tokyo score fitted better in the training sample than in the testing sample may indicate that there remained some overfitting bias. Another possible reason why the Tokyo score did not surpass the CLIP score in the testing sample was the presence of AFP in the latter but not in the former. Over 20% of patients in the testing sample had AFP levels >400 ng/ml compared with 10% in the training sample. It is reasonable to assume that AFP plays a more important role in advanced disease.

BCLC staging, developed from several independent studies on both early and advanced patients, includes treatment strategy, indicating that a single HCC without portal hypertension should be resected and that patients with no more than three nodules not exceeding 3 cm in diameter have indications for ablation therapy. Recently, Cillo *et al* found that BCLC was the best among staging systems, including CLIP, in patients treated with radical therapies.[29] One possible reason why BCLC staging did not show greater ability in the testing and training samples may be that our patients were not always treated according to the strategy.

The relative prognostic importance of each factor depends on the features of the patients in the training sample, as do the independent variables remaining after stepwise selection. Child-Pugh classification and the model for end stage liver disease (MELD) are suitable for assessing the prognosis of patients with severely impaired liver function[30] while only tumour related factors are relevant in assessing outcome after liver transplantation for HCC patients.[31 32] Similarly, the applicability of the Tokyo score is limited by the fact that it was established and validated on the basis of HCC patients treated by medical ablation or surgical resection. However, with the growing realisation of high risk groups for HCC and rapid advances in imaging techniques, an increasing number of patients are being diagnosed at an earlier stage, and qualify for potentially curative treatments, such as medical ablation and surgical resection.

In conclusion, we established the Tokyo score by analysing survival time among HCC patients treated with medical ablation, and validated it in patients who underwent surgical resection. The Tokyo score may be useful in predicting the prognosis of HCC patients who are candidates for these curative treatments.

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Original Paper

Factors Predisposing to Poorly Differentiated Hepatocellular Carcinoma and its Recurrence

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ABSTRACT

Background/Aims: We investigated the clinical factors predisposing moderately or poorly differentiated hepatocellular carcinoma and analyzed which clinical and histological factors are associated with poorly differentiated hepatocellular carcinoma (HCC) recurrence.

Methodology: Percutaneous fine-needle biopsy was taken from the liver tumor of 191 consecutive patients between January 1994 and September 1996. The histological degree of differentiation of hepatocellular carcinoma at the first time of initial treatment and at the time of second recurrence was classified according to the criteria of Edmondson and Steiner.

Results: At the time of the first therapy, 86 patients, 81, 24, and 0 patients had liver tumors classified as Edmondson (Ed), 1, 2, 3, and 4, respectively. The prognosis of patients with Ed-3/4 HCC was worse than and the tumor sizes were larger than that of Ed-1/2 HCC patients. Of the 167 patients classified as Ed-1/2 at the time of first therapy, HCC recurred in 95 of the patients during the mean follow-up period of 3.4 years. Multivariate analysis revealed that only tumor size ($P=0.035$) and TACE therapy ($P=0.0009$) were independently significant factors in predicting future Ed-3/4 or multiple HCC recurrence.

Conclusions: Tumor size and TACE therapy were clinical predisposing factors for Ed-3/4 or multiple HCC recurrences.

KEY WORDS: Hepatocellular carcinoma; Histological degree of differentiation; HCC recurrence; Tumor marker; Percutaneous ethanol injection therapy; Transcatheter arterial chemo-embolization

ABBREVIATIONS: Hepatocellular Carcinoma (HCC); Percutaneous Ethanol Injection Therapy (PEIT); Transcatheter Arterial Chemo-embolization (TACE); Hepatitis C Virus (HCV); Hepatitis B Virus (HBV); Ultrasonography (US); α-Fetoprotein (AFP); Percutaneous Microwave Coagulation Therapy (PMCT); Radiofrequency Ablation (RFA); Anti-Hepatitis C Virus (anti-HCV); Hepatitis B surface Antigen (HBsAg); Des-γ-Carboxy Prothrombin (DCP); Percutaneous Tumor Ablation (PTA)

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver, and the number of patients with HCC has reportedly been increasing in the United Kingdom, France and the United States over the past two decades (1-3). In Japan, the number of HCC cases has increased substantially (4-6). HCC incidence correlates with the frequencies of chronic hepatitis C virus (HCV) and hepatitis B virus (HBV) infection, and underlying cirrhosis is found in most patients with HCC (7). Screening programs using ultrasonography (US) and α-fetoprotein (AFP) level measurement in high-risk populations (8) have been established in Japan. As a result, small tumors are detected earlier, tumor stage is less advanced at the time of diagnosis, and patients generally have less severe underlying liver disease.

Reportedly, surgical mortality rates are low in cirrhotic patients undergoing resection (9). However, because of tumor extent and hepatic dysfunction, only a small percentage of patients are candidates for resection (10). In addition, tumor recurrence due to multifocality and progression of underlying liver disease has limited the efficacy of resection (9,10). At our hospital, patients with HCC have mainly undergone percutaneous ethanol injection therapy (PEIT) (8,11-13), percutaneous microwave coagulation therapy (14) (PMCT), or radiofrequency ablation (RFA) therapy (15).

Intrahepatic recurrence or multicentric occurrence is frequent even after successful PEIT or surgical resection (9,16-20). The high recurrence rate even after curative treatment is thus an important clinical problem. Several large-scale studies on the recurrence of and prognostic factors for HCC have emphasized clinical parameters, but few studies have examined the relationship between the pathological grade of primary HCC and recurrent HCC in patients treated curatively.

In this study, we investigated the clinical factors predisposing to Ed-3 or 4 HCC and analyzed which clinical and histological factors in Ed-1 or 2 HCC patients could potentially indicate the risk recurrence of HCC at the Ed-3 or Ed-4 level.

METHODOLOGY

Study Population

A series of 191 consecutive patients with HCC, admitted to Tokyo University Hospital for initial treatment from January 1994 through September 1996, underwent percutaneous fine-needle biopsy of the liver tumor and were treated by PEIT, PMCT or surgical resection. The patients comprised 139 men and 52 women, ranging in age from 41-83 years

Hepato-Gastroenterology 2005; 52:391-397

(mean, 64 years). The majority (155 patients, 81.2%) had anti-hepatitis C virus (anti-HCV) antibodies, 20 (10.5%) had hepatitis B surface antigen (HBsAg), four (2.1%) were positive for both HBsAg and anti-HCV, and 20 (10.4%) were negative for HBsAg and anti-HCV. Prior to the liver tumor biopsy, all patients underwent dynamic abdominal CT scan with contrast material enhancement during the arterial and portal phases and abdominal US with Doppler sound and power Doppler sound.

Pathological Diagnosis of HCC and Underlying Liver Disease

Prior to PEIT or PMCT, a tumor biopsy was performed. Biopsy samples were obtained by echo-guided needle biopsy using a 20-gauge biopsy needle (Bard monopty, Bard, Covington, GA). Tissue samples were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Sections of paraffin-embedded specimens were cut and stained with hematoxylin-eosin. Two blinded pathologists evaluated the histological characteristics of each specimen. Histological differentiation of HCC was graded according to the criteria of Edmondson and Steiner (21) **(Figure 1)**. When histological assessments differed between the two pathologists, a diagnosis was made in favor of malignancy. If the number of tumors was more than two, biopsy samples were obtained from each lesion. Pathological grading was classified according to the lowest grade of all samples. Fibrosis staging of the underlying liver disease was assessed in US-guided biopsy samples of non-cancerous tissue taken using a 14- or 16-gauge biopsy needle (Bard Monopty, Bard) according to the criteria of Desmet *et al.* (22).

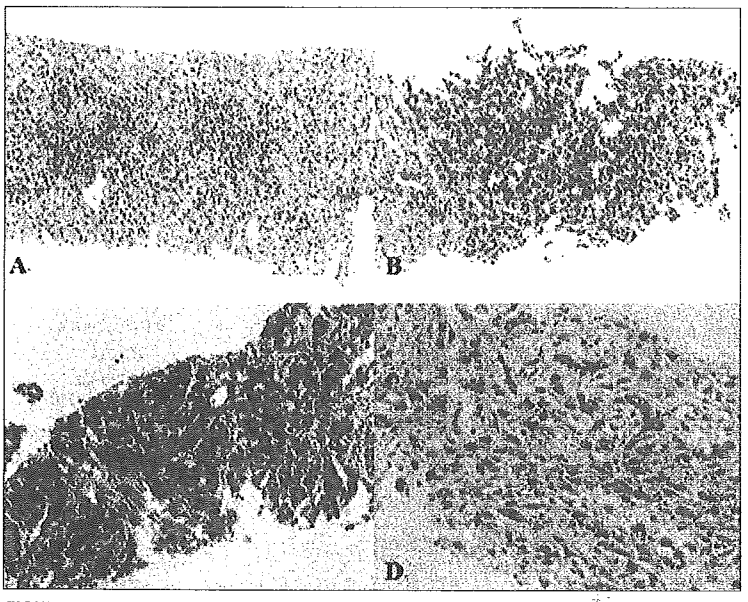

FIGURE 1 Histological differentiation of HCC according to criteria of Edmondson and Steiner. **(A)** Edmondson 1 HCC. The changes seen in a once normal liver cell are greater than one would expect in an ordinary adenoma or in an area of adenomatous hyperplasia. **(B)** Edmondson 2 HCC. The nuculei are larger and more hyperchromatic than normal, while the cytoplasm is abundant and acidophilic. **(C)** Edmondson 3 HCC. These nuculei occupy a relatively greater proportion of the cell. Some breakup or distortion of the usual trabecular pattern is present. **(D)** Edmondson 4 HCC. Growths in the liver are more medullary in character, the trabeculae are difficult to find, and many of the cell masses seen to lie loosely without cohesion in vascular channels.

Patient Follow-up

All patients were divided into either an Ed-1/2 HCC group (n=167) or an Ed-3/4 HCC group (n=24). Survival rates for the two groups were analyzed by the Kaplan-Meier technique and the differences in curves were tested with the log-rank test. The Ed-1/2 HCC group (n=167) treated by PEIT (n=126), PMCT (n=15), transcatheter arterial chemoembolization (TACE) and subsequent PEIT (PMCT) (n=19), surgical resection (n=3), or TACE (n=4), was followed until December 1998 for HCC recurrence. An early, 1- to 2-month, outpatient follow-up was conducted, taking blood tests and measuring serum tumor marker levels (α-fetoprotein [AFP]; lectin reactive AFP [AFP-L3]; des-γ-carboxy prothrombin [DCP]) (23). We examined each patient for HCC recurrence, by US every 3 months and by CT every 6 months. Patients were reclassified into the following four groups based on the detection of recurrence prior to December 1998: No recurrence (n=72), Ed-1/2 recurrence (n=48), Ed-3/4 recurrence (n=22), and the multiple recurrence (n=25).

The multiple recurrence group consisted of patients with more than five HCC lesions (n=15), portal vein tumor thrombi (n=6), or distant metastasis (n=4). If HCC recurrence was suspected, further imaging examinations, including dynamic computed tomography (CT), magnetic resonance (MR) imaging, angiography, and/or lipiodol CT were performed. A diagnosis of recurrent HCC was based mainly on the fine needle biopsy of tumors, and otherwise based on results of the above imaging modalities. When HCC recurrence was detected, patients received further treatment, mainly by PEIT and/or PMCT, but when multiple HCC recurrence was detected, the additional treatment was usually by TACE or TAI and no tumor biopsy was performed **(Figure 2)**. The follow-up period was defined as the interval from the date of the first HCC treatment after initial admission until the date of diagnosis of recurrence, the date of death, or the end of December 1998. If there was an Ed-3/4 recurrence before the end of December 1998, the follow-up period was defined as the time up to the diagnosis of Ed-3/4 HCC recurrence (Ed-3/4 recurrence group). In the event of no recurrence through December 1998, the follow-up period was defined as the time from initial treatment until the end of December 1998 or the date of death (no recurrence group). The mean follow-up period was 3.4 years ±0.8 years (range 2.3-5.0 years).

Confirmation of Treatment Efficacy

Complete necrosis of the lesion by PEIT and/or PMCT was confirmed on dynamic CT after completion of treatment in all patients. Complete necrosis was defined as hypoattenuation of the lesion, in both the arterial and the portal venous phase, on dynamic CT

and a necrotic area larger than the total areas of the pretreatment lesions (24). If tumor necrosis was incomplete as determined by the presence of a contrast-enhanced (hyperattenuation) area in the early phase, or if the necrotic area was smaller than pretreatment lesions, additional treatment was administered.

Statistical Analysis

The unpaired Student's *t*-test or the Mann-Whitney U test was used to compare differences between two groups. The chi-square test was used to compare categorical data, and differences among all three groups were evaluated by one-way ANOVA and the Post-hoc test. Baseline patient data were reported as the mean ± SD, or median and range. Cumulative incidence curves were determined by the Kaplan-Meier method, and the log-rank test was used to assess differences between groups. Twenty three possible predictors of Ed-3/4 recurrence were examined: age, sex, HBsAg, anti-HCV, HCV genotype, HCV load, interferon treatment history of eradicated HCC after treatment, alcohol consumption, tumor characteristics (diameter, number of tumors), fibrous staging of background tissue, Child classification, albumin level, total bilirubin level, ALT level, AST level, platelet count, prothrombin time (PT), $ICGR_{15}$ level, serum levels of tumor markers (AFP, AFP-L3, DCP), and history of TACE therapy. The data for each continuous variable, other than AFP-L3, were transformed into categorical data consisting of two ordinal numbers by median value. AFP-L3 was either <10% or ≥10%. Parameters that proved to be significant in the univariate analysis were tested by multivariate logistic regression in 70 patients. Possible predictors of Ed-3/4 and multiple HCC recurrence included the same 23 variables. In the 167 patients with Ed-1/2 HCC, we tested all parameters considered significant ($P<0.05$) in the univariate analysis by the multivariate Cox proportional hazards model. A *P*-value of less than 0.05 was considered significant.

RESULTS

Patient Characteristics

The demographic and clinical features of patients at the time of their enrollment are summarized in **Table 1**. Fifteen patients were positive for HBsAg, 151 were positive for HCV Ab, four were positive for both, and 20 were negative for both. The mean number of tumor foci was 2.2 (range, 1-12 foci), with a mean size of 30mm (range, 9-160mm). Tumor cells were classified as Edmondson 1, 2, 3 or 4 in 86, 81, 24 and 0 patients, respectively. With regard to background liver biopsy, one, 11, 27 and 138 patients had liver biopsy gradings of F1, F2, F3 and F4 respectively. The median serum AFP was 22ng/mL (range 2-35,151ng/mL) and median serum DCP was 0.0625 AU/mL (range 0.0625-80ng/mL).

One hundred and thirty-eight patients had previously undergone PEIT, 18 PMCT. Six patients had received both PEIT and PMCT, and 21 had undergone

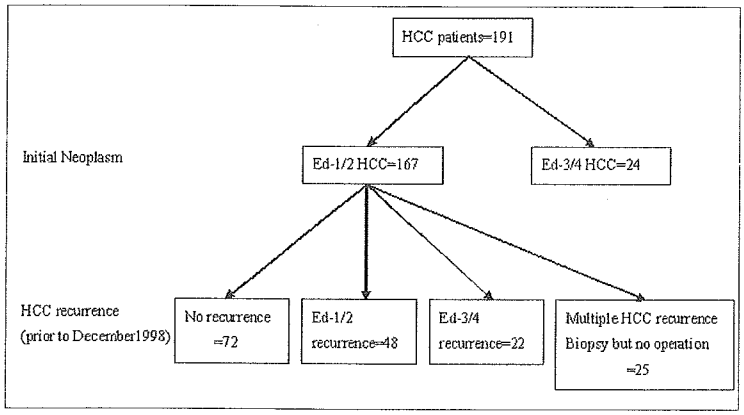

FIGURE 2 Schema for HCC patients.

TACE (18 patients; gelatin-sponge and lipiodol mixed with epirubicin hydrochloride, 2 patients; epirubicin hydrochloride and mitomycin-C, 1 patient; epirubicin hydrochloride and SMANCS) and subsequent PEIT (or PMCT). Since nearly all of the 21 patients had more than 3 lesions and/or tumors larger than 50 mm, we initially employed TACE. Three patients underwent surgical resection (2 of these patients had first been treated by TACE), another four aspiration biopsy for the purpose of diagnosis and treatment by TACE. The overall survival rates of our 191 HCC patients were 89.5%, 71.0% and 40.6% at one, three and five years, respectively.

Cumulative Survival Rates

Table 1 shows the profiles of patients in whom the first hepatocellular carcinoma was classified as either

TABLE 1 Patients Profile Classified according to the Pathologic Parameters of Initial Hepatocellular Carcinoma

	Ed-1/2 HCC (n=167)	Ed-3/4 HCC (n=24)	*P*-value
	median and range	median and range	
Age (years)	64 (41-83)	64.5 (41-82)	0.885
Sex (M:F)	(123:45)	(17:7)	0.807
Size of tumor (mm)	23 (9-160)	33 (19-160)	0.006*
Number of tumors	2 (1-12)	1 (1-8)	0.361
Albumin (g/dL)	3.5 (2.4-4.8)	3.7 (2.9-4.6)	0.607
T. Bil (mg/dL)	0.8 (0.4-3.1)	0.9 (0.3-4.6)	0.629
AST level (IU/L)	81 (25-287)	56 (29-138)	0.011*
ALT level (IU/L)	69 (16-340)	67 (27-148)	0.031*
$ICGR_{15}$(%)	27.7	25.9	0.622
Platelet count ($x10^4/mm^3$)	8.7 (3.2-43.9)	10.3 (3.7-22.9)	0.432
Prothrombin time (%)	67.6 (32.8-101)	67.6 (40.3-100)	0.436
AFP (ng/mL)	21.5 (2-35153)	21.5 (2-115280)	0.334
DCP (AU/mL)	0.0625 (0.0625-80)	0.0625 (0.0625-0.822)	0.222
HBsAg (positive/negative)	(17:150)	(3:21)	0.729
HCV Ab (positive/negative)	(134:33)	(21:3)	0.374
HCV serotype (1:2)	(95:29)	(17:3)	0.384
HCV load (copies/mL)	10^{5*} (0-$10^{6.5*}$)	10^{5*} ($10^{2.5*}$-$10^{6.5*}$)	0.536
Fibrosis staging (CH: LC)	(35:120)	(5:18)	0.928
Child classification (A:BC)	(86:81)	(13:11)	0.807
Alcohol abuse (yes:no)	(56:109)	(6:18)	0.384

*: *P*-value of less than 0.05.

TABLE 2 Initial Neoplasm Data and Recurrence Classified into 3 Groups (Histology of recurrence)

		No recurrence (N=72) median and range	Ed-1/2 recurrence (N=48) median and range	Ed-3/4 recurrence (N=22) median and range	*P*-value
AST (IU/L)	initial neoplasm	81.5 (25-278)	84 (27-219)	70 (29-210)	0.882
(IU/L)	recurrence	63 (20-340	72 (16-188)	78 (20-235)	0.380
ALT (IU/L)	initial neoplasm	85 (7-327)	72 (16-188)	81 (21-207)	0.662
(IU/L)	recurrence	55 (10-294)	75 (16-203)	64 (16-195)	0.415
PLT (IU/L)	initial neoplasm	7.8 (3.2-24)	9.1 (4.1-17.4)	9.4 (5.7-23.2)	0.142
(IU/L)	recurrence	7.5 (3.5-19.5)	7.6 (3.7-25.5)	9.8 (3-18.6)	0.197
PT (%)	initial neoplasm	68 (34.1-100)	65.9 (32.8-97)	74.3 (55.6-101)	0.138
	recurrence	72.7 (27.5-100)	66 (11.4-100)	70 (45-102)	0.351
T.B (mg/dL)	initial neoplasm	0.8 (0.4-3.1)	0.9 (0.4-2.8)	0.7 (0.4-1.9)	0.506
	recurrence	1.1 (0.3-44.8)	1.1 (0.5-2.8)	0.9 (0.3-2.9)	0.389
Alb (g/dL)	initial neoplasm	3.5 (2.5-4.2)	3.5 (2.5-4.4)	3.7 (2.5-4.8)	0.485
	recurrence	3.4 (2.2-4.6)	3.5 (1.9-4.2)	3.4 (2.1-4.8)	0.535
Tumor marker					
AFP (ng/mL)	initial neoplasm	15 (2-185)	22 (3-518)	71.3 (5-2698)	0.0002*
	recurrence	14 (1-2650)	22 (3-2505)	86 (3-13665)	<0.0001*
DCP (AU/mL)	initial neoplasm	0.0625 (0.0625-80)	0.0625 (0.0625-3.765)	0.0625 (0.0625-2.87)	0.407
	recurrence	0.0625 (0.0625-20)	0.0625 (0.0625-0.513)	0.0625 (0.0625-1.873)	0.033*
AFP-L3 (%)	initial neoplasm	0.5 (0.5-0.5)	0.5 (0.5-71.8)	10.5 (0.5-80.3)	0.0005*
	recurrence	0.5 (0.5-29.1)	0.5 (0.5-83.9)	26.1 (0.5-80.4)	<0.0001*

*: *P*-value of less than 0.05.

Ed-1/2 HCC or Ed-3/4 HCC. Ed-3/4 HCC patients had significantly larger tumors ($P=0.006$), and lower AST and ALT levels than the Ed-1/2 HCC patients ($P=0.011$ and $P=0.031$, respectively).

The survival curves of the Ed-1/2 HCC and Ed-3/4 HCC groups were comparatively analyzed by the Kaplan-Meier method. The 1-year, 2-year, and 3-year survival rates of Ed-1/2 HCC (n=168) patients were 89.9%, 81.5% and 73.2%, and the 1-year, 2-year and 3-year survival rates of Ed-3/4 HCC (n=24) patients were 87.5%, 75% and 56.8%, respectively. Thus, the survival rates for the Ed-1/2 HCC group were statistically superior to those of the Ed-3/4 HCC group ($P=0.021$). The patients with Ed-1/2 HCC died of tumor progression (26 patients-51.0%) or liver failure (10 patients-19.6%). The patients with Ed-3/4 HCC died of tumor progression (eight patiests-61%) or liver failure (2 patients-15.4%). There is no difference between the two groups ($P=0.862$).

Histological Recurrence Pattern of Ed-1/2 Primary HCC

Figure 2 illustrates the Ed-1/2 primary HCC recurrence pattern (n=167). Of the 167 patients in this category, 48 demonstrated an Ed-1/2 recurrence pattern and 22 an Ed-3/4 recurrence pattern during the observation period. Twenty-five patients demonstrated multiple HCC recurrence, although tumor biopsies were not performed. In 72 patients, no HCC recurrence was observed during the follow-up period.

Local Recurrence

In 18 of the 167 Ed-1/2 primary HCC patients, local recurrence was observed at rates of 5.5%, 7.8%, 13.4%, 17.5% and 17.5% at 1, 2, 3, 4 and 5 years, respectively after initial diagnosis. These recurrences were all detected less than 4 years after PEIT or PMCT. In the 24 Ed-3/4 primary HCC patients, local recurrence was observed in six of 56 PEIT or PMCT treated nodules. Local recurrence rates were 21.1%, 26.0 and 26.0% at 1, 3 and 5 years, respectively. Overall, local recurrence rates were higher for Ed-3/4 primary HCC than for Ed-1/2 primary HCC though the difference did reach statistical significance ($P=0.050$).

Differences in Clinical Data between the First Neoplasm and Recurrence

Table 2 shows clinical data for the first neoplasm and recurrence based on classification into three groups according to pathological findings (no recurrence, Ed-1/2-recurrence, Ed-3/4-recurrence). Average follow-up periods till recurrence or the defined endpoints of no recurrence group, Ed-1/2 recurrence group and Ed-3/4 recurrence group were 2.4 years, 2.4 years and 1.7 years, respectively. The recurrence period for the Ed-3/4 group was shorter than those for the no recurrence and Ed-1/2 recurrence groups, but the difference was not statistically significant ($P=0.065$).

For the first neoplasm, there was a difference in the serum AFP level. During the follow-up period, the mean serum AFP levels were 19.4ng/mL, 27.5ng/mL and 89.9ng/mL for the no recurrence, Ed-1/2 and Ed-3/4 recurrence groups, respectively ($P=0.0002$). Differences in tumor markers [AFP, DCP, AFP-L3] were also seen. Mean serum AFP levels were 16.1ng/mL, 29.4ng/mL and 140.3ng/mL, and mean serum DCP levels were 0.074 AU/mL, 0.080 AU/mL and 0.112 AU/mL for the no recurrence, Ed-1/2 and Ed-3/4

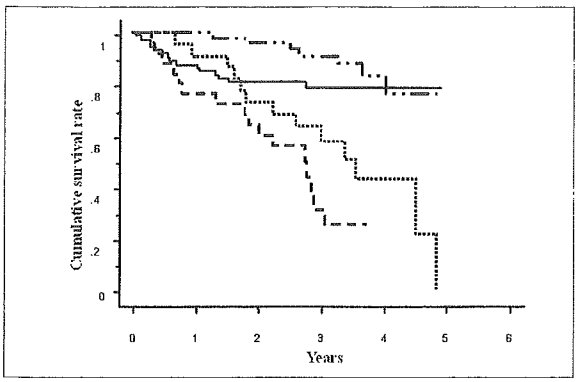

FIGURE 3 Cumulative survival for each group.
(A) (— · · — ·) represents the survival curve for the Ed-1/2 recurrence group. **(B)** (————) represents the survival curve for the no recurrence group. **(C)** (·····) represents the survival curve for the Ed-3/4 recurrence group. (D) (- - - - -) represents the survival curve for the multiple recurrence group.
$P<0.0001$ by log-rank test.

recurrence groups, respectively (AFP; $P<0.0001$, DCP; $P=0.033$). Mean AFP-L3 values were 3.14%, 13.6% and 34.7% for the no recurrence, Ed-1/2 and Ed-3/4 recurrence groups, respectively ($P<0.0001$).

Risk Factors for Ed-3/4 HCC Recurrence in Patients of Ed-1, 2 Primary HCC

Risk factors for future recurrence of Ed-3/4 HCC were studied in 70 patients with either Ed-1/2 recurrence (n=48) or Ed-3/4 recurrence (n=22). In the univariate analysis, the following factors had a statistically significant influence on Ed-3/4 recurrence rates: tumor size ($p=0.0524$), AFP ($P=0.0064$), PT ($P=0.0555$) and TACE therapy ($P=0.0122$). Multivariate analysis of ten significant factors in the multiple logistic regression model revealed only AFP to be an independently significant factor in predicting future Ed-3/4 recurrence ($P=0.045$) **(Table 3)**.

Relationship between Recurrence Pattern and Survival Rate

Figure 3 shows the cumulative survival rates of the 4 groups: no recurrence (n=72), Ed-1/2 recurrence (n=48), Ed-3/4 recurrence (n=22), and multiple recurrence (n=25). The Ed-3/4 and multiple HCC recurrence group survival rates were worse than those for the Ed-1/2 and no recurrence groups ($p<0.001$).

Cumulative survival rates of the recurrence positive subgroup (n=95) and the no recurrence group (n=72) were calculated using the Kaplan-Meier method. The recurrence positive subgroup contained patients with Ed-1/2 (n=48), Ed-3/4 (n=22), and multiple HCC (n=25) recurrences. While the survival rate for the no recurrence subgroup was higher than that of the recurrence groups, the log-rank test showed neither recurrence nor the absence of recurrence to be statistically significant ($P=0.093$). **Figure 3** shows the cumulative survival rates for the no recurrence and Ed-1/2 recurrence subgroups (n=120), and for the Ed-3/4 recurrence and multiple recurrence subgroups (n=47). The survival rates for the Ed-3/4 and multiple HCC recurrence subgroups were lower than those of the no recurrence and Ed-1/2 recurrence subgroups. The log-rank test showed the difference between these two subgroups to be statistically significant ($P<0.0001$).

Risk Factors for Ed-3/4 or Multiple HCC Recurrence

The risk factors for future Ed-3/4 or multiple recurrence were explored in Ed-1/2 HCC patients (n=167). In the univariate analysis, the following seven factors were found to be of statistical significance: gender ($p=0.029$), tumor size ($p<0.0001$), AFP value ($p=0.005$), DCP value ($p=0.0001$), TNM stage ($p=0.004$), alcohol abuse ($p=0.004$) and TACE therapy ($p<0.0001$).

Multivariate analysis of the seven significant factors in the Cox regression model demonstrated only tumor size ($P=0.035$) and TACE therapy ($P=0.0009$) to be independently significant factors in predicting future Ed-3/4 and multiple HCC recurrences **(Table 4)**.

DISCUSSION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver. At present, liver transplantation is the only form of curative treatment available (10,25), so the prognosis for patients with HCC remains dismal. Other treatments, such as surgical hepatic resection (9) or percutaneous tumor ablation (PTA) by PEIT, PMCT and RFA, have been employed for treating patients with small HCC (8,13,15). Among these treatment options, PEIT is advantageous because of its cost-effectiveness, low associated morbidity, and liver preservation (12,13). While factors predicting survival and tumor recurrence after PEIT and surgery have been reported (26-29), few studies have focused on how clinicopathological features relate to patient survival and tumor recurrence.

In our institution, when the tumor was angiographically demonstrated to be hypervascular, we carried out TACE before PEIT. Embolization is initially

TABLE 3 Risk Factors for Ed-3/4 HCC Recurrence

	P-value	Odds ratio (95% CI)
Size of tumor (>24mm)	0.108	2.612 (0.809-8.438)
TACE therapy	0.081	3.993 (0.842-18.941)
Prothrombin time (<66%)	0.599	0.720 (0.211-2.451)
AFP (39ng/mL)	0.045	3.516 (1.030-11.977)*

TABLE 4 Risk Factors for Ed 3/4 and Multiple HCC Recurrence (Multivariate analysis)

	P-value	Odds ratio (95% CI)
Size of tumor (>24mm)	0.035	2.38 (1.064-5.340)*
TACE therapy	0.0009	3.33 (1.635-6.782)*

*: *P*-value of less than 0.05.

performed to treat all lesions, some of which apparently remains viable. In this study, we classified all untreated HCC cases into two groups, Ed-1/2 and Ed-3/4 HCC. The prognosis for Ed-3/4 HCC patients was inferior to that of those with Ed-1/2, and tumors were larger in the former group. As to other clinical data, such as tumor markers, however, there were no differences between the Ed-1/2 HCC and Ed-3/4 HCC groups.

Ed-3/4 recurrences from Ed-1/2 primary HCC were investigated and compared to those from Ed-1/2 HCC recurrence and no recurrence patients. At the time of recurrence, tumor markers (AFP, AFP-L3, DCP) were higher in Ed-3/4 patients than in Ed-1/2 and no recurrence patients. Univariate analysis revealed the AFP level (≥39ng/mL), TACE therapy, PT (>66%), and tumor size (>24mm) to be factors predictive of Ed-3/4 recurrence. According to the multivariate analysis, the AFP level (≥39ng/mL) was a predictive factor for Ed-3/4 HCC recurrence. A previous study showed that the degree of tumor differentiation appears to be more important than tumor size in determining the level of HCC severity (30). In this study, AFP was not consistently associated with tumor differentiation, especially in the primary neoplasm. At the time of recurrence however, not only AFP, but also AFP-L3 and DCP were associated with tumor differentiation. The factors effecting HCC recurrence after PEIT treatment have yet to be clarified, although the size and number of lesions and basal AFP level do reportedly have an influence (17). Also, fibrous staging of the underlying liver disease is reportedly associated with intrahepatic recurrence in cases with HCV-associated HCC (30).

In this study, intrahepatic recurrence did not have a statistically significant effect on survival, while Ed-3/4 or multiple HCC recurrence exerted a strong influence. Tumor size (>24mm) and TACE therapy also affected Ed-3/4 and multiple HCC recurrences. TACE therapy influenced Ed-3/4 HCC recurrence according to univariate analysis, and affected Ed-3/4 and multiple HCC recurrences according to multivariate analysis. TACE, in this study, was performed by injecting a mixture of iodized oil with the anticancer drug, mainly epirubicin hydrochloride (average 30mg), followed by a gelatin sponge. Previous reports have shown the incidence of HCC with a sarcomatous appearance to increase remarkably with the use of anticancer therapies such as one shot injection and TAE (31,32). Since both univariate and multivariate analyses indicated an association between TACE and Ed-3/4 HCC recurrences, we can speculate that the epirubicin hydrochloride may have induced some sort of phenotypic change in HCC cells. Transcatheter embolization can produce sufficient ischemia, but, in general, HCC is not particularly sensitive to chemotherapeutic agents. Thus, the addition of an anticancer drug may be more harmful than beneficial. Further histological and biological studies are required to clarify the true nature of Ed-3/4 HCC recurrences.

There are some limitations in our analysis, related mainly to the histological staging of primary and recurrent lesions having been determined by fine-needle biopsies in most cases. The histological features of the entire tumor are not always represented in one biopsy specimen, as is apparent from detailed study of liver tumors obtained after hepatectomy or liver transplantation.

However, in analyzing the pathological grade of the primary and recurrent HCC of patients treated by PEIT, patients classified as Ed-3/4 HCC at the initial therapy were shown to have poor outcomes. Furthermore, tumor size and TACE were associated with either Ed-3/4 or multiple HCC recurrence in the patients who had undergone ablation therapy.

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Large-Scale Search of Single Nucleotide Polymorphisms for Hepatocellular Carcinoma Susceptibility Genes in Patients With Hepatitis C

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Hepatitis C virus (HCV) infection is a major risk factor for developing hepatocellular carcinoma (HCC). The host genetic factors that are involved in the development of HCC in patients with HCV infection remain to be investigated. To search for single nucleotide polymorphisms (SNPs) in HCC susceptibility genes, 393 SNPs in 171 candidate genes were examined in 188 Japanese patients with chronic HCV infection, including 77 patients with HCC. HCC-related SNPs were then examined in another 188 patients (including 93 patients with HCC) with chronic HCV infection. Haplotype analyses of HCC-related genes were performed in a total of 376 patients. Of the 393 SNPs, 31 SNPs in 29 genes were significantly associated with HCC based on an initial screening ($P < .05$). Of these 31 SNPs, 3 SNPs of 3 genes (SCYB14, GFRA1, and CRHR2) were significantly associated with HCC in a secondary screening. Haplotype analyses of these 3 genes identified 2 haplotype blocks associated with HCC. In conclusion, these SNPs and haplotypes located in the SCBY14, CRHR2, and GFRA1 genes will be used as markers to identify a subgroup of Japanese patients with chronic HCV infection who are at high risk of developing HCC. *Supplementary material for this article can be found on the HEPATOLOGY website (http://www.interscience.wiley.com/jpages/0270-9139/suppmat/index.html).* (HEPATOLOGY 2005;42:846-853.)

More than 170 million people worldwide are estimated to have chronic hepatitis C virus (HCV) infection (http://www.who.int/inf-fs/en/fact164.html). The most important sequelae of chronic HCV infection are progressive liver fibrosis leading to cirrhosis, and hepatocellular carcinoma (HCC), which is responsible for significant morbidity and mortality throughout the world.[1-4] Many factors, such as alcohol intake, older age at time of infection, male sex, and coinfection with hepatitis B virus, are reported to accelerate disease progression in HCV infection.[5-8] In addition, host genetic factors have been reported to affect the risk of developing HCC.[9-15]

Recently, we reported that genetic polymorphisms in interleukin-1β[11] and uridine 5′-diphosphate-glucuronosyltransferase 1A7[12] are associated with the development of HCC in Japanese patients with chronic HCV infection. Genetic polymorphisms in CYP enzymes,[13] the microsomal epoxide hydrolase gene,[14] and the aldehyde dehydrogenase 2 gene[15] also have been reported to be associated with HCC and the severity of HCV-related liver disease. The number of candidate genes that have been examined is, however, rather limited.

We performed a large-scale candidate-gene–based search of single-nucleotide polymorphisms (SNPs) to look for SNPs in genes associated with HCC susceptibility. A total of 393 SNPs in 171 candidate genes were examined in Japanese patients with chronic HCV infection.

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SNPs, single nucleotide polymorphisms; ALT, alanine aminotransferase; AFP, alpha fetoprotein; SCYB14, small inducible cytokine B14 precursor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFRA1, GDNF family receptor alpha 1; CRHR2, corticotropin-releasing hormone receptor 2.

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Patients and Methods

Patients. We studied 376 consecutive Japanese patients with chronic HCV infection who consulted the outpatient clinic of the University of Tokyo Hospital between August 2001 and June 2003 (208 men and 168 women, 22-84 years old, a median age of 62.5 years, 170 with HCC and 206 without HCC). The genomic DNA of these patients was made available after obtaining written informed consent for genotyping. The first 188 patients were enrolled in the initial screening for SNPs in HCC susceptibility genes. The second 188 patients were enrolled in the secondary screening. Approval was obtained from the institutional ethics committee, and all the procedures followed institutional guidelines.[16]

Patients selected for the study were those who tested positive for HCV antibody by the second-generation enzyme immunoassay (Ortho Diagnostics, Tokyo, Japan); HCV RNA was measured using the Amplicor HCV assay version 1 (Roche, Tokyo, Japan). All patients were negative for the hepatitis B surface antigen (Abbott Laboratories, North Chicago, IL). HCV genotypes were determined by using a genotyping assay (SRL Laboratory Co., Tokyo, Japan). Any patient with an ethanol intake of 80 g/d or above for longer than 10 years was considered to have a positive history of alcohol abuse. The following clinical parameters were obtained for each patient at the time of whole-blood collection: age, sex, serum albumin level, serum total bilirubin level, serum alanine aminotransferase (ALT) level, serum alpha fetoprotein (AFP) level, prothrombin time, platelet count, and serum viral load measured using the Amplicor-HCV monitor assay. The diagnosis of HCC was made by several imaging methods (ultrasonography, computed tomography, arteriography, or magnetic resonance imaging) and confirmed histologically by sonography-guided fine-needle biopsy specimens in all 170 patients.[17] All patients were shown not to have other cancers by an initial screening examination.

Polymorphism Genotyping. Genomic DNA was extracted from 100 μL whole blood using the SepaGene kit (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's instructions. Extracted DNA was dissolved in 20 μL Tris-HCl buffer (10 mmol/L, pH 8.0) containing 1 mmol/L EDTA and was stored at -30°C until use.

The genes and SNPs examined are shown in Supplementary Table 1. These genes were selected as possibly playing a role in hepatocarcinogenesis by modifying cell growth, hepatic inflammation, and hepatocyte apoptosis; they include growth factors, growth factor receptors, cytokines/chemokines, cytokine/chemokine receptors, genes related to apoptosis, genes related to interferon signals, and CD81 (a putative HCV receptor gene). SNPs of the selected genes were extracted from the JSNP database (http://snp.ims.u-tokyo.ac.jp),[18] a database for SNPs found in the Japanese population, or were identified using TaqMan Assays-on-Demand SNP Genotyping Products (Applied Biosystems, Foster City, CA). When more than 1 SNP was listed in the database, several SNPs located 10 to 30 kb apart from each other were chosen. Altogether, 393 SNPs derived from 171 genes were tested for an association with HCC in patients with chronic HCV infection. The genotyping was conducted with a fluorogenic polymerase chain reaction.[19, 20] The alleles and genotype frequencies of the SNPs were determined and combined with the clinical data to conduct statistical analyses.

Genotype Analysis. Clinical parameters were evaluated using the two-tailed t test, the Mann-Whitney U test, and the chi-square test to determine their associations with the presence of HCC. The association between different genotypes and the presence of HCC was evaluated using the chi-squared test. For all tests, a P value of less than .05 was considered significant. Possible confounding effects among the variables were adjusted using a multivariate logistic regression model, and odds ratios and 95% confidence intervals were calculated. All data analyses were performed using SPSS v. 12.0 (SPSS Inc., Chicago, IL). The Hardy-Weinberg equilibrium of alleles at individual loci was evaluated using HWE (ftp://linkage.rockefeller.edu/software).

Haplotype Analysis. The haplotype-based analyses consisted of 3 steps: (1) haplotype block partitioning, (2) haplotype reconstruction, and (3) haplotype-based association tests. In the first step, a set of consecutive SNPs was defined as a haplotype block if the P value derived from the logarithmic odds score was less than .01 for every combination of the SNPs. In the second step, for each haplotype block, haplotype frequencies were estimated from the genotypes of unrelated individuals, and the posterior probability distribution of the diplotype configuration for each subject was determined using the LDSUPPORT program,[21] which is based on the expectation-maximization (EM) algorithm. In the third step, an m $\times$ 2 (haplotypes $\times$ with/without HCC) contingency table was built for each haplotype block by counting haplotypes whose posterior probability was higher than 0.8. The empirical P value of the association between the haplotype distribution and HCC presence based on likelihood ratio test statistics was then obtained with 20,000 permutations using the FASTEHPLUS T5 test.[22] P values of less than .05 were considered significant. To identify risk haplotypes, Fisher's exact test was performed on the 2 $\times$ 2 (risk/non-risk haplotypes $\times$ with/without

Table 1. Patient Demographics

Variables	Initial Screening			Secondary Screening			Total		
	Without HCC* (n = 111)	With HCC (n = 77)	P	Without HCC (n = 95)	With HCC (n = 93)	P	Without HCC (n = 206)	With HCC (n = 170)	P
Sex (male)	46 (41%)	43 (56%)	.052*	51 (54%)	68 (73%)	.005*	97 (47%)	111 (65%)	.0004*
Cirrhosis	30 (27%)	56 (73%)	<.0001*	15 (22%)	59 (66%)	<.0001*	45 (25%)	115 (69%)	<.0001*
Alcohol > 80 g/d	6 (6%)	8 (11%)	.235*	0 (0%)	0 (0%)	1.000*	6 (3%)	8 (5%)	.404*
HCV serotype 1	67 (74%)	54 (81%)	.303*	62 (77%)	60 (76%)	.930*	129 (75%)	114 (78%)	.518*
Age (yrs)	62 (27-78)	65 (46-81)	.002†	58 (22-80)	65 (38-84)	<.0001†	60 (22-80)	65 (38-84)	<.0001†
HCV load (IU/mL)	395 (1-1600)	387 (10-1160)	.676†	498 (7-1460)	449 (1-1340)	.116†	436 (1-1600)	428 (1-1340)	.419†
Albumin (g/dL)	4 (2.3-4.7)	3.6 (2.6-4.7)	<.0001†	4.1 (3.3-4.8)	3.7 (2.3-4.5)	<.0001†	4 (2.3-4.8)	3.6 (2.3-4.7)	<.0001†
TB (mg/dL)	0.7 (0.2-2.3)	0.9 (0.3-2.2)	<.0001†	0.7 (0.3-1.8)	0.9 (0.3-3.3)	<.0001†	0.7 (0.2-2.3)	0.9 (0.3-3.3)	<.0001†
ALT (U/L)	70 (9-341)	58 (17-261)	.058†	61 (10-340)	65 (13-234)	.187†	66 (9-341)	63 (13-261)	.667†
AFP (ng/mL)	6 (2-425)	40 (3-3222)	<.0001†	6 (1-256)	31 (3-6107)	<.0001†	6 (1-425)	36 (3-6107)	<.0001†
PT (%)	84 (35-100)	75 (48-100)	<.0001†	84 (56-100)	71 (40-100)	<.0001†	84 (35-100)	72 (40-100)	<.0001†
Platelet count ($\times 10^4/\mu L$)	14.9 (2.6-34.4)	9.9 (2.9-24.6)	<.0001†	16.3 (4.0-29.4)	9.7 (1.7-38.8)	<.0001†	15.3 (2.6-34.4)	9.7 (1.7-38.8)	<.0001†

NOTE. Age, albumin, TB, ALT, AFP, PT, platelet count, and HCV load are shown as median (range). Male sex, alcohol > 80 g/d, and HCV serotype 1 are shown as frequency (percentage). Abbreviations: HCC, hepatocellular carcinoma; TB, total bilirubin; ALT, alanine aminotransferase; AFP, alpha fetoprotein; PT, prothrombin time; HCV, hepatitis C virus; IU, international unit.

*P values were calculated using the χ^2 test.

†P values were calculated using the Mann-Whitney *U* test.

HCC) contingency table for each haplotype in the significant blocks.

Quantification of Small Inducible Cytokine B14 Precursor mRNA Expression. Small inducible cytokine B14 precursor (SCYB14) mRNA expression was quantified in the cancerous and noncancerous liver tissues of 3 patients (53-year-old man, 59-year-old man, and 57-year-old woman) with chronic HCV infection who had been admitted to the University of Tokyo Hospital for surgical treatment of HCC. The diagnosis of HCC was made using several imaging methods and was confirmed histologically. Written informed consent according to the guidelines of the Helsinki Declaration was obtained from each patient. Approval for the study was obtained from the institutional ethics committee.

Total RNA samples were extracted from cancerous and noncancerous liver tissues using the Isogen RNA Extraction kit (Nippon-Gene, Tokyo, Japan) according to the manufacturer's instructions. The quality of the total RNA was judged from the ratio of the 28S to 18S ribosomal RNA after agarose gel electrophoresis. Total RNA was reverse transcribed to cDNA using Taqman Reverse Transcription reagents (Applied Biosystems). SCYB14 cDNA was then quantified in triplicate using the Assay-on-Demand Gene Expression product (Applied Biosystems) with the ABI PRISM 7000 sequence detection system (Applied Biosystems). As an endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was quantified in a similar manner.

Results

Initial Screening. The characteristics of the group of 188 subjects of the initial screening study for the 393 SNPs are shown in Table 1. No significant difference was found in the proportion of male subjects, alcohol abuse, HCV genotype, viral load, or serum ALT level between the groups of patients with and without HCC. In the group of patients with HCC, the patient age, proportion of patients with cirrhosis, serum total bilirubin level, and serum AFP level were higher; and serum albumin level, prothrombin time, and platelet count were lower, than in the group of patients without HCC.

Of the 393 target SNPs, 390 were successfully identified in patients. Of these 390 SNPs in 171 genes, 31 SNPs in 29 genes were found to be associated with the presence of HCC (Table 2): the genotype frequencies of 11 SNPs (11 genes) and allele frequencies of 15 SNPs (15 genes) were significantly different between the groups of patients with and without HCC. The at-risk alleles of 27 SNPs (27 genes) were associated with the presence of HCC in a dominant or recessive fashion.

Secondary Screening. The characteristics of the group of 188 subjects of the secondary screening study for the 31 SNPs identified in the primary screening are shown in Table 1. There was no significant difference in HCV genotype, viral load, or serum ALT level between the groups of patients with or without HCC. In the group of patients with HCC, patient age, the proportion of male patients, the proportion of patients with cirrhosis, serum total bilirubin level, and serum AFP level were higher,