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 itineration 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.[21] 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 ≥$10\times10^3/mm^3$ (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

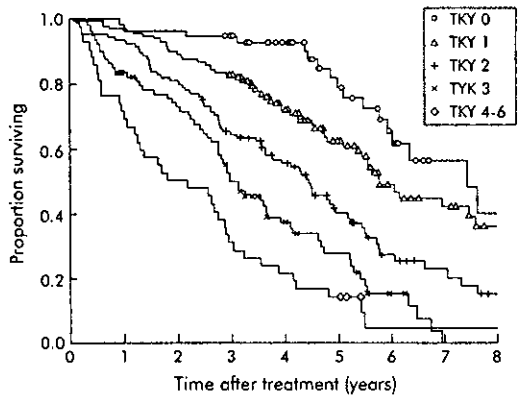
RESULTS

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 11 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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BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Acyclic Retinoid Inhibits Human Hepatoma Cell Growth by Suppressing Fibroblast Growth Factor–Mediated Signaling Pathways

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See editorial on page 228.

Background & Aims: Hepatocellular carcinoma (HCC) is one of the most common human malignancies. Its high mortality rate is mainly a result of high intrahepatic recurrence. The novel synthetic retinoid acyclic retinoid (ACR) has been reported to prevent the recurrence of human HCC after surgical resection of primary tumors, but the molecular mechanisms underlying its effects remain to be elucidated. In this study, we clarified the molecular targets of ACR. *Methods:* The inhibitory effects by ACR on growth were examined. Intracellular signaling induced by ACR was comprehensively studied by a reporter assay. Gene expression changes by ACR were examined using a microarray. From these results, a candidate signaling pathway modulated by ACR was determined and whether antagonizing this pathway reverses the effect was examined. *Results:* We show that ACR inhibits the growth of HCC cells through the down-regulation of fibroblast growth factor (FGF) receptor 3 expression and FGF-mediated signaling, which in turn suppresses the activity of Rho and serum response factor–mediated transcription. Conversely, overexpression of the active form of FGF receptor 3 or the addition of FGF reverses the ACR-mediated inhibition of growth. In addition, silencing the FGF receptor 3 gene by RNA interference inhibits cell growth. *Conclusions:* These studies show that ACR is a potent inhibitor of FGF signaling and that selective blocking of the FGF-mediated pathway could be a promising therapeutic approach for the management of patients with HCC.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, especially in Asian countries.[1,2] Recent progresses in the development of treatment modalities, such as hepatic resection, radiofrequency ablation therapy, transcatheter arterial embolization, and chemotherapy, have improved the immediate prognosis for patients with HCC.[2–5] However, the long-term prognosis of patients with HCC remains disappointing because of frequent recurrences and second primary tumors.[6,7] Therefore, strategies to prevent posttherapeutic recurrence of HCC are needed to improve long-term clinical outcomes.[8]

In 1996, a synthetic retinoid analogue, acyclic retinoid (ACR), was shown to prevent the occurrence of second primary tumors in patients with HCC.[9] In that study, oral administration of ACR for 12 months significantly reduced the recurrence of primary HCC after resection of the initial lesions. In addition, in contrast to other retinoid compounds, ACR showed no significant toxic effects.[9] Because the clinical studies are very promising, it is of interest to examine the underlying molecular effects of ACR on human hepatoma cells.

Recently, it was reported that the ACR-mediated inhibition of HepG2 cell growth was associated with the induction of p21 and the inhibition of cyclin D1 expression.[10,11] Although this may be one mechanism by which ACR inhibits HCC growth, the entire range of effects induced by ACR is yet to be elucidated.

The purpose of this study was to determine the molecular mechanism by which recurrence of HCC is prevented by ACR and to clarify its principal target molecule in comprehensive studies in order to facilitate the development of more effective therapies.

Abbreviations used in this paper: ACR, acyclic retinoid; EGF, epidermal growth factor; FGF, fibroblast growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA; SRE, serum response element; SRF, serum response factor; TGF, transforming growth factor.

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Materials and Methods

Cell Lines

Human liver tumor cell lines (HepG2, HLF, and HuH7) were obtained from the Riken cell bank (Tsukuba Science City, Japan). These cell lines were maintained in Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum in an atmosphere containing 5% CO_2.

Cell Growth Assays

To examine the effect of ACR on cell growth, 5×10^4 cells/well were seeded onto 6-well plates. The medium was changed the next day, and 0, 15, 30, 45, 60, or 90 μmol/L ACR (NIK-333; Nikken-Chemical Co, Tokyo, Japan) was added. To examine the effect that the blocking of fibroblast growth factor (FGF) receptor 3 has on HCC cell proliferation, 1 or 3 μg/mL anti–FGF receptor 3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit immunoglobulin G, a negative control, was added instead of ACR. To examine the effect of tyrosine kinase receptor ligand on cell proliferation, 100 ng/mL of FGF, 100 ng/mL of epidermal growth factor (EGF), 100 ng/mL of transforming growth factor (TGF)-α, and 200 ng/mL of hepatocyte growth factor was added to the medium of HepG2 cells with or without ACR. After 72 hours, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Chemical Co, St. Louis, MO)[12] or using the trypan blue dye exclusion method. The data are reported as the mean ± SD determined from triplicate wells.

Nuclear Extraction and Transcription Factor Array

Nuclear extracts were prepared using mini-nuclear extraction methods.[13] The activation levels of 54 transcriptional factors were compared simultaneously between ACR-treated and untreated HepG2 cells, using TranSignal Protein/DNA Arrays (Panomics, Redwood City, CA) according to the manufacturer's instructions.[14,15]

Reporter Plasmids

A series of vectors containing the *Photinus pyralis* (firefly) luciferase gene driven by a basic promoter element with an inducible *cis*-enhancer element was used as reporter plasmids. The serum response factor (SRF), nuclear factor κB, p53, and serum response element (SRE) luciferase reporters were obtained from Stratagene (La Jolla, CA). The activity of the adenomatous polyposis coli/β-catenin signaling pathway was assessed using pTOPFLASH and pFOPFLASH, generously provided by Dr Hans Clevers (Utrecht University Hospital, Utrecht, The Netherlands).[16] The activity of the FGF receptor 3 promoter was examined using pFR3-luc (B5) and pUFR3-luc, kindly provided by Dr D. M. Ornitz (Washington University Medical School, St. Louis, MO).[17] The pFR3-luc construct contained the 5′-flanking region of the FGF receptor 3 gene, spanning the region from base pair −2951 to base pair −27, and pUFR3-luc contained the region from base pair −2951 to base pair +609. These 2 constructs were used to test the effects of the 5′-untranslated region on gene expression.[17] The reporter plasmid c-fos-luc, kindly provided by Dr T. Ishikawa (University of Tokyo, Tokyo, Japan), contained a human c-fos promoter (base pair −456 to +47) with an SRE binding site.[18] A control plasmid, pCXN2-luc, which expresses luciferase driven by β-actin–based CAG promoter,[19] was also used. Transfection efficiency was monitored by cotransfection of pRL-TK (Toyo Ink, Tokyo, Japan), a control plasmid expressing *Renilla reniformis* (seapansy) luciferase driven by the herpes simplex virus thymidine kinase promoter.

Luciferase Reporter Assay

Separate cultures of HepG2 cells were transiently transfected with .4 μg of each of the reporter plasmids using Effectene transfection reagent (Qiagen, Hilden, Germany) as described previously.[20] ACR was added 8 hours after transfection. To examine the effects of anti–FGF receptor 3 antibodies, the antibodies (1 or 3 μg/mL) were added instead of ACR. The luciferase activities of total cell lysates were measured at 36 hours after transfection using the dual-luciferase reporter assay system (Toyo Ink).

Rho and Rac Activation Assays

Cells were treated with 60 μmol/L ACR for 0, 12, or 24 hours and then harvested. Cell extracts were clarified by centrifugation and were normalized for protein concentration to use 250 μg protein each for the assay with a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). For assessment of total Rho or Rac content, 25 μg (10%) of each extract was used. The remaining extract was reacted with agarose-coupled rhotekin Rho-binding domain or p21-activated kinase-1 binding domain. The precipitates were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis, transferred to Hybond-P (Amersham Pharmacia Biotech, Buckinghamshire, England), and probed with anti-Rho immunoglobulin G or anti-Rac immunoglobulin G, respectively, according to the instructions provided with the detection kits (Cytoskeleton, Denver, CO).

Microarray Procedures

The complementary DNA (cDNA) microarray analyses were performed using 2 microarray systems: (1) an in-house cDNA microarray system designed for analysis of digestive diseases, consisting of 4300 cDNAs derived mainly from liver and gastric tissues and cell lines,[21] and (2) the AceGene Human Oligo Chip 30K subset A (Hitachi Software Engineering Co, Yokohama, Japan), consisting of 10,800 open reading frame oligo probes, most of which have known functions. The microarray procedures were completed following our previously reported protocol[22,23] and the manufacturer's instructions, respectively. Briefly, after treatment with or without ACR for 48 hours, total RNA was isolated from HepG2 cells using the Isogen kit according to the manufacturer's instruc-

tions (Nippon-gene, Tokyo, Japan). Fluorescent cDNA probes labeled with Cy5 or Cy3 were prepared from 30 μg total RNA isolated from ACR-treated or untreated HepG2 cells, respectively. The 2 fluorescently labeled probes were mixed and applied to a microarray followed by incubation under humidified conditions at 60°C (in-house array) or 42°C (AceGene) overnight. Fluorescent images of the hybridized microarrays were scanned with a fluorescence laser confocal slide scanner (Affymetrix 428 Array Scanner; Affymetrix, Santa Clara, CA). The images were analyzed using ImaGene 4.2 software (BioDiscovery, Marina Del Rey, CA) according to the manufacturer's instructions. Full microarray data, following the MIAME guidelines issued by the Microarray Gene Expression Data Group,[24] are deposited and freely available from the Gene Expression Omnibus at the National Center for Biotechnology Information homepage (http://www.ncbi.nlm.nih.gov/geo/).

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction quantification was used to verify the microarray data. Total RNA was reverse transcribed to cDNA by Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Then, FGF receptor 3 cDNA was quantified in triplicate by Assay-on-Demand Gene Expression Product (Applied Biosystems) using the ABI PRISM 7000 sequence detection system (Applied Biosystems). As an endogenous control, glyceraldehyde-3-phosphate dehydrogenase cDNA was quantified similarly.

Western Blots

Cell extracts were normalized for protein concentration, resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and immunoblotted with anti-actin antibody (Chemicon, Temecula, CA) as described previously.[20] The bound antibody was detected using the ECL-Advance Western Blotting Detection System (Amersham Pharmacia Biotech).

Stable Cell Lines

To establish stable HepG2 cell lines expressing FGF receptor 3, HepG2 cells were transfected with pcDNA3 (control) or pcDNA3/myrR3-WT,[25] which expresses the myristylated form of the wild-type FGF receptor 3 (kindly provided by Dr D. J. Donoghue, University of California, San Diego, La Jolla, CA). The transfected cells were incubated in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 400 μg/mL G418 for 2–3 weeks. The surviving cell colonies were then pooled to establish the HepG2/pcDNA3 and HepG2/FGF receptor 3 stable cell lines. For the cell growth assays, stable cells were plated and incubated for 18 hours and then incubated with 60 μmol/L ACR for 4 days.

RNA Interference

RNA duplexes (small interfering RNA [siRNA]) directed against the FGF receptor 3 gene were synthesized (Qiagen). Four sequences were tested for FGF receptor silencing, and the most active oligonucleotide (siFGFR3) was used in this study. The sequence of siFGFR3 was GACGAUGCCACUGACAAGG, which had 2-nt (2′deoxy) uridine 3′ overhangs. Negative control RNA duplexes (Silencer Negative Control siRNA) and RNA duplexes against EGF receptor gene (siEGFR, Silencer Validated siRNA) were purchased from Ambion (Austin, TX). Oligonucleotides (15 μL of 20 μmol/L oligonucleotide solutions) were transfected into HepG2 cells in 12-well dishes with Oligofectamine (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Western blotting and MTT assays were performed at 72 hours after oligonucleotide transfection.

Statistical Analysis

The results of the luciferase assays and the cell counts were analyzed using analysis of variance with a post-hoc Scheffe test (StatView J; Abacus Concepts Inc, Berkeley, CA). Differences with P values of $<.05$ were considered significant.

Results

ACR Reduces the Growth of Human Hepatoma Cells

Because Muto et al had reported that an ACR prevented the occurrence of second primary tumors in patients with HCC,[9] we evaluated the function of ACR on liver tumor cell growth in vitro. ACR exerted a dose-dependent inhibition of the growth of HLF, HuH7, and HepG2 human hepatoma cell lines, with median inhibitory concentrations of 10 μmol/L, 45 μmol/L, and 45 μmol/L, respectively (Figure 1 and data not shown), which were consistent with a previous report.[10] The growth inhibitory effect was not observed until 48 hours after the addition of ACR (data not shown). Because HepG2 cells were well characterized in previous studies, they were used for additional investigations.

Identification of ACR-Mediated Signal Transduction Pathways

To clarify the mechanism by which ACR inhibits liver tumor cell growth, we comprehensively determined the transcriptional pathways modulated by ACR using protein/DNA arrays and luciferase reporter assays. The activities of 54 transcription factors were examined in parallel using array-based technology,[15,25] and 4 pathways (nuclear factor κB, p53, adenomatous polyposis coli/β-catenin, and SRF) were examined using luciferase reporter assays. Among the 4, only the activity of the SRF pathway was significantly reduced by ACR (Figure 2A and data not shown). Because the SRE is known to be composed of a CArG box, which binds the SRF, and a nearby Ets motif, which binds ternary complex factors such as Elk1,[26,27] we next examined the effect of ACR on SRE-driven promoter activity using a synthetic reporter plasmid containing 5 repeats of SRE and a reporter plasmid contain-

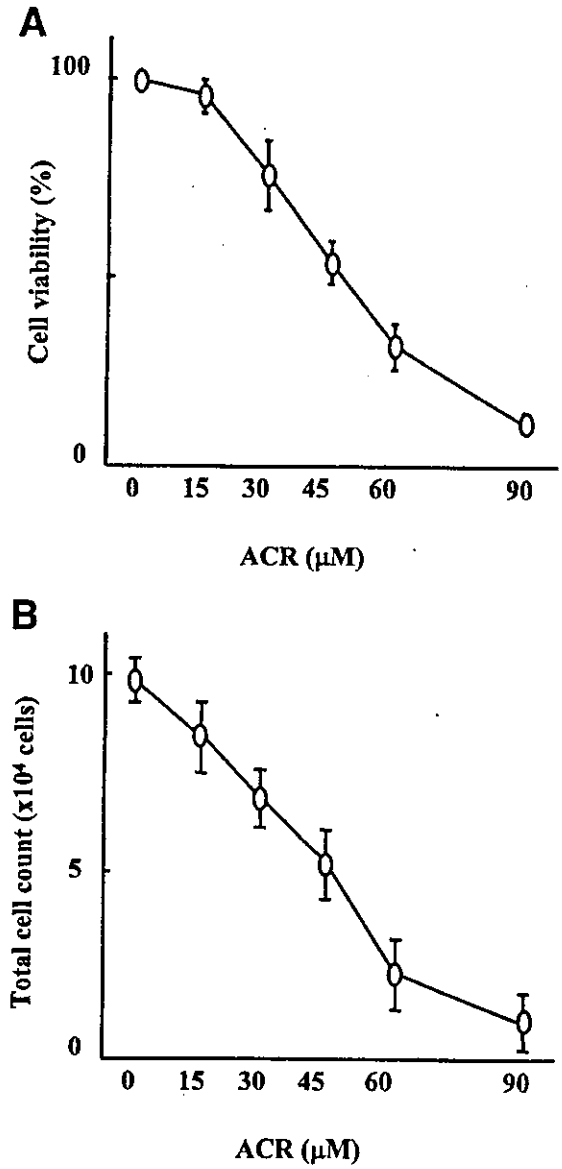

Figure 1. ACR reduces the growth of HCC cells. HepG2 cells were plated at a concentration of 5 × 10^4 cells/well in 6-well plates. Cells were treated with 0, 15, 30, 45, 60, or 90 μmol/L ACR at 24 hours after plating. After 4 days, cell growth was determined by (*A*) MTT assay or (*B*) counting cell numbers, as described in Materials and Methods. The data are shown as the mean (*bar*) ± SD (*line*) of 3 independent experiments.

ing the proto-oncogene c-fos promoter and SRE.[28] As shown in Figure 2*B*, ACR markedly inhibited not only synthetic SRE-driven promoter activity but also c-fos promoter activity.

ACR Inhibits Rho Activation

Because SRF activation is known to be mediated by members of the Rho family,[29] we determined whether ACR affects the levels of Rho A and Rac activity. As shown in Figure 3, ACR inhibited the activation of Rho A. In contrast, the activity of Rac was not changed by ACR (data not shown).

ACR Down-regulates Expression of FGF Receptor 3

To determine the mechanisms underlying the observed effects of ACR, we performed microarray experiments to identify changes in the expression of cellular genes after treatment with ACR. Of the genes investigated, the expression levels of approximately 6% were modulated by ACR when significant change was defined as a greater than 2-fold increase or decrease. (Full microarray data are deposited in the Gene Expression Omnibus at the National Center for Biotechnology Information. The platform ID numbers are GPL 511 and GPL 787, and the sample ID numbers are GSM13794 and GSM 13795.) Among the modulated genes, we focused our analyses on those associated with Rho activity. The expression of FGF receptor 3 was significantly down-regulated; the expression of FGF receptor 3 in ACR-treated cells was 16% of that in the control cells by our in-house microarray and 32% by a commercial oligonucleotide array. Because basic FGF is known to activate the Rho-SRF pathway,[30–32] we then further examined the relationship between FGF receptor 3 expression and the effects of ACR. To confirm the modulation of FGF receptor 3 expression by ACR at the messenger RNA and protein levels, real-time polymerase chain reaction was performed on HepG2 cell samples at 0, 12, 24, and 48 hours after the addition of ACR (Figure 4*A*). Samples were harvested at 0, 24, 48, and 72 hours after the addition of ACR and analyzed by Western blotting using an anti–FGF receptor 3 antibody (Figure 4*B*). We found a marked decrease in both the messenger RNA and protein expressions of FGF receptor 3 within 24 hours, which persisted up to 72 hours (Figure 4*A* and *B*).

To determine whether the ACR-induced changes in expression levels are regulated at the transcriptional or posttranscriptional level, the modulation of the activity of the FGF receptor 3 promoter by ACR was examined using reporter plasmids. We found that ACR down-regulates the activity of the FGF receptor 3 promoter in a dose-dependent manner (Figure 4*C*). This effect was mainly dependent on the promoter region itself, rather than the 5′ untranslated region of the FGF receptor gene, because ACR inhibited the luciferase activity generated by pFR3-luc (containing only base pairs −2951 to −27) to the same extent as that generated by pUFR3-luc (containing base pairs −2951 to +609).

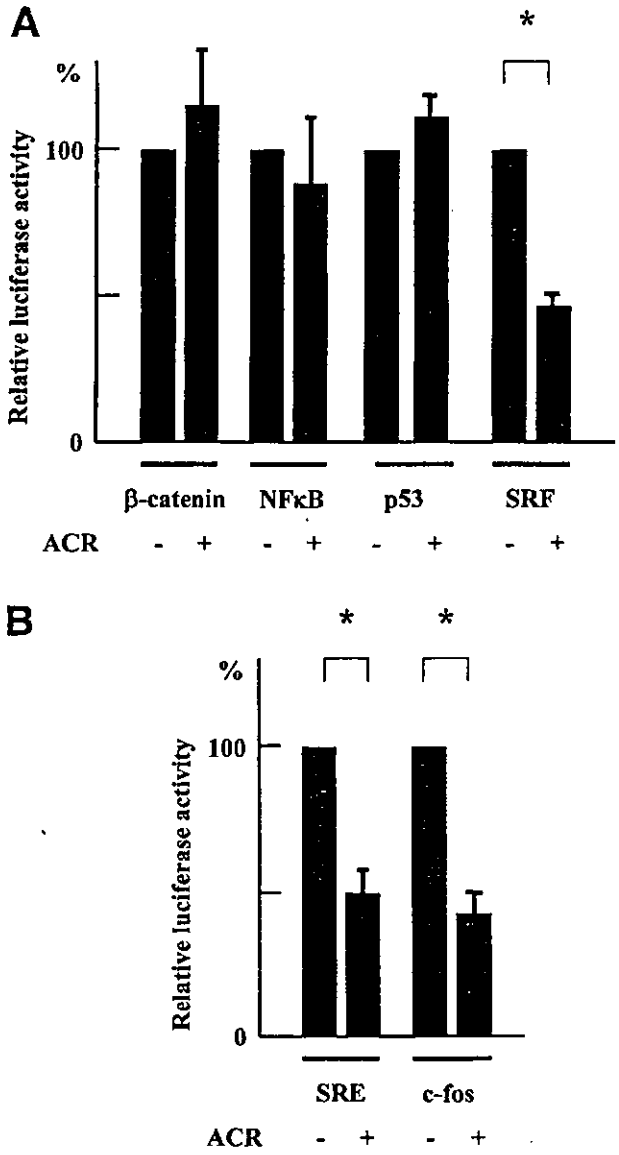

Figure 2. ACR inhibits the SRF-mediated signaling pathway. The luciferase activity of total cell lysates was measured using a dual-luciferase reporter assay system. Normalized luciferase activities are shown, using the activity of transfected cells without ACR treatment as 100%. The results are expressed as the mean ± SD of at least 3 experiments. ACR was added at 8 hours after transfection. $*P < .05$. (*A*) HepG2 cells were transiently transfected with β-catenin–, nuclear factor κB–, p53-, or SRF-driven luciferase reporter plasmids. (*B*) The reporter vectors, containing either 5 repeats of SRE or the c-fos promoter conjugated with the luciferase gene, were transfected into HepG2 cells.

Overexpression of Activated FGF Receptor 3 Reverses the Effects of ACR

Because FGF receptor 3 transduces stimulation by basic FGF,[33,34] which is a ligand that regulates cellular proliferation, differentiation, and motility, we examined whether the overexpression of activated FGF receptor 3 would neutralize the effects of ACR. To this end, we constructed a stable HepG2 cell line that overexpressed the membrane-localized wild-type kinase domain of the FGF receptor 3, which acts as a constitutively active form of FGF receptor 3.[25] As shown in Figure 5, the overexpression of the active form of FGF receptor 3 reversed the inhibitory effects of ACR on cell growth. This result suggested that the effects of ACR might be mediated mainly by the inhibition of the FGF pathway through the down-regulation of FGF receptor 3 expression.

Addition of FGF Reverses the Effects of ACR

Because FGF is a ligand of FGF receptor 3, we examined whether the addition of FGF to the medium of HepG2 cells would neutralize the effects of ACR. As a control, the addition of EGF, TGF-α, and hepatocyte growth factor was also examined. As shown in Figure 6, only the addition of FGF reversed the inhibitory effects of ACR on cell growth. Again, this result suggested the effects of ACR might be mediated by the inhibition of the FGF pathway.

Effects of Anti–FGF Receptor 3 Antibody on HCC Cell Proliferation

To examine whether FGF receptor 3–mediated pathways play a major role in the proliferation of HCC cells, cell proliferation assays were performed after adding anti–FGF receptor 3 antibodies to the culture medium. As shown in Figure 7, this anti–FGF receptor antibody functions as an antagonist, resulting in the suppression of SRF activation and cell proliferation similar to the effects of ACR. In cells cultured with 3 μg/mL of anti–FGF receptor 3 antibodies, SRF activation was reduced to 55.7% of control levels and cell numbers were decreased to 51.8% relative to controls. These results support the notion that FGF receptor 3–mediated sig-

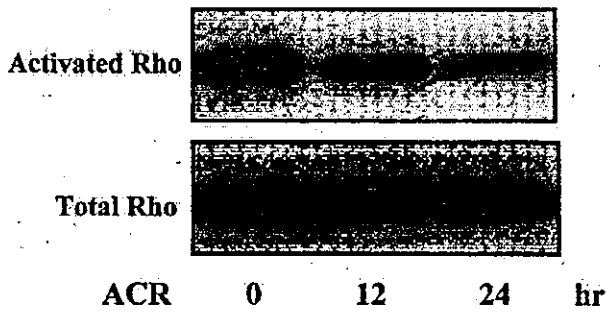

Figure 3. ACR inhibits Rho A activity. HepG2 cells were treated with 60 μmol/L ACR for 12 or 24 hours. Cells were harvested and cell extracts were assayed for Rho activity as described in Materials and Methods. Representative immunoblots of total and active Rho A are shown.

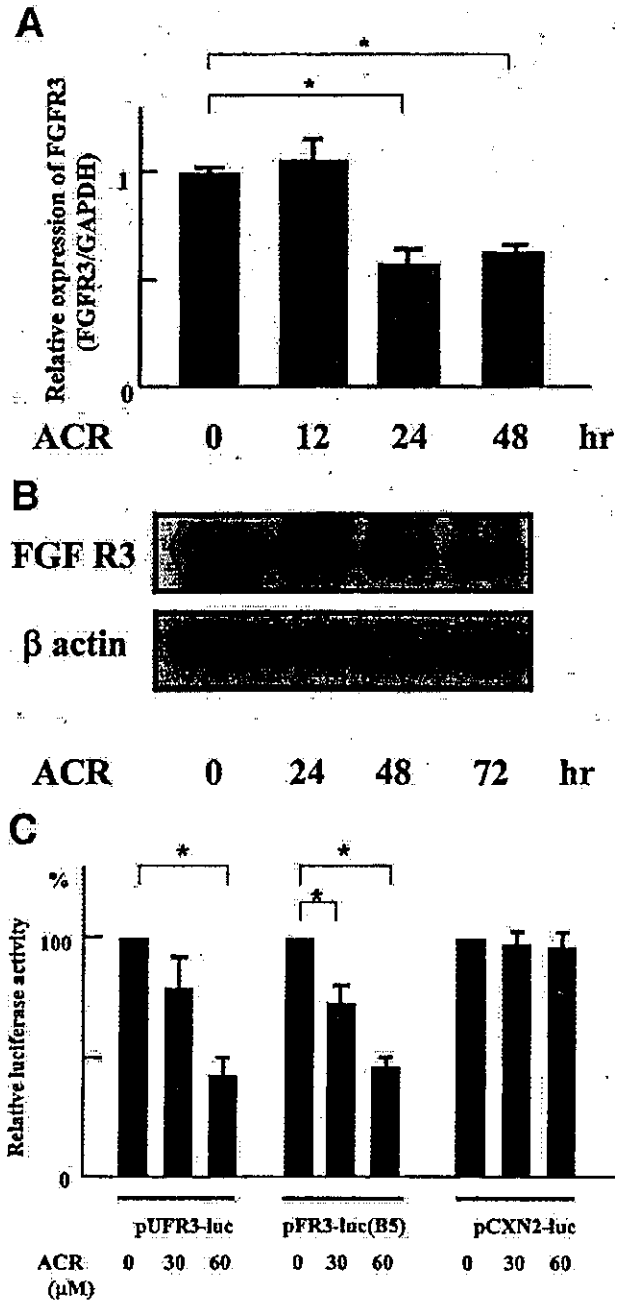

Figure 4. ACR decreases the expression of FGF receptor 3. (*A*) HepG2 cells were treated with ACR (60 μmol/L) for 0, 12, 24, and 48 hours and subjected to real-time polymerase chain reaction analyses for the expression of FGF receptor 3 messenger RNA. The results are expressed by taking the relative amount of FGF receptor 3 expression normalized by glyceraldehyde-3-phosphate dehydrogenase expression without ACR as 1. Data represent the mean ± SD of 3 experiments. *$P < .05$. (*B*) HepG2 cells were treated with ACR (60 μmol/L) for 0, 24, 48, and 72 hours and subjected to Western blot analysis for the expression of FGF receptor 3 protein. (*C*) HepG2 cells were transiently transfected with pFR3-luc, pUFR3-luc, or pCXN2-luc reporter plasmids. ACR was added at 8 hours after transfection, and luciferase activities were measured at 36 hours after transfection. Normalized luciferase activities are shown, setting the activity without ACR treatment as 100%. The results are expressed as the mean ± SD of at least 3 experiments. *$P < .05$.

naling pathways are essential for the proliferation of HCC cells and that ACR down-regulates these pathways.

FGF Receptor 3 Gene Silencing Reduces Growth of HepG2 Cells

To further confirm the role of FGF receptor 3 in the proliferation of HCC cells, cell proliferation assays were performed after posttranscriptional silencing of the FGF receptor 3 gene using RNA interference. As shown in Figure 8, the successful knockdown of FGF receptor 3 protein expression resulted in the suppression of cell proliferation. In contrast, the successful knockdown of EGF receptor protein expression resulted in no suppression of cell proliferation. These results further support the finding that FGF receptor 3–mediated signaling pathways are closely associated with the proliferation of HCC cells.

Discussion

ACR is the object of increasing interest because it has been shown to prevent the recurrence of human HCC after treatment of primary tumors.[35] However, the underlying mechanisms of the clinical effects have not been fully elucidated. In this study, we found that ACR inhibits FGF receptor 3 expression, Rho activity, and SRF-mediated transcription, resulting in the inhibition

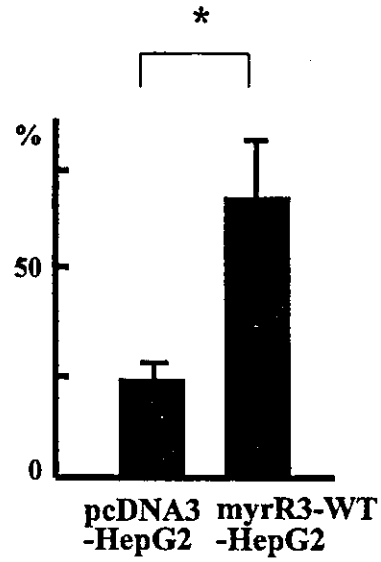

Figure 5. The antiproliferative effect of ACR is neutralized by the overexpression of FGF receptor 3. HepG2 cells stably expressing the active form of FGF receptor 2 (plasmid myrR3-WT) and the control (pcDNA3) were treated with ACR (60 μmol/L). After 4 days of treatment, the cell number was estimated by the MTT assay. The results are expressed as the percentage of cell growth compared with that of untreated HepG2 cells. The means ± SD of quadruplicate determinations from 3 separate experiments are shown. *$P < .05$.

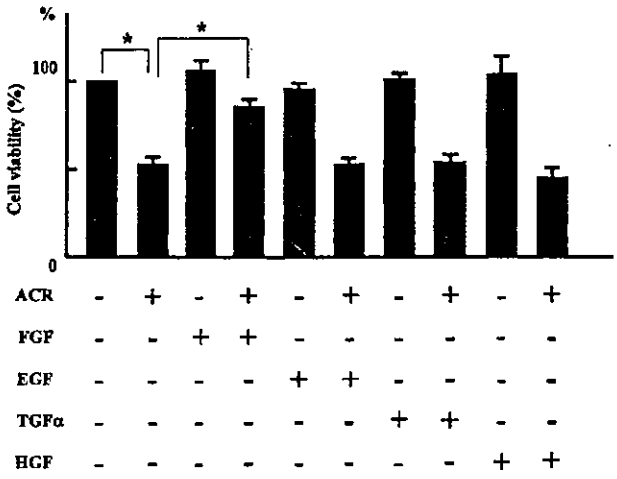

Figure 6. The antiproliferative effect of ACR is neutralized by the addition of FGF. FGF, EGF, TGF-α, or hepatocyte growth factor was added to the medium of HepG2 cells with or without ACR. After 4 days, the cell number was estimated by the MTT assay. The results are expressed as the percentage of cell growth compared with that of untreated HepG2 cells. The mean ± SD of triplicate determinations from 3 separate experiments are shown. $*P < .05$.

of tumor cell growth. Conversely, these effects were completely neutralized by the overexpression of activated FGF receptor 3 or the addition of FGF. Therefore, the mechanism of tumor inhibition by ACR might be in large part attributable to the blocking of the FGF signaling pathway.

ACR is a very promising anticancer agent because it is known to inhibit chemically induced hepatocarcinogenesis in rats, spontaneously occurring hepatoma in mice, and the recurrence of primary hepatocarcinoma in humans.[9] Recently, it was reported that the ACR-induced growth inhibition of human hepatoma cells was associated with the inhibition of cyclin D1 expression.[10] Indeed, our microarray study showed that cyclin D1 was suppressed about 82% at 12 hours after the addition of ACR compared with that in the control cells (data not shown). Although the down-regulation of cyclin D1 expression might be associated with the inhibition of tumor growth, it is not known whether this is a direct effect of ACR. It has been reported that basic FGF up-regulates the expression of cyclin D1.[36] c-fos is also known to activate cyclin D expression, and its binding site exists in the promoter of cyclin D1.[37] Therefore, the down-regulation of the FGF pathway by ACR might lead to down-regulation of cyclin D1; thus, the down-regulation of the FGF pathway is believed to be a primary underlying mechanism of the effects of ACR.

We showed that ACR inhibits FGF receptor 3 promoter activity and expression. The FGF family is a group of structurally related, multifunctional, mitogenic polypeptides that were originally identified as an activity that stimulated the proliferation of NIH3T3 cells.[38] Among the FGF family, basic FGF acts as a potent mitogen and as a differentiation factor.[39] To date, the expression of basic FGF and FGF receptors has been identified in epithelial cells, endothelial cells, fibroblasts, macrophages, and extracellular matrix in various organs, and the presence of basic FGF and FGF receptors has been confirmed in various tumor tissues, where they

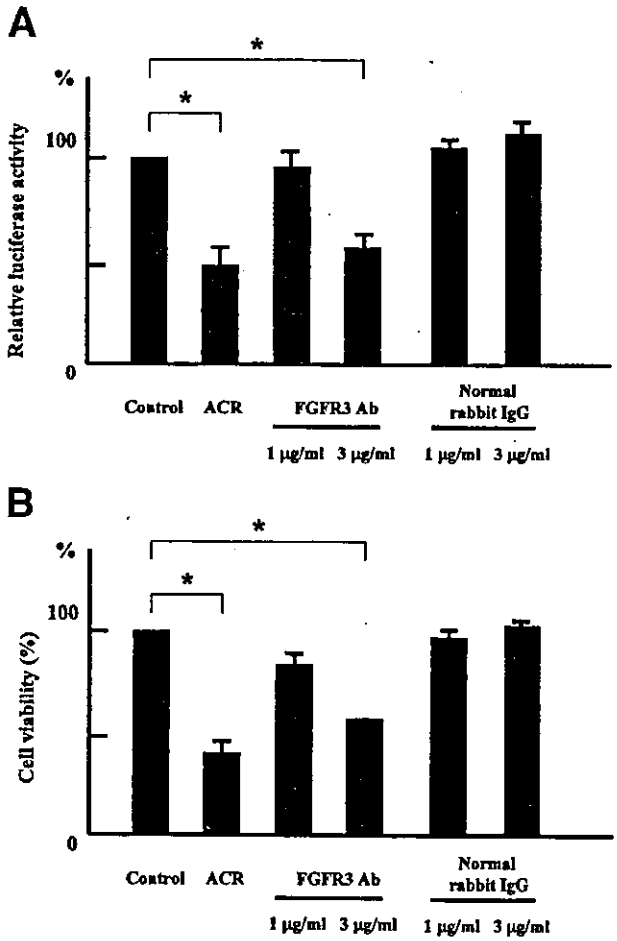

Figure 7. The effects of anti–FGF receptor 3 antibody on HCC cells. (*A*) HepG2 cells were transiently transfected with SRF-driven reporter plasmids. ACR (60 μmol/L), anti–FGF receptor 3 antibodies (1 or 3 μg/mL), or normal rabbit immunoglobulin G (1 or 3 μg/mL) were added at 8 hours after transfection. The luciferase activity of total cell lysates was measured at 36 hours after transfection using the dual-luciferase reporter assay system. Normalized luciferase activities are shown, setting the activity without treatment as 100%. The results are expressed as the means ± SD of at least 3 experiments. $*P < .05$. (*B*) HepG2 cells were treated with ACR (60 μmol/L), anti–FGF receptor 3 antibodies (1 or 3 μg/mL), or normal rabbit immunoglobulin G (1 or 3 μg/mL) at 24 hours after plating. After 4 days, cell growth was determined using the MTT assay. The results are expressed as the percentage of growth relative to that of untreated cells. The means ± SD of quadruplicate determinations from 3 separate experiments are shown. $*P < .05$ vs control.

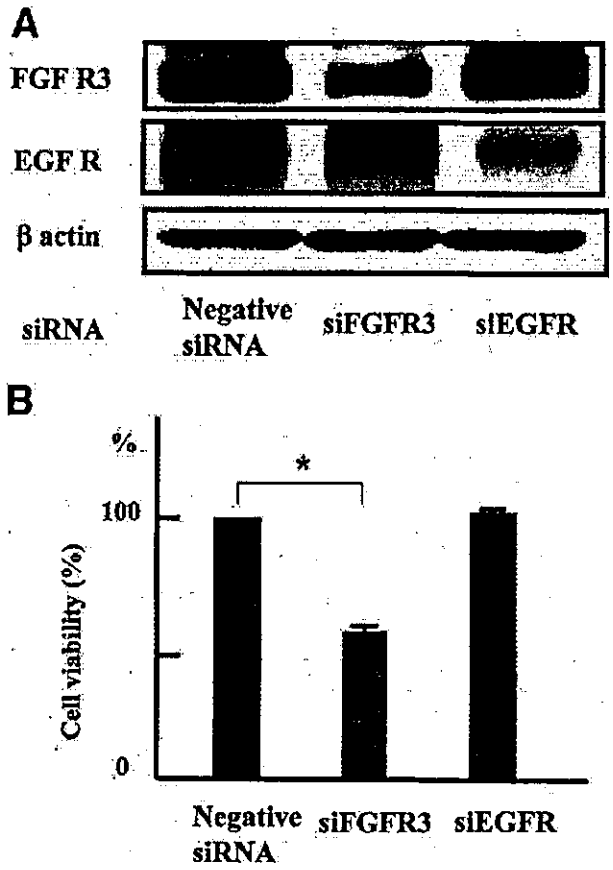

Figure 8. FGF receptor 3 gene silencing reduces HepG2 cell growth. (*A*) HepG2 cells were transfected with siRNA against FGF receptor 3 (siFGFR3), EGF receptor (siEGFR), or negative control siRNA. Cells were harvested at 72 hours after transfection, protein concentrations were normalized, and samples were analyzed by Western blotting using antibodies to FGF receptor 3, EGF receptor, and β-actin. (*B*) HepG2 cells were transfected with siRNA (siFGFR3, siEGFR, or negative control siRNA). After 72 hours, cell growth was determined using the MTT assay. The results are expressed as the percentage of growth in cells treated with siFGFR3 or siEGFR compared with that of cells treated with a negative control siRNA. The data represent the results of 3 separate experiments. $*P < .05$.

contribute to tumor infiltration and metastasis.[40–42] Similarly, in HCC, basic FGF and its receptors are necessary for proliferation.[43–45] The subcutaneous injection of anti-basic FGF antibody in nude mice transplanted with HCC cells suppresses tumor growth.[43] We also showed here that RNA interference targeted to FGF receptor 3 inhibits HCC cell growth. Therefore, FGF signaling must be an essential pathway for HCC tumor growth. Although the results of this study indicate that ACR could be a very promising drug for the treatment of patients with HCC because of its inhibitory effect on the FGF signaling pathway, this drug also had a moderate inhibitory effect on the proliferation of normal hepatocytes (median inhibitory concentration, 75 μmol/L; data not shown). Therefore, the development of more specific, more effective, and safer inhibitors of FGF signaling that act selectively on cancer cells may be an effective strategy for the treatment of patients with HCC.

Our study showed that RNA interference with FGF receptor 3 expression effectively suppresses HCC cell proliferation. RNA interference is emerging as a strategy for the highly specific suppression of gene expression, both in vitro and in vivo.[46] Our findings indicate that RNA interference targeted to FGF receptor 3 may become a promising therapeutic option for HCC in the future.

During the preparation of this report, it was shown that ACR inhibits the expression of TGF-α and cell proliferation.[47] In this study, our microarray analysis showed that the expression of TGF-α was about 89% at 12 hours after the addition of ACR compared with that in the control cells (data not shown). However, the mechanism of the inhibition of TGF-α expression by ACR is unknown and, more importantly, as the investigators stated in their report, it should be clarified whether this phenomenon is the cause or the result of the suppression of HCC proliferation.

We found that ACR down-regulated FGF receptor promoter activity, but how ACR exerts its effects on the promoter remains to be elucidated. It was reported that ACR binds to the cellular retinoic acid-binding protein, but the molecular effects of ACR are not necessarily dependent on this binding.[48] ACR, which is a lipid-soluble compound like corticosteroids, might bind to proteins in the nucleus and modulate gene-specific transcription. The identification of specific proteins that bind ACR is an interesting issue to pursue.

In summary, our data show that ACR inhibits FGF receptor 3 expression and suppresses Rho activity and SRF-mediated transcriptional activation. This might be a mechanism by which ACR exerts its inhibitory effect on tumor cell growth, resulting in the prevention of secondary tumors after the treatment of primary HCC. In the future, the use of blockers or modulators of the FGF signaling pathway, such as ACR, could be a promising approach to the treatment of patients with HCC.

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Percutaneous Radiofrequency Ablation for Hepatocellular Carcinoma

An Analysis of 1000 Cases

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BACKGROUND. Radiofrequency ablation (RFA) was introduced recently as a therapeutic modality for hepatocellular carcinoma (HCC), an alternative to percutaneous ethanol injection therapy (PEIT), which is coming into use worldwide. Previously reported treatment efficacy and complication rates have varied considerably.
METHODS. Between February 1999 and February 2003, the authors performed 1000 treatments of RFA to 2140 HCC nodules in 664 patients with a cooled-tip electrode at the University of Tokyo Hospital (Tokyo, Japan). Short-term and long-term complications were analyzed by treatment and session basis. Cumulative survival was also assessed in 319 patients who received RFA as primary treatment (naive patients) and 345 patients who received RFA for recurrent tumor after previous treatment including resection, PEIT, microwave coagulation therapy, and transarterial embolization (nonnaive patients).
RESULTS. A total of 40 major complications (4.0% per treatment, 1.9% per session) and 17 minor complications (1.7% per treatment, 0.82% per session) were observed during the observation period until March 31, 2004. There were no treatment-related deaths. Surgical intervention was required in one case each of bile peritonitis and duodenal perforation. The cumulative survival rates at 1, 2, 3, 4, and 5 years were 94.7%, 86.1%, 77.7%, 67.4%, and 54.3% for naive patients, whereas the cumulative survival rates were 91.8%, 75.6%, 62.4%, 53.7%, and 38.2% for nonnaive patients, respectively.
CONCLUSIONS. The authors confirmed the safety and efficacy of RFA for HCC in a large-scale series and long-term prognosis was satisfactory. *Cancer* **2005;103: 1201–9.** © *2005 American Cancer Society.*

KEYWORDS: hepatocellular carcinoma, interventional radiology, postoperative complications, survival analysis, cirrhosis.

Hepatocellular carcinoma (HCC) is a common malignancy worldwide, with an increasing incidence in the United States.[1,2] Current options for the treatment of this cancer consist of surgical resection, transcatheter arterial embolization (TAE), and percutaneous ablation therapy. Although surgical resection usually is considered to be the first-choice treatment,[3,4] it is not infrequently contraindicated by underlying chronic liver diseases based on hepatitis B or C virus (HCV) infection.[5,6] Orthotopic liver transplantation (OLT) is a strategy that can treat both cancer and liver dysfunction, and indeed has shown excellent survival in patients at an early stage of the cancer (e.g., a single nodule of ≤ 5 cm in dimension or < 3 nodules of ≤ 3 cm in dimension).[7,8] However, with an increasing demand for donor tissue but a limited supply, the waiting time for an OLT is now > 1 year in Europe and the United States.[8,9] TAE is a widely performed

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procedure for patients with multiple hypervascular nodules. However, complete necrosis rarely is achieved.[10] Percutaneous ethanol injection therapy (PEIT) continues to play an important role in the treatment of small HCCs, and is considered to be a bridge treatment for OLT. Long-term outcomes after PEIT, such as 5-year survival, are comparable to those of surgery.[11,12] However, the efficacy of PEIT is dependent on tumor size, and a local tumor progression rate of > 20% was reported for tumors > 3 cm.[13,14]

Radiofrequency ablation (RFA) is a recently introduced alternative technique to PEIT and is rapidly gaining use worldwide.[15–19] An area of ≤ 3 cm in diameter can be ablated with a single application of RFA.[20,21] The predictability of the ablation area is one of the merits of RFA compared with PEIT. The treatment efficacy and complication rate of RFA have been described in various studies.[22,23] We have performed 1000 treatments of RFA to 2140 HCC nodules in 664 patients and we assessed the early and late complication rates of RFA as well as long-term outcomes in this large case series.

MATERIALS AND METHODS

Patients

Since we introduced RFA to our department in February 1999, we had treated 664 patients with HCC by RFA in 4 years by February 28, 2003. All those patients were included in the current study. Ablation therapy was used either because patients were considered not to be suitable for resection (n = 419) by consideration of liver function impairment, number and distribution of the tumors, and cardiopulmonary dysfunction, or because they voluntarily preferred ablation with informed consent (n = 245) despite surgery also being feasible. Inclusion criteria for RFA were as follows: total bilirubin concentration < 3 mg/dL, platelet count ≥ $5 \times 10^5/mm^3$, and prothrombin activity ≥ 50%. Ascites should be controlled beforehand by diuretics. Patients with portal vein tumor thrombosis or extrahepatic metastasis were excluded. We also excluded patients who had a history of bilioenteric anastomosis or sphincterotomy that are considered as high risk for hepatic abscess formation. Written informed consent was obtained from all enrolled patients, and the protocol was approved by the ethics committee of the University of Tokyo Hospital (Tokyo, Japan).

Technical Terms

We defined a session as a single intervention episode that consists of one or more ablations performed on one or more tumors and a treatment as the completed effort to ablate one or more tumors that consists of one or more sessions according to the working party report on image-guided tumor ablation.[24]

Diagnosis of Hepatocellular Carcinoma

HCC was diagnosed based on typical findings by ultrasonography and computed tomography (CT) scans (hyperattenuation in the arterial phase and hypoattenuation in the portal-venous phase). The diagnosis of HCC also was confirmed histopathologically with ultrasound-guided biopsy in 302 of 319 naive patients. Ultrasonography was performed with a 3.5–6.0-MHz convex probe using an Aloka SSD-5500 (Aloka, Tokyo, Japan) or Toshiba SSA-370A machine (Toshiba Medical Systems, Tokyo, Japan). The dimension of the tumor nodule was measured by visualization of the largest dimension of the tumor.

Using dual-phase dynamic CT scans, arterial and portal phase images were obtained 31–33 seconds and 120 seconds, respectively, after the initiation of contrast material injection. Spiral CT scans were performed with 5-mm–thick sections at a table feed speed of 5–7 mm/sec. Using CT scans during arterial portography, scanning began 30 seconds after contrast medium injection. Using CT scans during hepatic arteriography, images were obtained after injection of 30 mL of contrast medium at a rate of 1.2 mL/sec. When > 3 hypervascular nodules were detected or the largest nodule was > 3 cm on these examinations, we subsequently performed TAE. Immediately after injection of 2–10 mL of iodized oil (Lipiodol Ultra-Fluid, Shering Japan, Osaka, Japan), feeding arteries were embolized selectively with gelatin sponge particles (Spongel, Yamanouchi Pharmaceutical Co., Tokyo, Japan). Otherwise, 1–2 mL of iodized oil only was injected to intensify the radiologic visibility of the target nodules. In February 2003, we changed the size criterion for performing TAE from > 3 cm to > 2 cm in diameter.

Specimens for histologic evaluation were obtained by ultrasound-guided needle biopsy using a 20-gauge needle (Bard Monopty, C.R. Bard, Inc., Covington, GA). Histopathologic grading of tumor differentiation was done according to the criteria of Edmondson and Steiner.[25]

Electrode Insertion and Radiofrequency Ablation

A 17-gauge, cooled-tip electrode was inserted under real-time ultrasound guidance. For the intercostal approach, we adjusted the operating table so that patients had a rotated or head-up position to allow for electrode insertion as square to the thoracic wall as possible. For the subcostal approach, patients usually remain in the head-up position to allow the electrode to be inserted without the patient taking a deep

breath. We also utilized an intrapleural infusion technique when the tumor was located adjacent to the diaphragm to visualize the entire image of the tumor.[26] A glucose solution (5%, 500 mL) was infused into the right pleural cavity under ultrasonographic guidance before needle insertion.

An electrode with a 2-cm or 3-cm exposed tip was connected to a 500-kHz RF generator (Radionics, Burlington, MA), which produces 200 W at 50Ω of impedance.[20,27] The equipment also allows the measurements of generator output, tissue impedance, and electrode tip temperature. A tip temperature of 10–20 °C is maintained by a peristaltic pump infusing chilled saline solution. After insertion of the electrode into the lesion, we started ablation at 60 W for the 3-cm exposed tip and 40 W for the 2-cm exposed tip. The power was increased to 140 W at a rate of 20 W/min. When a rapid increase in impedance was observed during thermal ablation, we minimized the output for 15 seconds and restarted the emission at a lower output.[28] The duration of a single ablation was 12 minutes for the 3-cm electrode and 6 minutes for the 2-cm electrode. After RF exposure, the pump was stopped and the temperature of the needle tip was measured. When the temperature was < 65 °C, additional ablation was performed. When the target nodule was > 2 cm in diameter, we performed multiple ablations. When the total ablation time in a treatment was > 60 minutes, we divided a treatment into ≥ 2 sessions in consideration of burden to the patient.

Antibiotics were administered before and after the procedure on the treatment day and on the morning of the next day. We continued to administer antibiotics when the patients had a fever > 37.5 °C.

Assessment of Treatment Efficacy and Follow-Up

After 1–2 sessions of RFA, dynamic CT scans with section thickness of 5 mm were performed to evaluate the ablation. Complete ablation of HCC was defined as hypoattenuation of the lesion including the surrounding liver parenchyma. Patients received additional sessions of RFA until the treatment was judged as complete. Follow-up consisted of monthly blood tests and monitoring of tumor markers at the outpatient clinic, and ultrasonography and dynamic CT scans were performed every 3–4 months. Intrahepatic HCC recurrence was classified as either tumor recurrence at a site distant from the primary tumor or adjacent to the treated site (local tumor progression). If HCC recurrence was suspected, further examinations including dynamic CT scans and CT angiography were performed. When recurring HCC tumors were identified, RFA was performed if the same initial inclusion criteria were again satisfied.

Complications were assessed on the basis of the number of treatments and sessions. Major complications were defined as those that, if left untreated, might threaten the patient's life, lead to substantial morbidity and disability, or result in hospital admission or substantially lengthen hospital stay according to the previously described guidelines.[24] All other complications were considered minor. Complications were classified into 3 categories according to the time after the last ablation: immediate complications (≤ 6–24 hours after the procedure), periprocedual complications (within 30 days), and delayed complications. In assessing delayed complications, follow-up was censored either on March 31, 2004 or in the event of death.

Survival analysis was performed on patient basis. Survival time was defined as the interval between the first RFA and the death or the last visit to outpatient clinic until March 31, 2004. Six-hundred sixty-four patients were divided into 2 groups: 319 patients who received RFA as the initial treatment for HCC (naive patients) and 345 patients who received RFA as a treatment for tumor recurrence after primary treatments including hepatic resection, PEIT, percutaneous microwave coagulation therapy (PMCT), and TAE. A cumulative survival curve was plotted using the Kaplan–Meier method. Survival was also assessed in naive patients among subgroups divided by age, gender, etiology of background liver disease, Child-Pugh class, tumor size and number, pathologic grade of tumor, and tumor markers. Difference between these subgroups was tested by the log-rank test.

Tumor recurrence was analyzed among 306 of the 319 naive patients, excluding 13 in whom several nodules were left untreated with the initial RFA therapy. The observation period for tumor recurrence was defined as the interval between the first RFA and either of the detection of tumor recurrence, death, or the last visit before March 31, 2004, whichever came first. As death and tumor recurrence are competing risks, we used cumulative incidence estimation with competing risks for the analysis of cumulative tumor recurrence as described by Gray.[29] Differences with $P < 0.05$ were statistically significant. All statistical analyses were performed with S-plus 2000 software (MathSoft, Inc., Seattle, WA).

RESULTS

Patient Profile

The mean age of the patients was 67 years (range, 44–90 years). Patients were male dominant (69%) and the majority were HCV positive (Table 1). The mean tumor size was 2.6 cm (range, 0.8–9.7 cm). As 227 of 664 patients received ≥ 2 treatments during the period

TABLE 1
Characteristics of 664 Patients Treated by RFA

Variables	Naive patients (n = 319) (%)	Nonnaïve patients (n = 345) (%)
Mean age (yrs)*	67.4 ± 7.8	66.6 ± 8.4
Male gender	212 (66.5)	247 (71.6)
Etiology		
HBs-Ag positive only	31 (9.7)	36 (10.4)
HCV-Ab positive only	251 (78.7)	284 (82.3)
Both positive	5 (1.6)	6 (1.9)
Both negative	32 (10.0)	19 (5.5)
Alcohol consumption >80 g/day	52 (16.3)	59 (17.1)
Child-Pugh class		
A	221 (69.3)	225 (65.2)
B	94 (29.5)	111 (32.1)
C	4 (1.3)	9 (2.6)
Tumor size (cm)		
≤2.0	87 (27.2)	113 (32.8)
2.1–5.0	215 (67.4)	209 (60.6)
>5.0	17 (5.3)	23 (6.7)
Tumor no.		
Single	193 (60.5)	138 (40.0)
2–3	105 (32.9)	141 (40.9)
>3	21 (6.6)	66 (19.1)
Tumor markers		
AFP (ng/mL)		
≤20	152 (47.6)	156 (45.2)
21–100	86 (27.0)	97 (28.1)
101–400	47 (14.7)	43 (12.5)
>400	34 (10.7)	49 (14.2)
DCP (mAU/mL)		
≤40	225 (70.5)	226 (65.5)
41–100	36 (12.9)	49 (14.2)
101–400	37 (11.6)	40 (11.6)
>400	21 (6.6)	30 (8.7)

RFA: radiofrequency ablation; HBs-Ag: hepatitis B surface antigen; HCV-Ab: hepatitis C virus antibody; AFP: α-fetoprotein; DCP: des-gamma carboxyprothrombin.
* Values are expressed as mean ± standard deviations.

for tumor recurrence, the total number of treatments (cases) received was 1000.

Treatment Efficacy and Complications

One thousand treatments to 664 patients consisted of 2082 sessions of RFA to 2140 nodules. The distribution of nodules was as follows: 367 nodules (17.1%) in the lateral segment, 238 (11.1%) in the medial segment, 891 (41.6%) in the anterior segment, 609 (28.5%) in the posterior segment, and 35 (1.6%) in the caudate lobe. The mean number of electrode insertions according to tumor size are as follows: 1.5 times for nodules < 2 cm, 2.3 times for nodules 2.1–3.0 cm, 4.2 times for nodules 3.1–5.0 cm, and 11.7 times for nodules > 5 cm. One hundred thirty-seven of 319 naive patients and 145 of 345 nonnaive patients received TAE before RFA. All nodules were ablated completely in 934 treatments. RFA was performed for debulking the tumor burden in the remaining 66 treatments. The α-fetoprotein (AFP) declined to < 100 ng/mL in 184 of 247 treatments (74.4%) in which the pretreatment AFP level was > 100 ng/mL. DCP normalized (≤ 40 mAU/mL) in 279 of 326 treatments (85.6%).

A total of 40 major complications (4.0% per treatment, 1.9% per session) and 17 minor complications (1.7% per treatment, 0.82% per session) were observed within the follow-up period (Tables 2, 3). There were no complication-related deaths. Side effects such as moderate pain controlled by analgesics, nausea, or infection-unrelated fever relieved by antifebriles were

TABLE 2
Major Complications after RFA

Complications	No. of complications	Prevalence (%) Per treatment	Per session
Immediate (within 24 hrs)			
Intraperitoneal hemorrhage requiring blood transfusion	4	0.4	0.19
Pleural effusion requiring drainage	4	0.4	0.19
Hepatic infarction	2	0.2	0.096
Pneumothorax requiring drainage	1	0.1	0.048
Hemothorax requiring drainage	1	0.1	0.048
Bile peritonitis	1	0.1	0.048
Periprocedual (within 30 days)			
Hepatic abscess requiring drainage	7	0.7	0.34
Bronchobiliary fistula	2	0.2	0.096
Duodenal perforation	1	0.1	0.048
Colonic penetration	1	0.1	0.048
Gastric penetration	1	0.1	0.048
Delayed			
Neoplastic seeding	15	1.5	0.72
Total	40	4.0	1.9

RFA: radiofrequency ablation.

TABLE 3
Minor Complications after RFA

Complications	No. of complications	Prevalence (%) Per treatment	Per session
Immediate (within 24 hrs)			
Self-limiting hemobilia	3	0.3	0.14
Skin burn (entrance point)	3	0.3	0.14
Periprocedual (within 30 days)			
Self-limiting portal vein thrombosis	4	0.4	0.19
Delayed			
Biloma	7	0.7	0.34
Total	17	1.7	0.82

RFA: radiofrequency ablation.