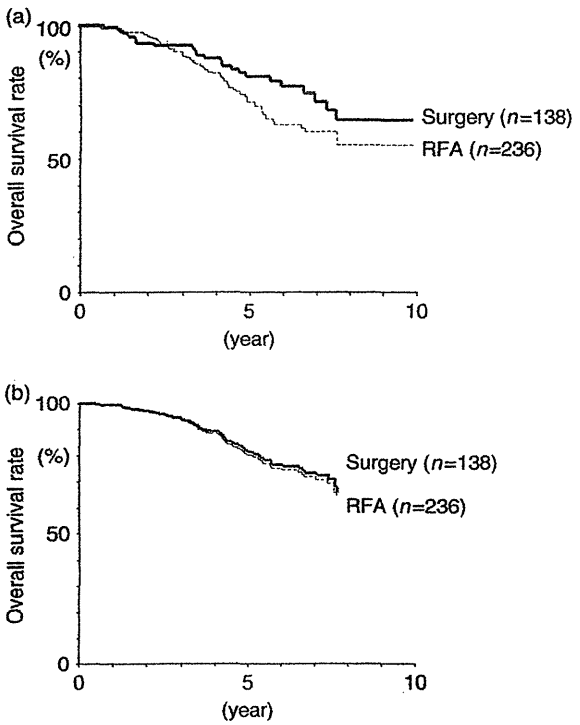

Fig. 4. (a) Crude survival rates in patients receiving radiofrequency ablation and those undergoing surgery as the initial therapy. (b) Adjusted survival rates in the radiofrequency group and surgery group, using proportional hazard analysis. RFA, radiofrequency ablation.

Table 4. Independent factors associated with the survival rate after the initial treatment for hepatocellular carcinoma

Factors	Category	Hazard ratio (95% confidence interval)	*P*
HBsAg	1: negative	1	
	2: positive	0.43 (0.19–0.94)	0.034
ICG R15*	1: < 30%	1	
	2: ≥30%	1.96 (1.20–3.20)	0.0070
α-fetoprotein	1: < 40 mg/ml	1	
	2: ≥40 mg/ml	1.71 (1.09–2.68)	0.020
Prothrombin time	1: < 80%	1	
	2: ≥80%	0.60 (0.37–0.96)	0.035
Initial therapy	1: surgery	1	
	2: RFA	1.09 (0.66–1.81)	0.73

*ICG R15, indocyanine green retention rate at 15 min.
RFA, radiofrequency ablation.

In the matrix of age of < 60 years, 2.34% of the patients in the early stage developed to the intermediate stage annually, 1.40% to the advanced stage and 0.93% died. The remaining 95.33% of the patients remained in the early stage after 1 year. The probability for the transition from an early stage to an intermediate stage

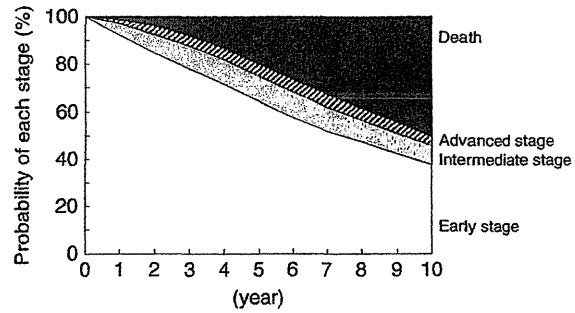

Fig. 5. Illustrated transition probabilities of patients, from the early stage of hepatocellular carcinoma, to the intermediate stage, the advanced stage and to death.

Table 5. One-year state-transition probability matrices for subsets of hepatocellular carcinoma*

	Early	Intermediate	Advanced	Death
All Patients of all age groups				
Early	92.17	4.81	1.73	1.29
Intermediate		69.32	27.27	3.41
Advanced			24.77	75.23
Death				100.00
Age < 60 years				
Early	95.33	2.34	1.40	0.93
Intermediate		58.33	37.50	4.17
Advanced			23.53	76.47
Death				100.00
Age 60–69 years				
Early	91.40	5.90	1.35	1.35
Intermediate		68.18	30.30	1.52
Advanced			22.21	78.79
Death				100.00
Age ≥70 years				
Early	90.68	5.49	2.33	1.50
Intermediate		74.42	22.09	3.49
Advanced			27.91	72.09
Death				100.00

*Early stage, solitary or multiple up to three nodules 3 cm or less each; Intermediate stage, four nodules or more, or larger than 3 cm; Advanced stage, portal vein invasion, extrahepatic metastasis, or Child–Pugh score C.

was significantly lower in young patients < 60 years of age (2.34%) than that in patients 60 years of age or older (5.70%)($\chi^2 = 7.76$, $P = 0.0053$). From the matrix stratified by three age groups, the transition probability from an intermediate to an advanced stage decreased with age: 37.50% in patients < 60 year of age, 30.30% in patients 60–69 year of age and 22.09% in patients 70 year of age or older ($\chi^2 = 10.57$, $P = 0.0011$).

Probabilities for transition according to the initial treatment

We also evaluated the transition probabilities among the four states in the subgroups of RFA and surgery as the initial mode of therapy.

In the matrix of patients receiving RFA therapy, the transition probability from early to intermediate stage was 5.40%, probability to the advanced stage was 1.63% and to death was 1.73%. In the patients undergoing surgery, the transition probability from an early to an intermediate stage was 3.90%, probability to an advanced stage was 1.87% and to death was 0.62%.

The probability for the transition from an early stage to an intermediate stage was slightly higher in the RFA group (5.40%) than that in the surgery group (3.90%), but statistical significance was not found ($\chi^2 = 1.90$, $P = 0.17$).

Discussion

Radiofrequency ablation has been considered as a less curative mode of therapy than surgical resection, because local tumour progression sometimes occurs after conservative treatment with relatively small ablative margins. As those patients with loco-regional therapy are generally followed up for tumour recurrence with a short time interval of 3–6 months using CT or MRI, we can usually ablate a newly appeared or a locally progressed tumour within a small size and few numbers. In order to elucidate the efficacies and usefulness of RFA compared with surgical resection, we analysed many HCC patients receiving RFA or surgical therapy regarding tumour progression and survival.

Fortunately, in Japan, where highly socialized medicine is practiced with everyone covered by some form of health insurance, almost all of the patients can receive any extensive medical services including surgery, RFA, embolization and repeated imaging diagnosis, regardless of the cost. Under intensive check-up and treatment repetition, the Markov model showed the probability of remaining at the early stage as 92.17% per year: the transition rate from the early to the intermediate stage was 4.81%, to the advanced stage 1.73 and to death 1.29% respectively. Similarly, the probabilities of remaining at the intermediate and advanced stages were 69.32 and 24.77% per year respectively.

Because young patients with HCC usually have better liver function and a relatively low carcinogenesis rate, younger patients are more likely to undergo radical methods of therapy for a recurrent tumour repeatedly. The reason for the low transition rate from the early to the intermediate stage was convincingly explained in the young patient group (Table 4). In contrast, the transition rate from the intermediate to the advanced stage was significantly higher in the young patient group. Although the exact reason was not known, multiple tumours of younger patients possibly progressed rapidly or were resistant to TACE. Hence, the Markov model would be eligible for simulating the outcomes of patients with the early stage of HCC. It is also helpful in planning strategies for the management of small HCC, for the eventual prolongation of a patient's life and for ideal cost-effective guidelines on a national basis, not only in Japan but also elsewhere in the world where the prevalence of HCC is increasing. Although we once generated a 'five-state model' consisting of no tumour, early stage, intermediate stage, advanced stage and death, we finally adopted the current 'four-state model' because of good mathematical fit and statistical robustness. Molinari and Helton (20) and Cho *et al.* (21) described a progression model of HCC after RFA and/or hepatectomy by the Markov model. Both authors performed a meta-analysis-like study using heterogeneous sources of patients from varied published articles, and estimated progression models of HCC in hypothetical patient cohorts. We analysed the actual clinical courses of patients in a single institution, where the same diagnostic and therapeutic procedures were adopted for every patient. Sufficient medical procedures and resources under a universal medical insurance system of the country seemed to give rise to better outcomes and survival, but an exact comparison cannot be carried out using the current data and the previous literatures.

In this study, we also compared RFA and surgery as an initial therapy for the early stage of HCC. Understandably, older patients, patients with severe cirrhosis and those with a concomitant disease other than liver disease tended to undergo non-surgical therapy. In addition, young patients with HBV-related HCC were likely to receive surgery because of good liver function, relatively low potential of recurrence and young age. Although the crude recurrence rate and the crude progression rate from the early stage to the intermediate stage were higher in patients receiving RFA therapy, multivariate analysis with adjustment of background biases showed that the initial mode of therapy did not affect the progression rate and did not affect the overall survival rate. When a regular check-up of imagings with an interval of 3–4 months was conducted, an additional ablation therapy was usually performed successfully for a small locally progressed tumour. Under intensive medical care for liver disease, the initial mode of therapy therefore did not affect the overall survival of a patient with an early stage of HCC. When a careful check-up with imagings and adequate application of repeated ablative procedures for HCC were performed, the choice of ablative manners was insignificant compared with the background liver features of aetiology of liver disease (hepatitis virus) and severity of liver disease (platelet count). The choice of ablative therapy for small-sized HCC should also be assessed from the viewpoints of conservation of liver function, cost-effectiveness and quality of life (9, 10, 12, 22).

Since it seemed to require at least 5 years to obtain a statistical difference in the recurrence rates and survival rates between RFA-treated and surgically treated groups, a prospective randomized trial is actually difficult to perform from both ethical and medical viewpoints. One of the significant results of the current study is that highly socialized medical circumstances with sufficient medical practice can attain a high survival rate of 71–80% at the end of the fifth year in patients at an early stage.

Further studies are required to determine the relationship between patient's age and stage transition. Because HCV-related chronic hepatitis often progresses to HCC during the clinical course, this kind of staging model with analyses of medical intervention will be necessary in the future from the viewpoints of epidemiological assessment and medical politics, together with patient's quality of life and feeling of satisfaction.

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Heterogeneous Type 4 Enhancement of Hepatocellular Carcinoma on Dynamic CT Is Associated With Tumor Recurrence After Radiofrequency Ablation

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OBJECTIVE. The aim of this study was to predict recurrence of hepatocellular carcinoma (HCC) from baseline dynamic CT images.

MATERIALS AND METHODS. This retrospective study included 191 consecutive patients who underwent surgical resection or radiofrequency ablation (RFA) between January 2005 and September 2009 for the treatment of HCC. Enhancement on pretreatment arterial and portal phase dynamic CT images was classified into one of the four following enhancement patterns: Types 1 and 2 are homogeneous enhancement patterns without or with increased arterial blood flow, respectively; type 3 is a heterogeneous enhancement pattern with septations; and type 4 is an irregularly shaped ring structure enhancement pattern. Predictive factors for tumor recurrence including dynamic CT enhancement pattern were also evaluated. Moreover, risk factors including recurrence type (i.e., tumor number ≥ 10, portal vein invasion, or both) were evaluated in RFA-treated cases.

RESULTS. Among 60 patients who underwent surgical resection, no statistical association was observed between dynamic CT enhancement patterns and recurrence rate. In contrast, in the 131 patients who underwent RFA, cumulative recurrence rates for each enhancement pattern were significantly different: Recurrence rates 2 years after RFA for patients with type 1, 2, 3, and 4 were 26.6%, 46.9%, 38.6%, and 77.8%, respectively ($p = 0.042$). Recurrence, which was defined as the presence of 10 or more nodules, portal vein invasion, or both occurred in nine of 131 patients (6.9%) in the RFA group. A multivariate Cox proportional hazards analysis revealed that the type 4 dynamic CT enhancement pattern is an independent factor for HCC recurrence (hazard ratio, 27.68; 95% CI, 6.82–112.33; $p < 0.001$).

CONCLUSION. The pretreatment type 4 dynamic CT enhancement pattern can potentially be used to predict recurrence of HCC after RFA treatment.

Keywords: dynamic CT, hepatocellular carcinoma, radiofrequency ablation, recurrence, surgical resection

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WEB
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Hepatocellular carcinoma (HCC) is a common malignancy worldwide, and the incidence rate is increasing in Japan as well as in the United States [1–3]. Chronic viral hepatitis and liver cirrhosis after infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) play important roles in the development of HCC [4, 5]. The incidence of HCC in patients with HCV-related cirrhosis is estimated to be 5–10% per annum in Japan, and HCV-related cirrhosis is one of the major causes of death particularly in Asian countries [5]. Among the available treatment options for HCC, surgical resection is generally considered to be a local eradication method that can provide a satisfactory long-term outcome [6–13]. Advances in imaging procedures have led to the increased detection of early stage HCC and have improved survival because more patients in whom hepatic resection is possible are being identified [14, 15].

For patients who are not eligible for surgical treatment for various reasons (e.g., lack of sufficient liver function for surgical resection), percutaneous local therapy is a viable therapeutic option. A number of local ablation therapies are available including percutaneous ethanol injection, percutaneous acetic acid injection, cryotherapy, percutaneous microwave coagulation therapy, and radiofrequency ablation (RFA). In addition to surgical resection, local ablation therapies, particularly RFA, are considered to be local eradication methods for HCC that can provide good long-term outcomes [16]. However, despite the high complete necrosis rate in RFA-treated HCC, some patients experience tumor recurrence within 1 year of RFA, either as local recurrence or new tumor formation. A series of studies have

identified factors predictive of HCC tumor recurrence and seeding including tumor size, tumor location relative to the hepatic capsule (presence or absence of tumor on subcapsular portion), α-fetoprotein (AFP) level, tumor stage, and histopathologic grade [17, 18]. For the reasons stated earlier, it is important to determine the histopathologic grade of HCC before administering local ablation therapy.

We previously reported that a "heterogeneous enhancement pattern with irregular ring-like structures" [19] in the arterial phase of dynamic CT analysis accurately predicts the histopathologic grade of poorly differentiated HCC, and we named this enhancement pattern "type 4" [19]. Therefore, one aim of the current study was to examine the risk factors for tumor recurrence after local eradication, including differences between treatment procedure (surgical resection vs RFA) and dynamic CT enhancement pattern. Moreover, in a previous study, investigators reported an association between tumor seeding after RFA and histopathologic grade of HCC [17, 18]. Therefore, the other aim of the current study was to evaluate the relationship between the type 4 dynamic CT enhancement pattern and HCC recurrence in patients who undergo RFA.

Materials and Methods

Study Population

From January 2005 to September 2009, 705 patients were diagnosed with HCC and underwent surgical resection or RFA as the initial treatment in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan. Among the 705 patients, 191 patients satisfied the following criteria for inclusion in our study: triple-phase dynamic CT study performed before surgical resection or RFA; pretreatment diagnosis of a solitary HCC with a maximum tumor diameter of 30 mm or less; no evidence of extrahepatic metastases as confirmed on pretreatment imaging studies (CT, sonography, or chest radiography); no history of other malignancies; and no pretreatment chemotherapy, including transcatheter arterial chemoembolization (TACE). Accordingly, these 191 patients were retrospectively evaluated for an association between arterial and portal phase dynamic CT enhancement pattern and recurrence of HCC. The observation starting point was the time of the first surgical resection or RFA session for HCC.

Contrast Infusion and CT Protocol

All patients received nonionic contrast material with an iodine concentration of 350 mg I/mL (iomeprol [Iomeron 350, Bracco-Eisai]). CT was performed with a 64-MDCT scanner (Aquilion 64, Toshiba Medical Systems) with the following scanning parameters: rotation time, 0.5 second; beam collimation, 64 × 0.5 mm; section thickness and interval, 5 mm; beam pitch, 0.83; tube voltage, 120 kV; and tube current, 150 mAs. All helical scans were started at the top of the liver and proceeded in a cephalocaudal direction. Unenhanced and three-phase contrast-enhanced helical scans of the whole liver were obtained. Patients were instructed to hold their breath with exhalation during scanning.

An automatic bolus-tracking program (Sure Start, Toshiba Medical Systems) was used to time the start of acquisition in each phase after contrast injection. The attenuation at the axis of the celiac artery level was monitored by one radiology technician; the region-of-interest cursor (1 cm^2) was placed in the abdominal aorta. Real-time low-dose (120 kV, 25 mAs) serial monitoring studies were initiated 5 seconds after the start of contrast injection. The trigger threshold level was set at 100 HU. A double arterial phase acquisition was started 15 and 20 seconds after triggering, and portal phase and delayed phase acquisitions were started 70 and 180 seconds after the start of the contrast injection, respectively.

Diagnosis of HCC

Diagnosis of HCC was predominantly based on image analysis. If a hepatic nodular lesion was identified on screening sonography, the patient underwent dynamic CT, dynamic MRI, or both. Furthermore, when a liver nodule either showed hyperattenuation in the arterial phase of the dynamic study and washout in the portal or delayed phase or showed typical hypervascular staining on digital subtraction angiography, the nodule was diagnosed as HCC. In accordance with the American Association for the Study of Liver Diseases guidelines [20], we obtained at least two dynamic images before treatment. When a nodule did not appear to show the mentioned typical imaging features, fine-needle aspiration biopsy was performed followed by histologic examination and diagnosis.

Imaging Analysis of Hepatocellular Carcinoma and Definition of Enhancement Pattern

Before treatment was administered, triple-phase contrast-enhanced CT was performed of all patients. The enhancement pattern on the arterial and portal phases of dynamic CT was classified into one of four types, and the four enhancement types on the original images were converted into simplified images (Fig. 1 [19]). The type 1 pattern represented a homogeneous enhancement pattern with no increase in arterial blood flow; the entire image was uniform during the arterial phase and portal phase. The type 2 pattern represented a homogeneous enhancement pattern with increased arterial blood flow; the entire image was uniform during the arterial phase and portal phase. The type 3 pattern represented a heterogeneous enhancement pattern with septations with heterogeneous enhancement and septations in the arterial phase, whereas the septations resembled a near-uniform tumor tissue periphery in the portal phase. The type 4 pattern represented a heterogeneous enhancement pattern with irregular ring-like structures; the arterial phase was marked by the presence of irregularly shaped ring areas of enhancement and areas of little blood flow relative

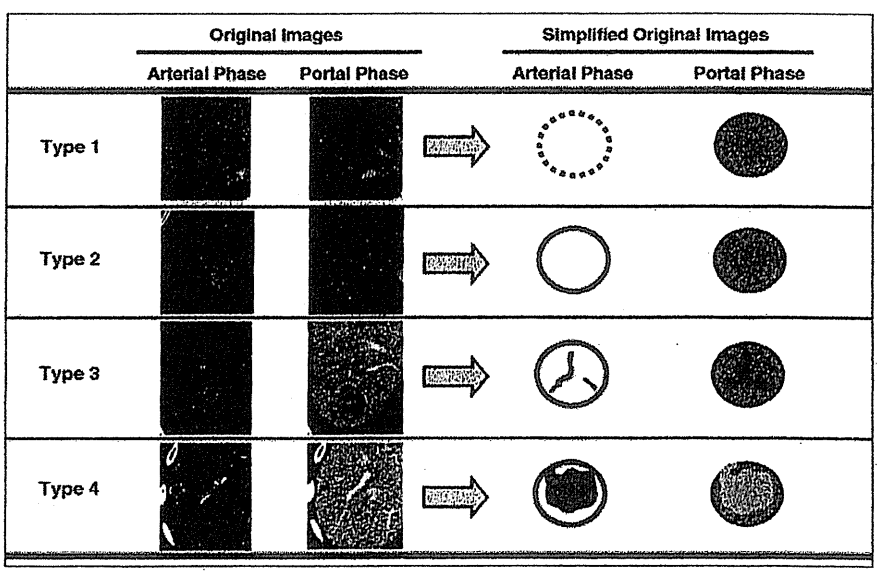

Fig. 1—Sample of original dynamic CT images and simplified images for each enhancement pattern. (Reprinted and modified with permission from John Wiley and Sons [19])

to the periphery of the tumor tissue, and the portal phase was characterized by areas of reduced blood flow.

The enhancement pattern on the arterial and portal phases of dynamic CT was determined by consensus of three expert hepatologists who were blinded to the pathologic results.

Treatment Methods

Physicians and surgeons generally discussed the preferred choice of therapy in individual patients. Hepatic resection was performed under intraoperative sonographic monitoring and guidance. For small and superficial HCCs, arterial and portal vein clamping at the hepatic hilum was not usually required to maintain liver perfusion. RFA was performed using three different devices: a multitined expandable electrode with a 3-cm array with a 150-W radiofrequency generator (model 1500 series, RITA Medical Systems), an internally cooled electrode with a 3-cm active tip with a 200-W radiofrequency generator (Cool-tip Radiofrequency System, Covidien), and a multitined expandable electrode with a 200-W radiofrequency generator (LeVeen Needle Electrode and Radiofrequency 3000 Generator, RTC System, Boston Scientific Japan). For the first two systems, treatment procedures were performed according to the protocols recommended by the manufacturers. However, treatment using the RTC System was performed by adopting the "stepwise hook extension technique" [21].

The needle was inserted into the tumor percutaneously under sonographic guidance. In the case of RFA, dynamic CT was performed 1–3 days after therapy, and the ablated area was evaluated. The goal of treatment was to obtain an ablative margin larger than the original tumor, with a surrounding treatment margin of 5 mm or greater in all directions. When this margin was not achieved or a residual tumor was found, additional ablation was considered.

In this study, 93 of 131 procedures (71%) were performed using the multitined expandable electrode (LeVeen), 28 of 131 procedures (21%) were performed using the internally cooled electrode (Cool-tip), and 10 of 131 procedures (8%) were performed using the multitined expandable electrode (RITA).

Definition of Multinodular Recurrence of HCC

In this study, we defined "multinodular" as follows: the appearance of 10 or more lesions at the time of first recurrence after surgical resection or RFA.

Follow-Up Protocol

Physicians examined the patients every 4 weeks after treatment, and liver function tests and tumor

TABLE 1: Clinical Profile and Laboratory Data of 191 Patients With Hepatocellular Carcinoma Treated by Surgical Resection or Radiofrequency Ablation (RFA)

Parameter	Surgical Resection	RFA	p
Patient characteristics			
No. of patients	60	131	
Sex (no. of patients)			0.922
M:F ratio	38:22	82:49	
Age (y)			0.021
Median	66	69	
Range	35–80	37–83	
Background liver disease (no. of patients)			0.003
Hepatitis C virus	34	100	
Hepatitis B virus	22	19	
Others	4	12	
Laboratory data			
Platelet count ($\times 10^4/\mu L$)			0.153
Median	13.3	11.8	
Range	5.1–27.2	2.7–39.6	
Albumin (g/dL)			0.019
Median	3.7	3.7	
Range	2.9–4.7	2.7–4.4	
Total bilirubin (mg/dL)			0.030
Median	0.8	0.9	
Range	0.3–2.2	0.3–2.7	
Prothrombin activity (%)			0.218
Median	94.5	89.9	
Range	60.4–124.0	56.7–124.0	
AST (IU/L)			0.423
Median	41	48	
Range	16–163	16–191	
AFP (μg/L)			0.561
Median	12.0	10.5	
Range	1.6–5541.0	1.0–993.7	
DCP (AU/L)			0.137
Median	20.5	17.0	
Range	9.0–556.0	6.0–314.0	
Tumor characteristics			
Diameter (mm)			<0.001
Median	20.0	16.0	
Range	10.0–30.0	7.0–30.0	
Tumor location (no. [%] of patients)			
Subcapsular	48/60 (80)	52/131 (40)	<0.001
Subphrenic	24/60 (40)	58/131 (44)	0.579
Dynamic CT enhancement pattern (no. [%] of patients)			<0.001
Type 1	4 (7)	46 (35)	
Type 2	27 (45)	52 (40)	
Type 3	21 (35)	24 (18)	
Type 4	8 (13)	9 (7)	

Note—AFP = α-fetoprotein, AST = aspartate aminotransferase, DCP = des-γ-carboxy prothrombin.

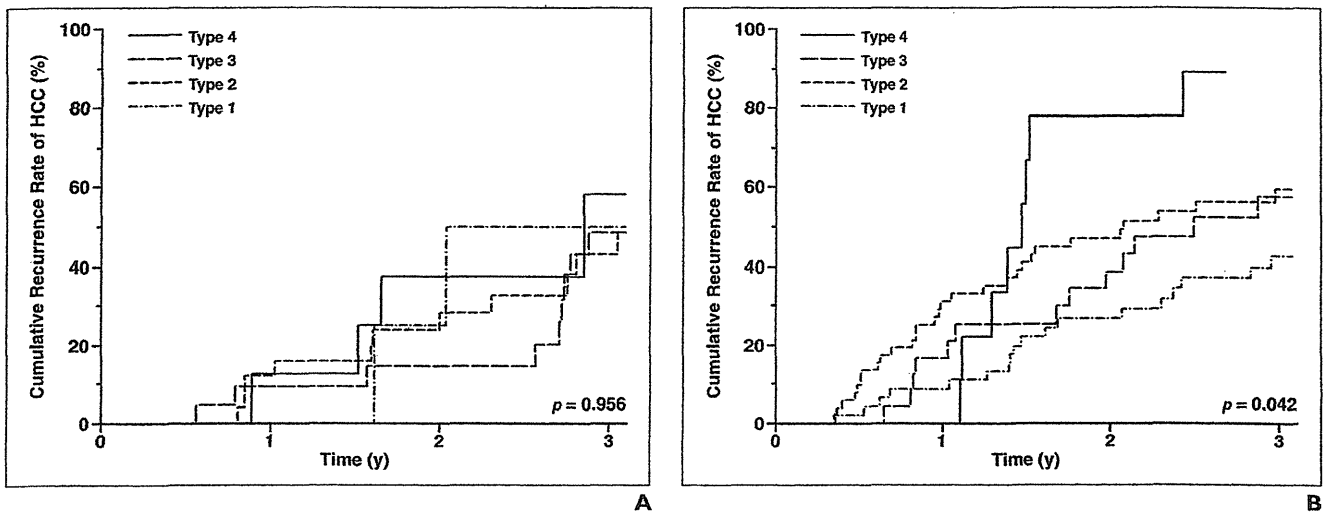

Fig. 2—Correlation between cumulative recurrence rates and enhancement patterns of pretreatment dynamic CT after each treatment procedure.
A and **B**, Graphs show associations between cumulative hepatocellular carcinoma (HCC) recurrence rate after surgical resection (**A**) and after radiofrequency ablation (**B**) and pretreatment dynamic CT enhancement pattern.

markers were also measured once every month. After completion of HCC treatment, patients underwent contrast-enhanced three-phase CT survey every 3 months for recurrence. Local tumor progression was defined as tumor recurrence adjacent to the resected or ablated area.

Statistical Analysis and Ethical Considerations

Differences in background features and laboratory data between the surgical resection and RFA groups were analyzed by the chi-square test and Mann-Whitney *U* test. Recurrence was analyzed using the Kaplan-Meier technique, and differences in curves were tested using the log-rank test. Independent factors associated with overall recurrence and recurrence characterized by multiple nodules, portal vein invasion, or both were studied using stepwise Cox regression analysis. Potential risk factors for overall recurrence after surgical resection and RFA included the following 15 variables: age, sex, cause of background liver disease, serum albumin level, bilirubin level, aspartate aminotransferase (AST) level, platelet count, prothrombin time, AFP level, des-γ–carboxy prothrombin (DCP) level, diameter of the HCC, tumor location relative to the hepatic capsule (presence or absence of tumor on subcapsular portion), tumor location relative to the diaphragm (presence or absence of tumor on subphrenic portion), treatment procedure, and enhancement pattern of pretreatment dynamic CT analysis.

Potential risk factors for recurrence characterized by multiple nodules, portal vein invasion, or both after RFA included the following 15 variables: age, sex, cause of background liver disease, serum albumin level, bilirubin level, AST level,

TABLE 2: Predictors of Tumor Recurrence in Patients With Hepatocellular Carcinoma Treated by Surgical Resection or Radiofrequency Ablation (RFA)

Category	Univariate Analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	*p*	Hazard Ratio (95% CI)	*p*
Sex				
1: Female	1			
2: Male	1.26 (0.84–1.89)	0.274		
Age				
1: < 65 y	1		1	
2: ≥ 65 y	1.50 (0.10–2.26)	0.050	1.85 (1.16–2.94)	0.010
Background liver disease				
1: Hepatitis C virus	1			
2: Hepatitis B virus	0.84 (0.51–1.39)	0.503		
3: Others	1.29 (0.66–2.49)	0.458		
Platelet count				
1: ≥ 10 ´ 10^4/μL	1		1	
2: < 10 ´ 10^4/μL	1.65 (1.10–2.49)	0.016	1.61 (1.04–2.48)	0.033
Albumin				
1: ≥ 3.5 g/dL	1			
2: < 3.5 g/dL	1.72 (1.15–2.58)	0.008		
Total bilirubin				
1: < 1.0 mg/dL	1			
2: ≥ 1.0 mg/dL	1.64 (1.11–2.42)	0.013		
Prothrombin activity				
1: ≥ 70%	1			
2: < 70%	1.95 (1.01–3.75)	0.046		
AST				
1: < 40 IU/L	1		1	
2: ≥ 40 IU/L	1.65 (1.09–2.49)	0.018	1.66 (1.04–2.66)	0.035

(Table 2 continues on next page)

TABLE 2: Predictors of Tumor Recurrence in Patients With Hepatocellular Carcinoma Treated by Surgical Resection or Radiofrequency Ablation (RFA) (continued)

Category	Univariate Analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	p	Hazard Ratio (95% CI)	p
AFP				
1: < 100 μg/L	1		1	
2: ≥ 100 μg/L	2.21 (1.40–3.50)	0.001	2.25 (1.30–3.89)	0.004
DCP				
1: < 30 AU/L	1		1	
2: ≥ 30 AU/L	1.82 (1.15–2.88)	0.011	1.77 (1.05–2.99)	0.032
Tumor diameter				
1: < 20 mm	1			
2: ≥ 20 mm	1.13 (0.76–1.67)	0.544		
Tumor on subcapsular portion				
1: Yes	1		1	
2: No	1.37 (0.93–2.00)	0.115	1.72 (1.10–2.70)	0.019
Tumor on subphrenic portion				
1: No	1			
2: Yes	1.01 (0.68–1.49)	0.984		
Treatment				
1: Surgical resection	1			
2: RFA	1.52 (0.98–2.36)	0.062		
Dynamic CT enhancement pattern				
1: Type 1	1			
2: Type 2	1.33 (0.81–2.18)	0.261		
3: Type 3	1.15 (0.66–2.01)	0.628		
4: Type 4	1.95 (0.98–3.89)	0.058		

Note—AFP = α-fetoprotein, AST = aspartate aminotransferase, DCP = des-γ-carboxy prothrombin.

platelet count, prothrombin time, AFP level, DCP level, tumor diameter, tumor location relative to capsule (subcapsular portion), tumor location relative to diaphragm (subphrenic portion), type of RFA device, and dynamic CT enhancement pattern. Several variables were transformed into categoric data consisting of two to four simple ordinal numbers for univariate and multivariate analyses. All factors that were at least marginally associated with overall recurrence and recurrence characterized by multiple nodules, portal vein invasion, or both ($p < 0.15$) in univariate analysis were entered into a stepwise Cox regression analysis. Significant variables were selected by the stepwise method. A two-tailed $p < 0.05$ was considered to be statistically significant. Data analysis was performed using statistics software (SPSS, version 11.0, SPSS Inc.).

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

Results

Clinical Background, Laboratory Data, and Distribution of Enhancement Patterns on Pretreatment Dynamic CT

Table 1 summarizes the clinical profile and laboratory data of 191 HCC patients who were treated by surgical resection or RFA. The RFA group included significantly older individuals and significantly more patients with less preserved liver function compared with the surgical resection group. The cause of background liver disease was also significantly different between the two treatment groups: Patients in the surgical resection group had larger tumors that were more likely to have a subcapsular location.

The type 2, 3, and 4 enhancement patterns were more commonly observed in the surgical resection group than the type 1 enhancement pattern. In contrast, in the RFA group, the type 1 enhancement pattern was more commonly observed than the type 2, 3, or 4 pattern. In addition, the distribution of enhancement patterns on pretreatment dynamic CT was significantly different for each treatment procedure.

Distribution of Each Enhancement Pattern and Frequency of Poorly Differentiated Hepatocellular Carcinoma by Histologic Examination in the Surgical Resection Group

In 60 surgical resection patients, four patients (7%) had the type 1 enhancement pattern, 27 patients (45%) had the type 2 pattern, 21 patients (35%) had the type 3 pattern, and eight patients (13%) had the type 4 pattern. Pathologic HCC diagnoses by enhancement pattern were as follows: type 1 enhancement pattern, all patients had well-differentiated HCC; type 2 enhancement pattern, five of 27 patients (19%) had well-differentiated HCC and 21 of 27 (78%) patients had moderately differentiated HCC; type 3 enhancement pattern, one of 21 patients (5%) had well-differentiated HCC and 19 of 21 (90%) patients had moderately differentiated HCC; and type 4 enhancement pattern, five of eight patients (63%) had moderately differentiated HCC. Rates of poorly differentiated HCC by enhancement pattern were as follows: type 1 enhancement pattern, zero of four patients (0%); type 2 enhancement pattern, one of 27 patients (4%); type 3 enhancement pattern, one of 21 patients (5%); and type 4 enhancement pattern, three of eight patients (38%).

Correlation Between Cumulative Recurrence Rates and Enhancement Patterns on Pretreatment Dynamic CT After Each Treatment Procedure

In the surgical resection group, cumulative recurrence rates were not significantly different between each pretreatment dynamic CT enhancement pattern (types 1, 2, 3, and 4: 0.0%, 12.0%, 9.5%, and 12.5% at the first year after treatment, respectively, and 25.0%, 28.2%, 14.6%, and 37.5% at the second year) (Fig. 2A). However, in the RFA group, the cumulative recurrence rate was significantly different between each enhancement pattern (types 1, 2, 3, and 4: 8.7%, 31.1%, 16.7%, and 0.0% at the first year, respectively, and 26.6%, 46.9%, 38.6%, and 77.8% at the second year, respectively; $p = 0.042$) (Fig. 2B).

Predictive Factors for Initial Recurrence After Surgical Resection or Radiofrequency Ablation

Multivariate Cox proportional hazards analysis revealed that the following independent

factors are predictive for recurrence of HCC treated by surgical resection or RFA: AFP ≥ 100 μ g/L (hazard ratio [HR], 2.25; 95% CI, 1.30–3.89; p = 0.004), age ≥ 65 years (HR, 1.85; 95% CI, 1.16–2.94; p = 0.010), DCP ≥ 30 AU/L (HR, 1.77; 95% CI, 1.05–2.99; p = 0.032), tumor not present in subcapsular portion (HR, 1.72; 95% CI, 1.10–2.70; p = 0.019), AST ≥ 40 IU/L (HR, 1.66; 95% CI, 1.04–2.66; p = 0.035), and platelet count < 10×10^4/μL (HR, 1.61; 95% CI, 1.04–2.48; p = 0.033) (Table 2).

Association Between the Frequency of Recurrence Characterized by Multiple Nodules, Portal Vein Invasion, or Both and Clinical Features for Each Treatment Procedure

The frequency and clinical features of recurrence characterized by multiple nodules, portal vein invasion, or both are presented in Table 3. Such recurrences occurred in 10 of 191 patients (5.2%). In the surgical resection group, recurrence occurred in one of 60 patients (1.7%), and in the RFA group, recurrence occurred in nine of 131 patients (6.9%). Notably, in the RFA group, six of nine patients (66.7%) had a pretreatment type 4 enhancement pattern. Among the type 4 patients, recurrence of HCC occurred more than 1 year after treatment in six of six patients (100%) after RFA.

Regarding the needles used for RFA of HCC in these nine patients, an internally cooled electrode (Cool-tip) was used in case 2 (Table 3), a RITA multitined expandable electrode was used in case 4, and a LeVeen multitined expandable electrode was used in the other seven patients.

Figure 3 shows a case of recurrence after RFA (case 7 in Table 3). Figures 3A and 3B show that the pretreatment dynamic CT and digital subtraction angiography (DSA) images revealed a type 4 dynamic CT enhancement pattern. In Figures 3C and 3D, dynamic CT and DSA images acquired at the time of recurrence after RFA are shown: Multiple hepatic tumors are apparent surrounding the previously ablated area.

Association Between Cumulative Hepatocellular Carcinoma Recurrence Rate After Radiofrequency Ablation and Pretreatment Dynamic CT Enhancement Patterns: Type 4 Versus Other Enhancement Patterns

In the RFA group, the cumulative recurrence rate was significantly higher in tumors displaying a pretreatment type 4 dynamic CT enhancement pattern than in tumors showing other enhancement patterns (type 4 vs other enhancement patterns, 0.0% vs 2.8% at the first year, 74.6% vs 2.8% at the second year; $p < 0.001$).

Predictive Factors for Hepatocellular Carcinoma Recurrences Characterized by Multiple Nodules, Portal Vein Invasion, or Both After Radiofrequency Ablation

The Multivariate Cox proportional hazards analysis revealed that the type 4 pretreatment dynamic CT enhancement pattern is an independent predictive factor for HCC recurrence characterized by multiple nodules, portal vein invasion, or both in patients with HCC treated by RFA (HR, 27.68; 95% CI, 6.82–112.33; $p < 0.001$) (Table 4).

Discussion

A number of local eradication therapies are currently available for HCC. However, with the exception of surgical resection, the potential risk of tumor dissemination always exists in patients who receive such therapies. Therefore, to properly select the most suitable therapy for an individual patient, it is important to predict the potential risk of HCC before treatment.

As others have previously reported [17, 18], identification of poorly differentiated HCC is particularly important for making good therapeutic

TABLE 3: Frequency of Hepatocellular Carcinoma Recurrence Characterized by 10 or More Nodules, Portal Vein Invasion, or Both by Treatment Procedure and Clinical Features

Case No.	At the Time of First Treatment[a]					At the Time of Tumor Recurrence				Survival Period (y)	Patient Status at End of Follow-Up Period
	Age (y)	Type of Enhancement Pattern	Tumor Diameter (mm)	AFP (μg/L)	DCP (AU/L)	AFP (μg/L)	DCP (AU/L)	Treatment of Recurrence	First Recurrence Period (y)		
1[b]	72	Type 3	26	3	12	8	14	Radiation	2.7	4.1	Alive
2[c]	65	Type 4	9	37	12	34	12	TAI	1.4	1.5	Dead
3	53	Type 4	13	117	94	10	978	TACE and radiation	1.3	2.9	Alive
4	70	Type 4	16	3	14	3	10	TACE	1.5	4.0	Dead
5	77	Type 4	20	4	33	5	19	TACE and RFA	1.1	5.0	Alive
6	60	Type 4	20	27	32	23	4313	TACE	1.5	1.9	Dead
7	83	Type 4	21	6	34	6	70	TACE	1.5	3.0	Alive
8	53	Type 3	10	64	8	141	10	TACE	0.8	4.3	Alive
9	69	Type 2	18	55	10	85	16	TACE	0.6	5.3	Alive
10	73	Type 2	21	7	12	3	34	TACE	0.8	4.0	Alive

Note—AFP = α-fetoprotein, DCP = des-γ-carboxy prothrombin, TACE = transcatheter arterial chemoembolization, and TAI = transcatheter arterial infusion chemotherapy. RFA = radiofrequency ablation.

[a]Disease was Child-Pugh class A in all 10 patients.

[b]For surgical resection, advanced recurrence occurred in one of 60 patients (1.7%) (case 1).

[c]For RFA, advanced recurrence occurred in nine of 131 patients (6.9%) (cases 2–10).

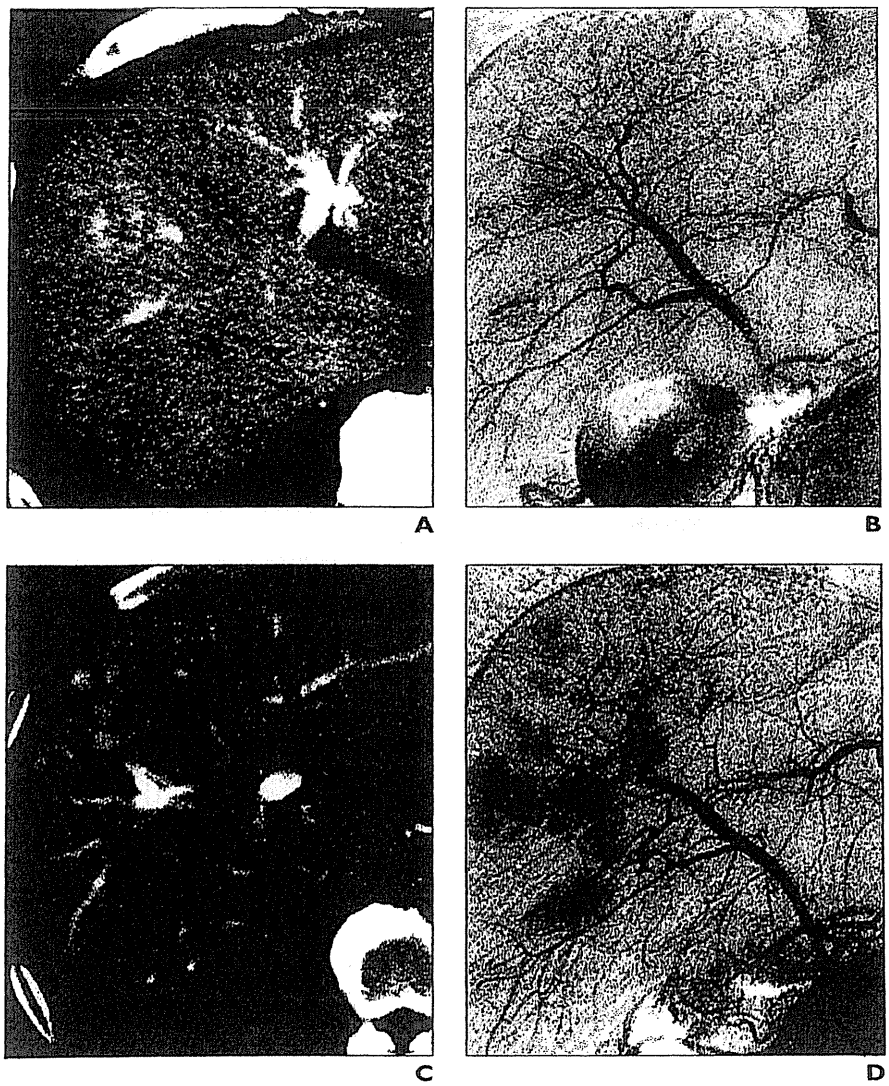

Fig. 3—83-year-old man with hepatocellular carcinoma (case 7 in Table 3).
A, Pretreatment dynamic CT (arterial phase) image. Tumor shows heterogeneous enhancement pattern with irregular ringlike structures—that is, type 4 enhancement pattern.
B, Pretreatment digital subtraction angiography (DSA) image shows single hypervascular nodule, so radiofrequency ablation (RFA) was performed.
C, Dynamic CT study (arterial phase) image obtained at time of recurrence after RFA (1.5 years after treatment) shows multiple hepatic tumors are observed around previously ablated area.
D, DSA image at time of recurrence shows multiple hepatic tumors are observed around original tumor.

progress. In one of our previous studies, we identified the type 4 enhancement pattern as an independent factor that is predictive of poorly differentiated HCC [19]. The results of that study revealed that the risk of a pathologic diagnosis of poorly differentiated HCC in patients with a preoperative type 4 dynamic CT enhancement pattern is approximately 13 times higher than that of patients with a type 1 or 2 enhancement pattern.

Therefore, the first aim of this study was to evaluate the clinical outcomes of patients with HCC treated by surgical resection and of those with HCC treated by RFA in association with dynamic CT enhancement patterns. In the surgical resection group, no significant differences in recurrence rates were observed between patients with different enhancement patterns. We presume that no significant differences were observed because surgical resection is the most effective local eradication therapy for HCC. In contrast, in the RFA group, significant differences in recurrence rates were observed between patients with different enhancement patterns. This result is surmised to reflect the association between each enhancement pattern and histopathologic diagnosis based on the results of these associations in the surgical resection group. However, in multivariate analysis, pretreatment dynamic CT enhancement pattern was not identified as an independent factor predictive for recurrence of HCC. Therefore, a larger-scale examination is required in the future; depending on the results of that study, it may be necessary to reclassify these enhancement patterns.

The second aim of this study was to investigate the relationship between recurrence characterized by 10 or more nodules, portal vein invasion, or both and pretreatment dynamic CT enhancement pattern in the RFA group. Significant differences between the enhancement patterns and recurrence rates were observed, and in multivariate analysis, a pretreatment type 4 dynamic CT enhancement pattern was identified as an independent factor predictive of this type of HCC recurrence after RFA treatment. The risk of this type of recurrence in patients with a pretreatment type 4 dynamic CT enhancement pattern was approximately 28 times higher than that of other enhancement patterns. Based on these results, this new classification of dynamic CT enhancement pattern—particularly the type 4 enhancement pattern—appears to be very useful for avoiding RFA treatment likely to recur.

In addition, among the six patients with a pretreatment type 4 dynamic CT enhancement pattern who underwent RFA, this type of HCC recurrence occurred more than 1 year after treatment in all six patients (100%). Histopathologic tumor features and adhesion molecules may have contributed to this long interval between the initial treatment and this type of recurrence after RFA. However, in this study, we were not able to perform tumor biopsies of nodules in patients with the type 4 enhancement pattern. Further studies, including histopathologic and molecular biologic examinations, are required to confirm this hypothesis.

This study has some limitations. First, there were more HCC patients with HCV in the RFA group than in the surgical resection group; this difference might have been a potential source of bias. This difference may be because patients with HBV-related HCC usually have a better liver reserve than those with HCV-related HCC at the time of initial hepatocarcinogenesis and that patients with

TABLE 4: Predictors of Recurrence Characterized by Multiple Nodules, Portal Vein Invasion, or Both in Patients With Hepatocellular Carcinoma Who Underwent Radiofrequency Ablation (RFA)

Category	Univariate Analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	*p*	Hazard Ratio (95% CI)	*p*
Sex				
1: Female	1			
2: Male	0.50 (0.14–1.87)	0.305		
Age				
1: < 65 y	1			
2: ≥ 65 y	1.31 (0.33–5.24)	0.703		
Background liver disease				
1: Hepatitis C virus	1			
2: Hepatitis B virus	0.41 (0.05–3.30)	0.405		
3: Others	1.23 (0.15–9.87)	0.843		
Platelet count				
1: ≥ 10 ´ 10^4/μL	1			
2: < 10 ´ 10^4/μL	0.58(1.17–2.86)	0.499		
Albumin				
1: ≥ 3.5 g/dL	1			
2: < 3.5 g/dL	1.22 (0.30–4.88)	0.783		
Total bilirubin				
1: < 1.0 mg/dL	1			
2: ≥ 1.0 mg/dL	1.36 (0.36–5.06)	0.649		
Prothrombin activity				
1: ≥ 70%	1			
2: < 70%	2.04 (0.25–16.39)	0.505		
AST				
1: < 40 IU/L	1			
2: ≥ 40 IU/L	4.99 (0.62–39.93)	0.130		
AFP				
1: < 100 μg/L	1			
2: ≥ 100 μg/L	1.01 (0.13–8.02)	0.998		
DCP				
1: < 30 AU/L	1			
2: ≥ 30 AU/L	3.73 (1.00–13.89)	0.050		
Tumor diameter				
1: < 20 mm	1			
2: ≥ 20 mm	1.62 (0.43–6.03)	0.473		
Tumor on subcapsular portion				
1: Yes	1			
2: No	2.44 (0.50–11.11)	0.272		
Tumor on subphrenic portion				
1: No	1			
2: Yes	1.60 (0.43–5.96)	0.484		
Type of RFA needle				
1: LeVeen Needle Electrode[a] (Boston Scientific Japan)	1			
2: Cool-tip[b] (Covidien)	0.46 (0.06–3.75)	0.470		
3: Model 1500 series[a] (RITA Medical Systems)	1.36 (0.17–11.05)	0.774		
Type of enhancement pattern				
1: Types 1, 2, and 3	1		1	
2: Type 4	29.52 (7.28–119.82)	< 0.001	27.68 (6.82–112.33)	< 0.001

Note—AFP = α-fetoprotein, AST = aspartate aminotransferase, DCP = des-γ-carboxy prothrombin.
[a]Multitined expandable electrode.
[b]Internally cooled electrode.

HCV-related HCC generally have smaller tumors than those with HBV-related HCC. Thus, more patients with HCV-related HCC were treated by RFA. Another limitation is that diagnosis of HCC was essentially based on image analysis, and heterogeneous enhancement resembling the type 4 enhancement pattern is recognized in other hepatic tumors (e.g., cholangiocellular carcinoma and fibrolamellar HCC). However, these other tumors that show the type 4 enhancement pattern are rare in patients with chronic hepatitis or liver cirrhosis compared with HCC: Cholangiocellular carcinoma comprises 4.4% of primary liver cancers [22] and fibrolamellar HCC represents only 0.68% of liver tumors in Japan. Thus, detection of a heterogeneous enhancement pattern on dynamic CT images should be considered first to represent HCC with a highly malignant potential. Moreover, regarding HCC nodules that have a type 4 enhancement pattern, MRI (T1- and T2-weighted images, contrast-enhanced MRI, and comparison of diffusion-weighted images obtained with different b values) is considered to contribute to improved tumor characterization. Adoption of these advanced techniques is expected to increase moving forward.

In our opinion, in patients with a type 4 enhancement pattern on dynamic CT images who have adequate liver reserve to allow any treatment, including surgical resection, we believe that the information about recurrence in this population could be used as an index to prioritize surgical resection. If surgical resection cannot be performed, we recommend up-front embolic therapies (e.g., TACE, radioembolization) rather than RFA monotherapy alone.

In conclusion, the current study showed a strong relationship between the type 4 enhancement pattern and HCC recurrence characterized by 10 or more nodules, portal vein invasion, or both after RFA treatment. The management of HCC with a type 4 enhancement pattern should include a thorough therapeutic approach including surgical resection.

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Total and High Molecular Weight Adiponectin and Hepatocellular Carcinoma with HCV Infection

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Abstract

Background: Adiponectin is shown to be inversely associated with development and progression of various cancers. We evaluated whether adiponectin level was associated with the prevalence and histological grade of hepatocellular carcinoma (HCC), and liver fibrosis in patients with hepatitis C virus (HCV) infection.

Methods: A case-control study was conducted on 97 HCC patients (cases) and 97 patients (controls) matched for sex, Child-Pugh grade and platelet count in patients with HCV infection. The serum total and high molecular weight (HMW) adiponectin levels were measured by enzyme-linked immunosorbent assays and examined in their association with the prevalence of HCC. In addition, the relationship between these adiponectin levels and body mass index (BMI), progression of liver fibrosis, and histological grade of HCC was also evaluated. Liver fibrosis was assessed using the aspartate aminotransferase to platelet ratio index (APRI).

Results: There were no significant differences in the serum total and HMW adiponectin levels between cases and controls. Moreover, there were no inverse associations between serum total and HMW adiponectin levels and BMI in both cases and controls. On the other hand, serum total and HMW adiponectin levels are positively correlated with APRI in both cases ($r=0.491$, $P<0.001$ and $r=0.485$, $P<0.001$, respectively) and controls ($r=0.482$, $P<0.001$ and $r=0.476$, $P<0.001$, respectively). Interestingly, lower serum total (OR 11.76, 95% CI: 2.97–46.66 [$P<0.001$]) and HMW (OR 10.24, CI: 2.80–37.40 [$P<0.001$] adiponectin levels were independent risk factors of worse histological grade of HCC.

Conclusions: Our results suggested that serum total and HMW adiponectin levels were predictors of liver fibrosis, but not prevalence of HCC in patients with HCV infection. Moreover, low these adiponectin levels were significantly associated with worse histological grades.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. The incidence of HCC has increased in Eastern Asia and Africa during the past several decades, and has also increased in the United States [1]. In many countries, this trend is attributed to hepatitis C virus (HCV) infections, and in Japan, over 70% of all HCC are related to chronic liver disease with HCV infection [2]. In order to prevent and treat this malignancy, it is important to understand the pathogenesis of HCC in patients with HCV infection.

Obesity is widely recognized as a significant risk factor for the development of various cancers [3]. It also is suggested that obesity is associated with the progression of chronic liver disease [4,5] and with HCC [6]. Especially, nonalcoholic fatty liver disease is recognized to be a hepatic manifestation of the metabolic syndrome and obesity and insulin resistance play a major role in the pathogenesis [7]. Nonalcoholic fatty liver disease represents a spectrum of conditions that are histologically characterized from simple steatosis to nonalcoholic steatohepatits, which is associated with increased risk of advanced liver fibrosis, cirrhosis and development of HCC [8]. Similarly, in patients with HCV infection, several studies have shown that obesity was associated with disease progression [4,5] and with HCC development [9,10]. However, it is unclear how obesity is linked to development of HCC in patients with HCV infection.

Adiponectin is a peptide hormone secreted by adipocyte and hepatocyte [11]. Adiponectin exists primarily in three forms: low molecular weight trimers, medium molecular weight hexamers, and high molecular weight (HMW) multimers [12]. Among three forms, HMW adiponectin is thought to have more biological activity than other forms of adiponectin [12,13]. Adiponectin has

antiatherogenic, antiinflamatory and insulin-sensitizing actions and is inversely associated with body mass index (BMI); therefore it is also linked to the various metabolic abnormalities associated with obesity [14]. Recently, hypoadiponectinemia has been shown to be an important risk factor for the development of various cancers associated with obesity such as breast cancer [15], endometrial cancer [16], colorectal cancer [17], and gastric cancer [18]. However, it is still unknown whether adiponectin contribute to the development of HCC in patients with HCV infection. Moreover, adiponectin has been shown to be associated with progression of liver fibrosis in patients with chronic liver disease [19,20,21]. However, in patients with HCV infection, the association between adiponectin and liver fibrosis is uncertain.

Histological grade is known to be one of the most important risk factor for patients with HCC. Several studies have shown that histological grade affected recurrence and survival after curative resection and liver transplantation for HCC [22,23]. Although it is known that worse histological grade is associated with high cell proliferation and angiogenesis of HCC, these molecular mechanisms is not unclear. Several clinical studies have demonstrated that serum adiponectin levels were negatively associated with increasing of histological grades of several cancers [15,24,25].

Therefore, in case-control study, we investigated the association of serum total and HMW adiponectin levels with prevalence of HCC in chronic liver disease with HCV infections. In addition, the associations between these adiponectin levels and BMI, progression of liver fibrosis, and histological grades of HCC were also examined.

Materials and Methods

Ethics Statement

The study protocol was approved by The Ethical Committee of Kurume University, and written informed consent for participation in the study was obtained from each subject and conformed to the guidelines of the 1995 Declaration of Helsinki.

Patients

Between January 1997 and December 2007, 97 Japanese cases with chronic HCV infection at the Kurume University School of Medicine were diagnosed with HCC and enrolled in this study. All cases had no medical history of previous or present neoplastic disease at any other site. The case patients were histologically confirmed with HCC by needle biopsy, and with a single tumor $\leq$5 cm or three or fewer tumors each $\leq$3 cm seen on ultrasonography and computed tomography. Histological classification was based on Liver Cancer Study Group of Japan [26]. According to Edmondson-Steiner classification [27], well differentiated corresponds to grade I and a part of grade II, and moderately differentiated corresponds to grade II and grade III with a clear trabecular pattern, and poorly differentiated corresponds to grade III with an indistinct trabecular pattern and part of grade IV. Tumor sizes were determined based on the largest dimension of the tumor. Between January 2005 and December 2007, 97 patients with chronic HCV infection, who matched for sex, Child-Pugh grade and platelet count ($\pm 20\times10^9$/L), were randomly selected as controls at the same hospital. The control patients had no medical history of previous or present neoplastic disease, including HCC. In all cases and controls, hepatic functional reserve was determined using the Child-Pugh scoring system. Diabetes was defined as a fasting blood glucose $\geq$126 mg/dl, and/or a random blood glucose $\geq$200 mg/dl. BMI was calculated as body weight in kg divided by the square of the height in meters (kg/m^2).

Assessment of liver fibrosis

Liver fibrosis was assessed using the aspartate aminotransferase (AST) to platelet ratio index (APRI) in this study. APRI has been recognized as a noninvasive test to characterize the degree of liver fibrosis in chronic liver disease with HCV infection [28]. APRI was calculated for all study subjects as follows: AST/upper limit of normal (45 IU/L)$\times$100/platelet count (10^9/L).

Markers of hepatic virus

HCV infection was evaluated using anti-HCV antibody (HCV-Ab). The diagnosis of HBV infection was also based on detection of hepatitis B surface antigen (HBsAg). The presence of HCV-Ab and HBsAg was determined using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital). All cases and controls were positive for HCV-Ab and negative for HBsAg in this study.

Measurement of serum adiponectin

Fasting morning blood samples were obtained from all subjects and stored at -20°C for later analysis. Blood samples were collected by all cases before HCC therapy was initiated. Serum total and HMW adiponectin levels were measured by enzyme-linked immunosorbent assays using the Human Adiponectin Latex Kit (Eiken Chemical Co., Ltd., Tokyo, Japan) and High Molecular Weight Adiponectin Assay Kit (Fujirebio Inc., Tokyo, Japan), respectively.

Statistical Analysis

Continuous variables were expressed as mean $\pm$ standard deviation. Comparisons between the 2 groups were performed using the Mann-Whitney U test for continuous variables, and the chi-square test for discrete variables. Pearson correlation test was used to evaluate the association between plasma total and HMW adiponectin levels and BMI and APRI in each cases and controls. Comparison analysis between histological grades was performed by the one-way ANOVA with Bonferroni corrections for post hoc comparisons. The relationships between total and HMW adiponectin and HCC histological grades of were determined using multiple logistic regression models. Data were reported as odds ratios (ORs) and 95% confidence intervals (95% CIs). All P values were 2-tailed, and $P<0.05$ was considered to be statistically significant. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL).

Results

Serum adiponectin levels and prevalence of HCC

The baseline clinical characteristics of the 97 cases and 97 controls were shown in Table 1. No significant differences were found between cases and controls according to AST level, alanine transaminase (ALT) level, APRI, diabetes mellitus, or BMI. The mean age of the cases was significantly higher than the mean age of the controls. The associations between serum total and HMW adiponectin levels and prevalence of HCC were shown in Figure 1. There were no significant differences between the mean total adiponectin levels of the cases and controls (15.5$\pm$10.4 μg/ml and 16.6$\pm$12.8 μg/ml, respectively, $P=0.670$) (Figure 1A), or the mean HMW adiponectin levels (10.1$\pm$7.4 μg/ml and 10.8$\pm$9.0 μg/ml, respectively, $P=0.752$) (Figure 1B).

Association between serum adiponectin levels and BMI

We examined the associations between serum total and HMW adiponectin levels and BMI in cases and controls. In controls,

Table 1. Baseline clinical characteristics in case and control.

	Case	Control	P value
Number	97	97	
Age (years)	67.4±8.3	61.2±11.4	<0.001
Gender (Female/Male)	30/67	30/67	Matched variable
AST (U/L)	61.8±28.3	60.0±28.5	0.632
ALT (U/L)	58.4±33.4	60.0±31.8	0.513
Platelet (×10^9/L)	115.0±51.4	115.5±52.5	Matched variable
APRI	1.5±1.0	1.5±1.1	0.863
Child-Pugh grade (A/B+C)	82/15	82/15	Matched variable
Diabetes mellitus (Absent/Present)	64/33	71/26	0.275
BMI (kg/m^2)	22.5±3.2	23.1±3.3	0.228
Total adiponectin (μg/ml)	15.5±10.4	16.6±12.8	0.670
HMW adiponectin (μg/ml)	10.1±7.4	10.8±9.0	0.752

Continuous variables presented as mean ± standard deviation.
Abbreviation: AST = aspartate aminotransferase; ALT = alanine aminotransferase; APRI = aspartate aminotransferase-to-platelet ratio index; BMI = body mass index; HMW = high molecular weigh.
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there were no inverse associations between serum total and HMW adiponectin levels and BMI ($r=-0.142$, $P=0.166$ for total adiponectin [Figure 2A] and $r=-0.144$, $P=0.160$ for HMW adiponectin [Figure 2B]). Similarly, in cases, there were also no inverse associations between serum total or HMW adiponectin levels and BMI ($r=-0.129$, $P=0.208$ for total adiponectin [Figure 2C] and $r=-0.131$, $P=0.201$ for HMW adiponectin [Figure 2D]).

Association between serum adiponectin levels and APRI

We also evaluated the associations between serum total and HMW adiponectin levels and APRI in cases and controls. In controls, serum levels of total and HMW adiponectin were positively associated with APRI ($r=0.482$, $P<0.001$ for total adiponectin [Figure 3A] and $r=0.476$, $P<0.001$ for HMW adiponectin [Figure 3B]). Similarly, in cases, serum levels of total and HMW adiponectin were positively associated with APRI ($r=0.491$, $P<0.001$ for total adiponectin [Figure 3C] and $r=0.485$, $P<0.001$ for HMW adiponectin [Figure 3D]).

Comparison of serum adiponectin levels according to HCC histological grades

The baseline clinical characteristics of 97 HCC cases were also separately evaluated according to the histological grades (Table 2). No significant differences were found between HCC histological grades and age, gender, AST level, ALT level, platelet count, APRI, Child-Pugh grade, diabetes mellitus, BMI, alpha-fetoprotein (AFP) level, des-gamma-carboxy prothrombin (DCP) level, and number of tumor. Patients with moderately and poorly differentiated HCC had significantly larger tumor sizes than patients with well-differentiated HCC. The associations between serum total and HMW adiponectin levels and HCC histological grades were shown in Figure 4. The mean total adiponectin levels in patients with moderately (13.4±6.9 μg/ml, $P=0.001$) and

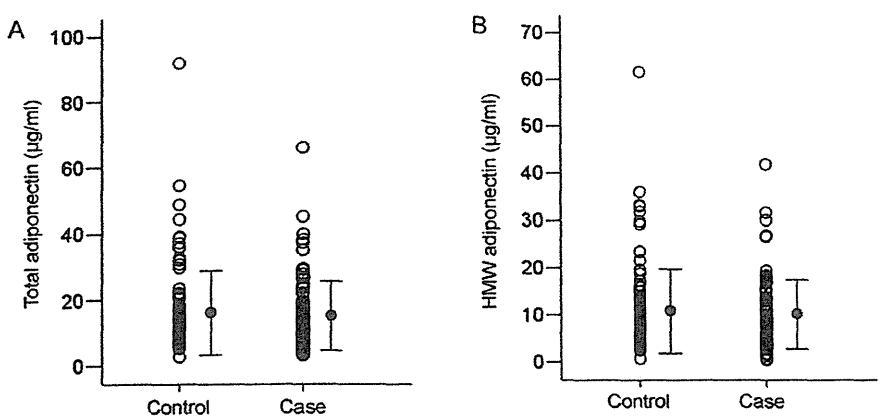

Figure 1. Comparison of adiponectin levels between 97 controls and 97 cases. A. Comparison of total adiponectin levels between patients with cases and controls ($P=0.670$). B. Comparison of HMW adiponectin levels between patients with cases and controls ($P=0.752$).
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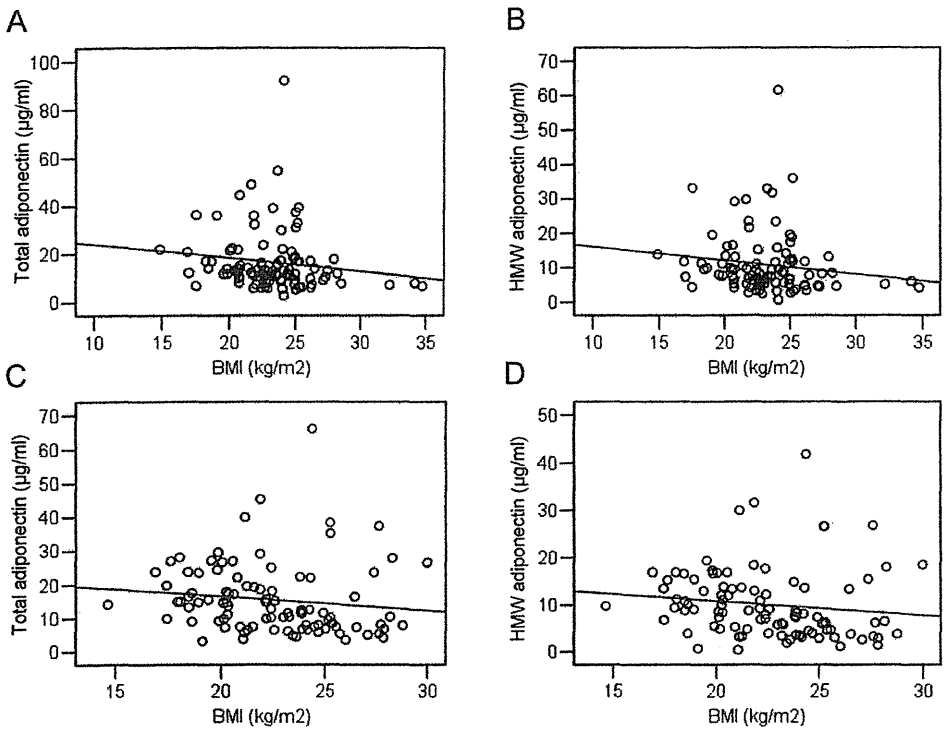

Figure 2. Serum adiponectin levels and body mass index (BMI). A. Correlation between serum total adiponectin levels and BMI in controls ($r=-0.142$, $P=0.166$). B. Correlation between serum high molecular adiponectin (HMW) adiponectin levels and BMI in controls ($r=-0.144$, $P=0.160$). C. Correlation between serum total adiponectin levels and BMI in cases ($r=-0.129$, $P=0.208$). D. Correlation between serum HMW adiponectin levels and BMI in cases ($r=-0.131$, $P=0.201$).
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poorly (11.5±7.3 μg/ml, $P<0.001$) differentiated HCC were significantly lower compared to those in patients with well-differentiated HCC (22.0±13.6 μg/ml). The mean HMW adiponectin levels in patients with moderately (8.6±4.7 μg/ml, $P<0.001$) and poorly (6.9±5.0 μg/ml, $P<0.001$) differentiated HCC were significantly lower compared to those in patients with well-differentiated HCC (14.9±9.5 μg/ml). In addition, multiple logistic regression analyses were performed to determine whether the serum total and HMW adiponectin levels were independently associated with HCC histological grades (Table 3). In the analysis adjusted for other variables, lower serum total adiponectin levels (12–24 μg/ml; OR: 9.33, 95% CI: 2.27–38.43 [$P=0.002$], <12 μg/ml; OR 11.76, 95% CI: 2.97–46.66 [$P<0.001$]) and serum HMW adiponectin levels (7–14 μg/ml; OR: 5.67, 95% CI: 1.66–19.33 [$P=0.006$], <7 μg/ml; OR 10.24, CI: 2.80–37.40 [$P<0.001$]) were independent risk factors for worse HCC histological grades.

Discussion

Obesity is known to be associated with various cancers and hypoadiponectinemia has been also shown to be a risk factor for these obesity-associated cancers [15,16,17,18]. Epidemiological evidence of the association between obesity and HCC is also rapidly increasing including patients with HCV infection. In a community-based cohort study, Chen et al. reported that obesity was an independent risk factor for HCC development in anti-HCV-seropositive subjects [9]. Therefore, we hypothesized that serum adiponectin level may be associated with the prevalence of HCC in patients with HCV infection. However, in this case-control study, serum total and HMW adiponectin levels were not significantly and inversely associated with the prevalence of HCC. Similarly, Nkontchou et al. also demonstrated that serum level of adiponectin is not predictive of HCC development in patients with compensated HCV cirrhosis [29]. In patients with HCV infection, it is well known that metabolic abnormalities such as obesity and diabetes are closely associated with hepatic steatosis [4] and severe fibrosis [5]; as a result, obesity affect as a partial factor of HCC development. On the other hand, several recent reports have shown that hypoadiponectinemia was associated with hepatic steatosis only in limited genotype [30] and association between hypoadiponectinemia and fibrosis progression was not found in patients with HCV infection [31]. In addition, we assessed the association between BMI and serum adiponectin levels in cases and controls. Our results showed that these adiponectin levels were not inversely associated with BMI, suggesting that adiponectin is not a cofactor in the development of HCC associated with obesity in patients with HCV infection.

Recently, several studies have been reported that serum adiponectin level was associated with progression of liver fibrosis in patients with chronic liver disease [19,20,21]. We also evaluated the association between serum total and HMW adiponectin levels and progression of liver fibrosis. APRI is a useful noninvasive marker for the prediction of liver fibrosis in chronic liver disease with HCV infection [28] and we assessed the degree of liver fibrosis by APRI in this study. As a result, serum total and HMW adiponectin levels were positively and significantly associated with APRI in both cases and controls. Tietge et al. reported that circulating adiponectin level increased in patients with liver

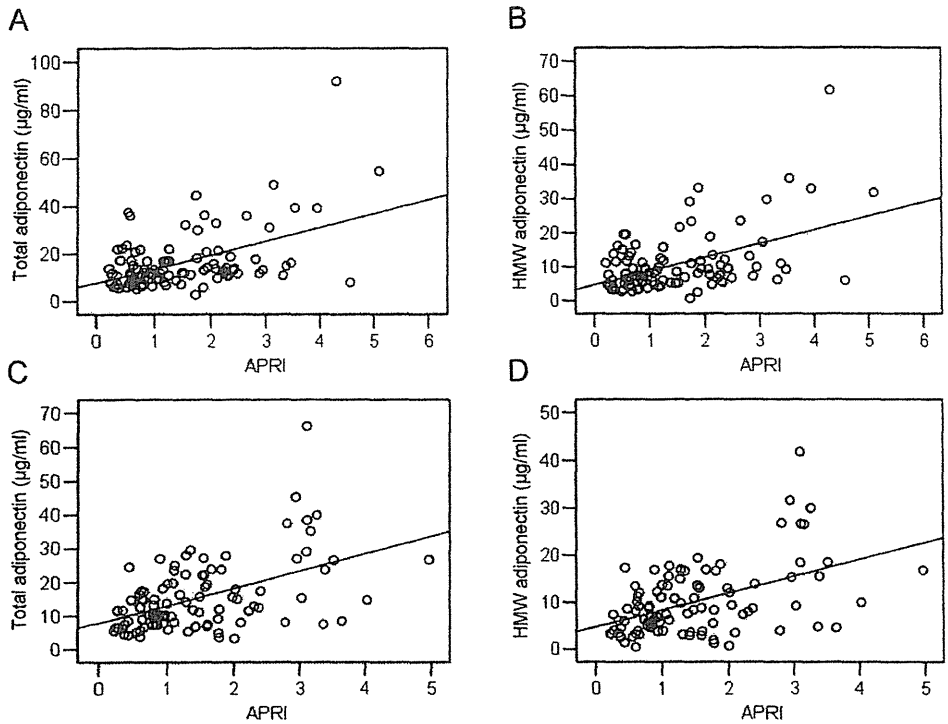

Figure 3. Serum adiponectin levels and aspartate aminotransferase-to-platelet ratio index (APRI). A. Correlation between serum total adiponectin levels and APRI in controls ($r=0.482$, $P<0.001$). B. Correlation between serum high molecular adiponectin (HMW) adiponectin levels and APRI in controls ($r=0.476$, $P<0.001$). C. Correlation between serum total adiponectin levels and APRI in cases ($r=0.491$, $P<0.001$). D. Correlation between serum HMW adiponectin levels and APRI in cases ($r=0.485$, $P<0.001$).
doi:10.1371/journal.pone.0026840.g003

Table 2. Association between clinical characteristics and histological grade in patients with hepatocellular carcinoma.

	Well	Moderate	Poor	*P* value
Number	30	39	28	
Age (years)	67.5±6.9	67.3±8.2	67.1±9.9	0.942
Gender (Female/Male)	11/19	12/27	7/21	0.630
AST (U/L)	67.2±23.8	59.5±28.8	59.3±25.9	0.409
ALT (U/L)	61.0±35.5	60.0±33.1	53.5±32.1	0.647
Platelet count ($\times10^9$/L)	103.1±46.5	116.9±47.6	125.1±54.0	0.228
APRI	1.8±1.1	1.3±0.9	1.3±0.9	0.213
Child-Pugh grade (A/B+C)	22/8	36/3	24/4	0.095
Diabetes mellitus (Absent/Present)	24/6	21/18	19/9	0.073
BMI (kg/m^2)	22.5±3.2	22.4±3.0	22.6±3.5	0.949
AFP (ng/ml)	51.3±74.4	165.0±448.4	560.1±1508.8	0.065
DCP (mAU/ml)	65.5±139.5	464.2±978.8	276.1±586.4	0.070
Tumor size (mm)	21.0±4.9	26.8±10.0†	26.6±11.8†	0.027
Tumor number (single/2–3)	19/11	29/10	19/9	0.609
Total adiponectin (μg/ml)	22.0±13.6	13.4±6.9†	11.5±7.3†	<0.001
HMW adiponectin (μg/ml)	14.9±9.5	8.6±4.7†	6.9±5.0†	<0.001

Continuous variables presented as mean ± standard deviation.
Abbreviation: AST = aspartate aminotransferase; ALT = alanine aminotransferase;
APRI = aspartate aminotransferase-to-platelet ratio index; BMI = body mass index;
AFP = alpha-fetoprotein; DCP = des-gamma-carboxy prothrombin; HMW = high molecular weigh.
†$P<0.05$ for Bonferroni corrected post hoc comparison with well-differentiated hepatocellular carcinoma.
doi:10.1371/journal.pone.0026840.t002

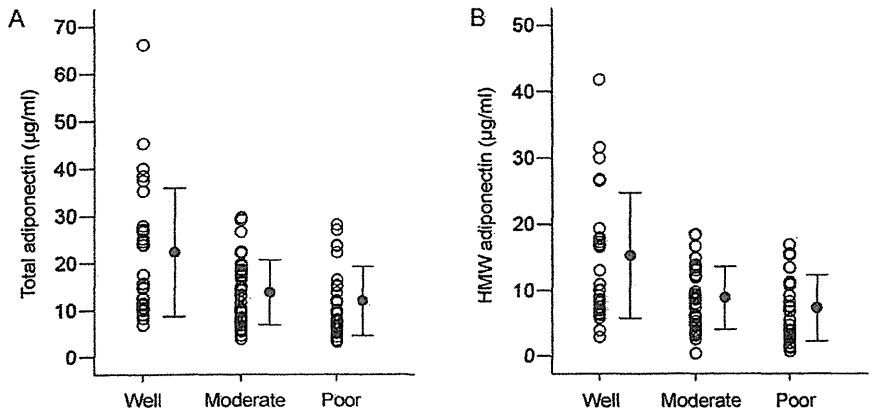

Figure 4. Comparison of adiponectin levels according to histological grades in 97 cases. A. The serum total adiponectin levels in patients with moderately ($P=0.001$) and poorly ($P<0.001$) differentiated hepatocellular carcinoma (HCC) were significantly lower compared to those in patients with well-differentiated HCC. B. The serum high molecular weight (HMW) adiponectin levels in patients with moderately ($P<0.001$) and poorly ($P<0.001$) differentiated HCC were significantly lower compared to those in patients with well-differentiated HCC.
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cirrhosis, because of reduced liver function as a major source of adiponectin extraction and altered hepatic hemodynamics [20]. Taken together serum total and HMW adiponectin levels may be a predictors of liver fibrosis in patients with HCV infection.

Next, we examined the relationship between serum adiponectin levels and HCC histological grades. Interestingly, low total and HMW adiponectin levels were independent risk factors for worse HCC histological grades. It is generally known that majority of HCC arises as very well-differentiated cancers and proliferate in a stepwise process of dedifferentiation. When small HCC of the early-stage reach around 1.5–2.0 cm, moderately or poorly differentiated cancer tissues develops within the well-differentiated cancer tissue, and well-differentiated cancer tissue are replaced by less differentiated cancer tissue in so-called advanced HCC [32]. In this study, the mean tumor size of well-differentiated HCC was 14.2±3.2 mm, which was significantly smaller than the mean size of moderately and poorly differentiated HCC. This result indicated that a dedifferentiation of HCC is associated with tumor proliferation.

This is a cross-sectional study and causal relationship between serum adiponectin level and dedifferentiation of HCC is unclear. One would think that dedifferentiation of HCC may trigger a mechanism which leads to decreased serum adiponectin level. However, it seems that hypoadiponectinemia may trigger dedifferentiation of HCC because of followings: Saxena et al. showed that adiponectin increased the phosphorylation of AMP-activated protein kinase (AMPK) and the TSC2 tumor suppressor, and inhibited phosphorylation of the mammalian target of rapamycin (mTOR) in vitro assay using Huh7 and HepG2 HCC cells [11]. Moreover, microarray analysis of tissue adiponectin expression levels in human HCC patients revealed that adiponectin expression was inversely correlated with tumor size [11]. Miyazaki et al. showed that adiponectin stimulated c-Jun NH2-terminal kinase (JNK) activation and suppressed signal transducer and activator of transcription 3 (STAT3) activation in HepG2 HCC cells [33]. Thus, these studies support that adiponectin level may inhibit proliferation and differentiation of HCC.

Table 3. Association between total and HMW adiponectin and histological grade in patients with hepatocellular carcinoma by multiple logistic regression analysis.

	Model 1		Model 2	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Total adiponectin (μg/ml)				
24<	Reference		Reference	
12–24	6.67 (1.83–24.3)	0.004	9.33 (2.27–38.43)	0.002
<12	9.87 (2.76–35.2)	<0.001	11.76 (2.97–46.66)	<0.001
HMW adiponectin (μg/ml)				
14<	Reference		Reference	
7–14	4.38 (1.40–13.64)	0.011	5.67 (1.66–19.33)	0.006
<7	9.92 (2.91–33.85)	<0.001	10.24 (2.80–37.40)	<0.001

Abbreviation: OR = odds ratio; 95% CI = confidence interval; HMW = high molecular weigh.
Model 1: adiponectin only.
Model 2: adiponectin and covariates in Table 2.
doi:10.1371/journal.pone.0026840.t003

Angiogenesis is also an important process for proliferation, dedifferentiation, and metastasis of HCC. In small sized and well-differentiated HCCs, artery-like vessels are not well developed [34]. On the other hand, in moderately or poorly differentiated HCCs with 2 cm or larger, artery-like vessels are well developed [35], and these tumors display high proliferation and metastasis so-called advanced stage. Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen, and is the most important factor in tumor angiogenesis [36]. Yamaguchi et al. showed that VEGF expression in well-differentiated HCC was higher than expression in moderately and poorly differentiated HCC [37]. Well-differentiated HCCs that are 1.0–1.5 cm would be in the transitioning from the portal blood supply to an arterial blood supply, which would result in increased VEGF expression because of relative hypoxia from low blood flow. Therefore, high VEGF expression in small-sized and Well-differentiated HCCs suggests that VEGF plays an important role during relatively early angiogenesis stages in HCC. Previous studies have reported on the the molecular mechanisms involved in the negative association of adiponectin with tumor angiogenesis [38,39]. Man et al. showed in an orthotopic liver tumor nude mouse model that adiponectin suppresses tumor growth through inhibition of tumor angiogenesis [39]. The molecular mechanism involves adiponectin downregulation of VEGF expression through inhibition of tumor-associated macrophages in tumor tissue. Moreover, the nude mice administrated adiponectin had significantly lower circulating VEGF levels than the control. This result suggests that adiponectin may inhibit dedifferentiation in well-differentiated HCC by inhibition of tumor angiogenesis-related VEGF.

Adiponectin exists mainly in three forms [12]. HMW adiponectin is thought to have higher biological activity than the other forms of adiponectin, especially in the liver [12,13]. Several studies have reported that HMW adiponectin levels or the ratio of HMW to total adiponectin is inversely and more strongly associated with metabolic risk factors than total adiponectin levels [40,41]. However, it remains unknown whether HMW adiponectin has more strongly potential actions on cancer pathophysiology than total adiponectin. In this study, we demonstrated that total and HMW adiponectin were independent risk factors for HCC histological grade. However, odds ratio of these factors were similar and serum total adiponectin levels were significantly associated with serum HMW adiponectin levels in this study (data not shown). Thus, one would think that impact of HMW adiponectin may be equal to total adiponectin for predicting HCC histological grade and testing for either total or HMW adiponectin levels may be as effective as testing both levels.

In conclusion, our data suggested that serum total and HMW adiponectin levels were predictors of liver fibrosis, but not prevalence of HCC in patients with chronic HCV infection. Moreover, we showed that low total and HMW adiponectin levels were independent risk factors for worse histological grades of HCC. Further study will be focused on the causal relationship between hypoadiponectinemia and dedifferentiation of HCC.

Author Contributions

Conceived and designed the experiments: SS TK M. Sata. Performed the experiments: SS MN. Analyzed the data: SS AT M. Satani. Contributed reagents/materials/analysis tools: MN M. Satani SY TN. Wrote the paper: SS TK RK M. Sata TT.

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