

CLINICAL STUDIES

HBcrAg is a predictor of post-treatment recurrence of hepatocellular carcinoma during antiviral therapy

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Keywords

covalently closed circular DNA – HBcrAg – HCC recurrence – nucleot(s)ide analogue – portal vein invasion

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Abstract

Background/Aims: The recurrence rate of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) is high even in patients receiving curative therapy. In this study, we analysed the risk factors for tumour recurrence after curative therapy for HBV-related HCC while under treatment with nucleot(s)ide analogues (NAs) by measuring serum HBcrAg and intrahepatic covalently closed circular DNA (cccDNA) levels to elucidate the viral status associated with HCC recurrence. **Methods:** We enrolled 55 patients who developed HCC during NA therapy and underwent either curative resection or percutaneous ablation for HCC. **Results:** Hepatocellular carcinoma recurred in 21 (38%) of the patients over a period of 2.2 (range, 0.2–7.4) years. In multivariate analysis, serum HBcrAg levels $\geq 4.8 \log U/ml$ at the time of HCC diagnosis (hazard ratio, 8.96; 95% confidential interval, 1.94–41.4) and portal vein invasion (3.94, 1.25–12.4) were independent factors for HCC recurrence. The recurrence-free survival rates of the high cccDNA group were significantly lower than those of the low cccDNA group only in patients who underwent resection ($P = 0.0438$). A positive correlation ($P = 0.028$; $r = 0.479$) was observed between the intrahepatic cccDNA and the serum HBcrAg levels at the incidence of HCC. **Conclusion:** HBcrAg is a predictor of the post-treatment recurrence of HCC during antiviral therapy. Serum HBcrAg and intrahepatic cccDNA suppression by NAs may be important to prevent HCC recurrence.

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, and one million people die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually (1, 2). Recently, oral nucleot(s)ide analogues (NAs) have been used as the mainstay therapeutic strategy against chronic hepatitis B. Five such antiviral agents have been approved, and range in the profundity and rapidity of HBV DNA suppression, barrier to resistance and side-effect profile (3–10). Lamivudine (LAM) was the first NA to be approved for treating chronic hepatitis B, followed by adefovir dipivoxil (ADV) and entecavir (ETV), in Japan. However, a major problem with long-term LAM treatment is the potential development of drug resistance, mainly caused by mutation of the tyrosine–methionine–aspartic acid–aspartic acid (YMDD) motif of reverse transcriptase (11, 12). For preventing breakthrough hepatitis induced by LAM-resistant mutants, additional ADV administration has been recommended (13, 14).

The methods for monitoring the treatment response include measurements of the serum alanine transaminase

(ALT) levels, HBV DNA levels, HBcAg and antibody levels, HBsAg and antibody levels and liver histology. Other serum markers have been reported to be useful for monitoring the effect of antiviral therapy (15, 16). Recently, a new assay was developed for detecting the HBcrAg, consisting of HBcAg, HBeAg and a 22 kDa precore protein coded with the precore/core gene (17, 18). Because NAs have no inhibiting action on the transcription and translation activities of viral mRNA, HBcAg- and HBeAg-related proteins continue to be produced for a certain period of time in spite of the achievement of adequate suppression of the viral DNA synthesis. Therefore, HBcrAg is a viral marker independent of HBV DNA for monitoring the antiviral effect of NAs (19). In addition, recent reports have indicated another interesting aspect of serum HBcrAg levels: these levels were found to be correlated with intrahepatic covalently closed circular DNA (cccDNA) levels and could be a surrogate marker of the intrahepatic cccDNA pool (20, 21). This phenomenon may be explained by the fact that the production of HBcrAg depends on the

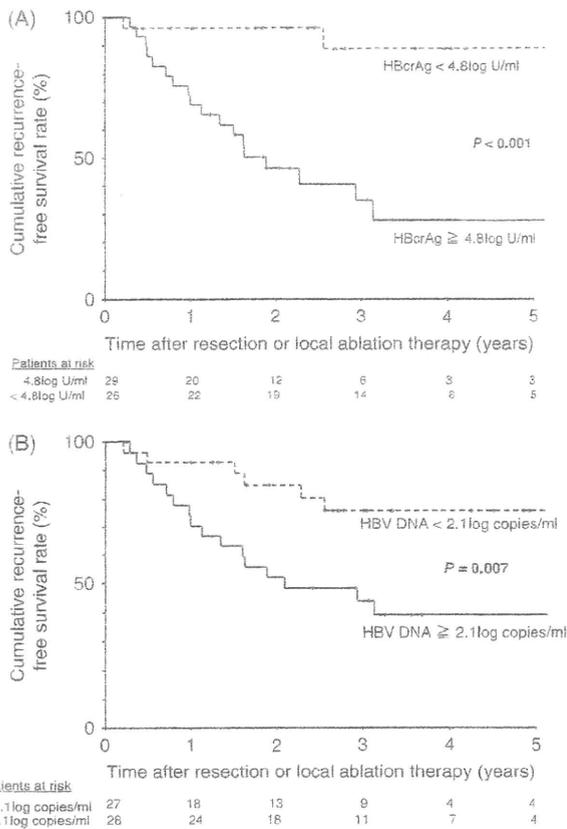


Fig. 3. (A) Kaplan-Meier life table for the cumulative recurrence-free survival rates by the serum HBcrAg levels and comparison by the log-rank test. (B) Kaplan-Meier life table for the cumulative recurrence-free survival rates by the serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient and comparison by the log-rank test.

developed HCC after the commencement of NA therapy and underwent radical therapy for HCC. The recurrence rates of HCC were high in patients with high levels of intrahepatic cccDNA and serum HBcrAg. In particular, HBcrAg levels were measurable by using serum samples and clinically useful.

Nucleot(s)ide analogues, including LAM, ADV and ETV, are widely used for the treatment of chronic hepatitis B, and reportedly reduce the development of HCC in such patients (22, 23). Although few events of HCC development occur during NA therapy (24–26), analysis of a large number of patients is needed to examine the risk factors for HCC. We could clarify the risk factors associated with the development of primary HCC after radical therapy by enrolling patients who underwent radical therapy for HCC in spite of their small number. High HBV loads in serum have been reported to be associated with HCC recurrence after resection or radical therapy in NA-naïve patients (27–31), but no study has demonstrated the viral risk factors of recurrence in patients receiving NAs. The novel finding of this study is that serum HBcrAg and

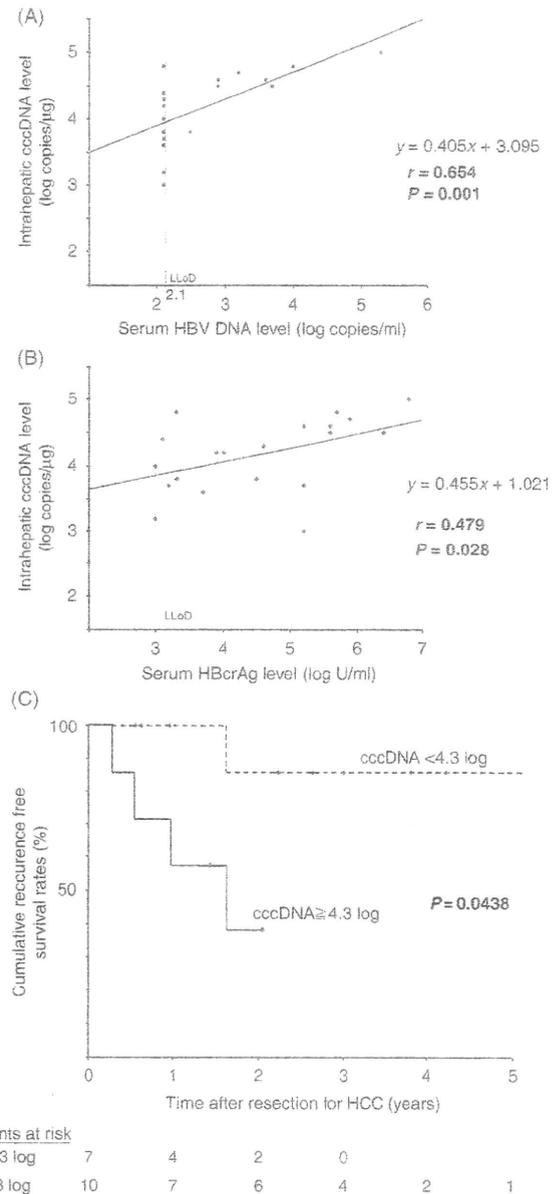


Fig. 4. (A) Correlation between intrahepatic covalently closed circular DNA (cccDNA) and serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient who underwent resection ($n = 22$). (B) Correlation between intrahepatic cccDNA and serum HBcrAg levels at the time of HCC diagnosis. (C) Kaplan-Meier life table for the cumulative recurrence-free survival rates by the intrahepatic cccDNA levels in patients with early- or intermediate-stage HCC ($n = 17$).

intrahepatic cccDNA levels are predictors of HCC recurrence in patients radically treated for HCC during NA therapy.

In this study, the serum HBV DNA levels at the time of HCC diagnosis were associated with recurrence by univariate analysis. However, the serum HBcrAg level was the only viral factor associated with recurrence in multivariate analysis. There are two possible reasons for the

intrahepatic cccDNA levels is slower than that of serum HBV DNA levels during NA administration (15, 16). We found that suppression of cccDNA by NAs could prevent the development of recurrent primary HCC. Because cccDNA provides the template for pregenomic and viral messenger RNA-encoded viral proteins (33–35), the transcriptional activity of cccDNA may induce carcinogenesis. Further research is required to validate this hypothesis. Serum HBcrAg can be a surrogate marker of the intrahepatic cccDNA pool because of the viral proteins transcribed through messenger RNA from cccDNA (20, 21). Therefore, we consider that serum HBcrAg reflects the intrahepatic viral status more accurately than serum HBV DNA. Recently, Chan *et al.* (36) showed that serum HBsAg quantification could reflect intrahepatic cccDNA in patients treated with peginterferon and LAM combination therapy. They also indicated that reduction in HBsAg had good correlation with reduction in cccDNA. We tried to measure HBsAg levels at the start of NA therapy and the time of HCC diagnosis using a commercial assay (chemiluminescent immunoassay). However, HBsAg levels declined very slowly during NAs monotherapy in this study (data not shown). Brunetto *et al.* (37) showed that mean reduction for 48 weeks in HBsAg was 0.02logIU/ml in patients treated with LAM monotherapy, different from peginterferon therapy. Meanwhile, the median reduction from the start of NA to the diagnosis of HCC in HBcrAg was 1.4logU/ml in this study (Table 1). It seems that HBcrAg is a superior on-treatment risk predictor (e.g. tumour recurrence) to HBsAg during NAs monotherapy in terms of reduction of titres in each assay. HBcrAg is also more useful in terms of needless to serum sample dilution. As HBcrAg levels can be measured from serum samples, they are clinically useful, compared with the measurement of cccDNA, which requires liver specimens. It is not practical to carry out liver biopsy and the measurement of cccDNA for patients who have normal AST/ALT levels and viral suppression during antiviral therapy. Liver specimens cannot be also taken from patients who undergo ablation therapy for HCC. The measurement of serum HBcrAg levels in these patients is helpful to indirectly estimate the status of intrahepatic cccDNA. In the future, it is necessary to investigate whether HBcrAg in patients receiving NAs can be a predictor of primary carcinogenesis.

Previous studies have indicated that the rates of intrahepatic cccDNA loss and serum HBcrAg loss differ from serum HBV DNA loss under NA therapy, with the former two being much slower (15, 16, 19). In this study, the period of serum HBV DNA loss was longer, with lower intrahepatic cccDNA and serum HBcrAg levels (Fig. 2B). Therefore, these findings suggest that a long period of time is required to prevent the development of recurrent primary HCC by viral suppression under antiviral therapy. In contrast, the serum HBcrAg levels at the time of HCC diagnosis were higher in patients with emergent LAM-resistant mutants and subsequent VBT

than in patients without mutants and VBT (Fig. 6). This result suggests that it is important to administer a potent NA early for drug-resistant strains and suppress viral replication to prevent subsequent carcinogenesis. Although we evaluated the relationship between the development of primary HCC and serum HBcrAg levels by a case-control study, the serum HBcrAg levels at the commencement of NA therapy and 1 year later were not associated with the development of primary HCC (unpublished data). This finding is attributable to the slow decline of the serum HBcrAg levels during antiviral therapy. The measurement of HBcrAg at intervals of 3–6 months may be helpful to predict the development of HCC. However, further studies are needed to confirm the finding.

In summary, HBcrAg is a predictor of the post-treatment recurrence of HCC during antiviral therapy. Measurement of the serum HBcrAg level is simple and useful because it reflects the intrahepatic viral status. Further, intrahepatic cccDNA and serum HBcrAg suppression by NAs is important to prevent HCC recurrence.

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References

1. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733–45.
2. Ganem D, Prince AM. Hepatitis B virus infection – natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118–29.
3. Lai CL, Chien RN, Leung NW, *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61–8.
4. Dienstag JL, Schiff ER, Wright TL, *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256–63.
5. Marcellin P, Chang TT, Lim SG, *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; **348**: 808–16.
6. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. *N Engl J Med* 2003; **348**: 800–7.
7. Chang TT, Gish RG, de Man R, *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; **354**: 1001–10.
8. Lai CL, Shouval D, Lok AS, *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; **354**: 1011–20.

Original Article

New classification of dynamic computed tomography images predictive of malignant characteristics of hepatocellular carcinoma

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Aim: The aim of this study was to elucidate whether the histopathological characteristics of hepatocellular carcinoma (HCC) can be predicted from baseline dynamic computed tomography (CT) images.

Methods: This retrospective study included 86 consecutive patients with HCC who underwent surgical resection between January 2000 and September 2008. The arterial- and portal-phase dynamic CT images obtained preoperatively were classified into four enhancement patterns: Type-1 and Type-2 are homogeneous enhancement patterns without or with increased arterial blood flow, respectively; Type-3, heterogeneous enhancement pattern with septum-like structure; and Type-4, heterogeneous enhancement pattern with irregular ring-like structures. We also evaluated the predictive factors for poorly-differentiated HCC, specific macroscopic type of HCC (simple nodular type with extranodular growth [SNEG] and confluent multinodular [CMN]) by univariate and multivariate analyses.

Results: The percentages of poorly-differentiated HCC according to the enhancement pattern were three of 51

nodules (6%) of Type-1 and -2, three of 24 (13%) of Type-3, and eight of 11 (73%) of Type-4. The percentages of SNEG/CMN according to the enhancement pattern were 12 of 51 nodules (24%) of Type-1 and -2, 13 of 24 (54%) of Type-3, and five of 11 (45%) of Type-4. Multivariate analysis identified Type-4 pattern as a significant and independent predictor of poorly-differentiated HCC ($P < 0.001$) while Type-3 pattern was a significant predictor of SNEG/CMN ($P = 0.017$).

Conclusion: Heterogeneity of dynamic CT images correlates with malignant characteristics of HCC and can be potentially used to predict the malignant potential of HCC before treatment.

Key words: confluent multinodular type, dynamic computed tomography, hepatocellular carcinoma, poorly-differentiated hepatocellular carcinoma, radiofrequency ablation, simple nodular type with extranodular growth type.

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is a common malignancy worldwide, and the incidence rate is increasing in Japan as well as in the USA.^{1–3} Chronic viral hepatitis and liver cirrhosis following 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 at 5–10% per annum, and it is one of the major causes of death, especially in Asian countries.⁵ Among the available treatment options for HCC, surgical resection is generally considered a potentially curative method and could provide a satisfactory long-term outcome.^{6–13} Recent advances in imaging procedures have led to increased detection of early-stage HCC and improved survival because of the greater number of patients identified in whom curative hepatic resection is possible.^{14,15} However, for patients who are not suitable for surgical treatment for several reasons (e.g. lack of sufficient liver function for surgical resection), percutaneous local therapy is another therapeutic option. Various methods, such as percutaneous ethanol

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injection (PEI), percutaneous acetic acid injection (PAI), cryotherapy, percutaneous microwave coagulation therapy (PMCT) and radiofrequency ablation (RFA) are available for local therapy. In addition to surgical resection, local ablation therapy especially RFA is considered potentially curative for HCC and provides better long-term outcome.¹⁶ However, despite the high complete necrosis rate in RFA, some patients show tumor recurrence within 1 year, either local recurrence or new tumor formation. A series of studies discussed the predictive factors involved in tumor recurrence and seeding including tumor size, subcapsular lesion, α -fetoprotein (AFP) levels, tumor staging and histopathological grading of HCC.^{17,18} Another study has reported that the specific macroscopic type of HCC relevant to microvascular invasion on histopathological examination could help predict recurrence, and that this is especially true for simple nodular type with extranodular growth (SNEG) and confluent multinodular type (CMN) tumors.¹⁹ For the above reasons, it is important to determine the histopathological grade and macroscopic type of HCC before the application of local ablation therapy.

One aim of the present study was to determine whether malignant characteristics of HCC (especially poorly-differentiated HCC, SNEG and CMN) can be diagnosed by dynamic computed tomography (CT) images obtained before treatment. We reported previously that angiographic hypervascularity corresponds with thick-walled, nuclei-rich, and slender-shaped non-triadal vessels (named "Type II vessels") identified by immunohistochemical staining for α -smooth muscle actin (α -SMA).²⁰ The other purpose of the present study was to correlate heterogenic enhancement pattern of the arterial- and portal-phase dynamic CT images with the distribution patterns of α -SMA-positive non-triadal vessels in HCC.

METHODS

Study population

FROM JANUARY 2000 to September 2008, 340 patients were diagnosed with HCC and received surgical resection as initial treatment in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan. Among the 340 patients, 86 patients satisfied the following criteria: (i) triple-phase dynamic CT study was performed before surgical resection; (ii) preoperative diagnosis of a solitary HCC with a maximum tumor diameter of 50 mm; (iii) no evidence of extrahepatic metastases as confirmed by imaging studies (CT, ultra-

sonography [US] and chest X-ray) before procedure; (iv) no history of other malignancies; and (v) no preoperative chemotherapy including transcatheter chemoembolization. Accordingly, 86 patients with HCC who were underwent surgical resection for HCC were retrospectively evaluated for the relationship between heterogeneous enhancement pattern of the arterial- and portal-phase dynamic CT images and histopathological malignant characteristics of HCC. The observation starting point was the time of the first surgical resection for HCC.

Imaging analysis of HCC and definition of enhancement pattern

Before surgery, triple-phase contrast-enhanced CT was performed in all patients. In these studies, 95 mL of 350 mg I/mL Iomeprol (Iomeron 350, Eisai, Tokyo), as the contrast medium, was rapidly injected i.v. at 0.06 mL/kg bodyweight/s. Phase-1, -2, and -3 imaging were performed at 25, 60 and 180 s after the start of injection, respectively. The axial images were reconstructed at intervals of 5 mm. The enhancement pattern on the arterial- and portal-phase 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). 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 "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 "heterogeneous enhancement pattern with septum-like structure"; with heterogeneous enhancement and septum-like formation in the arterial phase, while the septum-like structure resembled a near-uniform tumor tissue periphery in the portal phase. The Type-4 pattern represented "heterogeneous enhancement pattern with irregular ring-like structures" in the arterial phase; marked by the presence of irregularly-shaped ring areas of enhancement and areas of little blood flow relative to the periphery of the tumor tissue, and in the portal phase, by areas of reduced blood flow. The enhancement pattern on the arterial- and portal-phase dynamic CT was determined by three expert hepatologists blind to the pathological result.

Histopathological features

Tumor differentiation was graded histologically according to the classification of the Liver Cancer Study Group of Japan.²¹ Macroscopic classification of nodular type

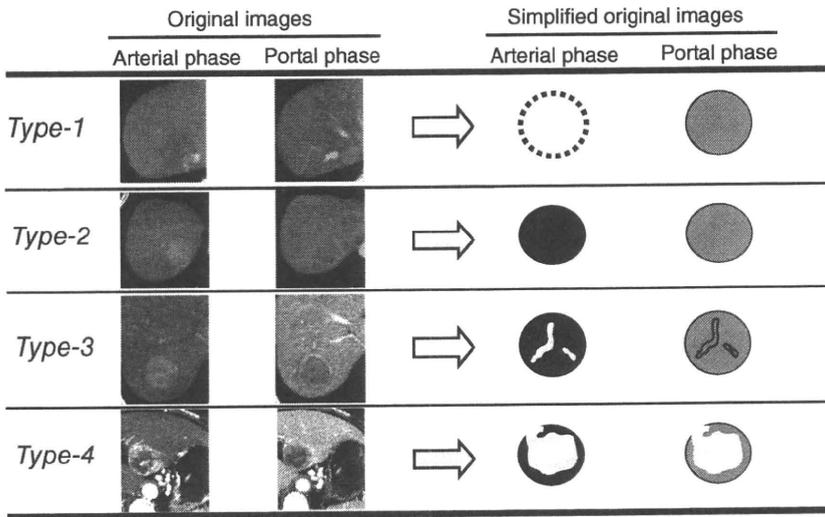


Figure 1 Sample of original dynamic computed tomography images and simplified images for each enhancement pattern.

HCC was based primarily on the definition of Kanai *et al.*²² and the Liver Cancer Study Group of Japan:²¹ small nodular type with indistinct margin (SNIM type, indistinct margins and containing portal tracts); simple nodular type (SN type, round nodule with clear margin); simple nodular type with extranodular growth (SNEG type, similar to the SN type with extranodular growth); confluent multinodular type (CMN type, nodular lesion consisting of a cluster of small and confluent nodules); and infiltrative type (IF type, nodular lesion with irregular and indistinct margins). The histopathological diagnosis of HCC was established by consensus of at least two pathologists and three hepatologists.

Immunohistochemical staining

Part of the liver specimen was soaked in neutral formalin solution immediately after resection and fixed for 24–48 h. Paraffin-embedded fixed specimens were sliced into 2 µm thick sections. After deparaffinization,

the sections were immunostained with α-SMA monoclonal antibody (Actin, α-smooth muscle, clone 1A4; Ventana Medical Systems, Tucson, AZ, USA) using an automated system (BenchMark; Ventana Medical Systems). When a round, oval or slender ring-shaped structure was identified in the α-SMA-immunostained specimen, it was regarded as an abnormal new blood vessel irrespective of its site in the liver, provided it was unrelated to the portal area. Therefore, we regarded such ring-shaped α-SMA-stained structures as non-triadal vessels and defined them as “positive neovascularity”. We have also reported thick-walled, nuclei-rich, and slender-shaped α-SMA-positive vessels (Fig. 2a “HE staining”, Fig. 2b “α-SMA staining”) in HCC tissue that were closely related to angiographic hypervascularity.²⁰ This type of blood vessel was termed “Type-II vessel”. To understand the relation between the findings on dynamic CT and immunohistochemical pattern, we analyzed the relationship between Type-4 pattern and the distribution of Type-II vessels.

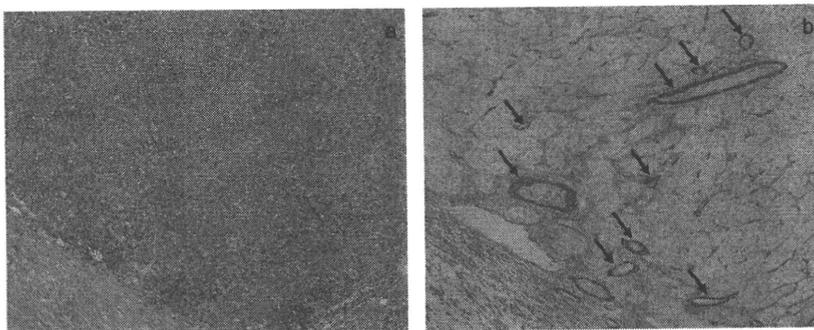


Figure 2 (a) Hematoxylin–eosin staining of hepatocellular carcinoma tissue. (b) Immunohistochemical staining for α-smooth muscle actin (α-SMA) of the same slice shown in (a). Solid arrows: typical Type II vessels. (Original magnifications: [a] ×100; [b] ×100.)

Table 1 Clinical profile and laboratory data of 86 patients with HCC

Sex (M : F)	57:29
Age (years)	62 (35–80)
Background of liver disease	
Hepatitis B surface antigen positive	26
Anti-HCV antibody positive	50
Both negative	10
Status of liver function	
Child–Pugh classification (A/B/C)	83/3/0
Preoperative image diagnosis of HCC	
Tumor diameter (mm)	23 (9.0–50)
Portal vein invasion (yes/no)	0/86
Laboratory data	
Platelet count ($\times 10^4/\mu\text{L}$)	13.0 (4.0–30.1)
Albumin (g/dL)	4.0 (3.1–4.3)
Bilirubin (mg/dL)	1.0 (0.3–1.7)
AST (IU/L)	43 (18–386)
Prothrombin time (%)	91 (60–124)
ICG-R15 (%)	19 (3.0–68)
AFP ($\mu\text{g/L}$)	13 (1.0–5541)
DCP (AU/L)	19 (4.0–1650)

AFP, α -fetoprotein; AST, aspartate aminotransferase; DCP, des- γ -carboxy prothrombin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ICG-R15, indocyanine green retention rate at 15 min.

Clinical background and laboratory data

Table 1 summarizes the clinical profile and laboratory data of 86 HCC patients in this study. The male : female ratio was 1.97:1. HCV antibody was detected in 58.1% of the patients, and 96.5% patients were classified as Child–Pugh A but none of the patients was classified as Child–Pugh C. Based on preoperative image analysis, the median tumor diameter was 23 mm and none of the patients had portal vein tumor invasion. Furthermore, the median levels of AFP and des- γ -carboxy prothrombin (DCP) were 13 $\mu\text{g/L}$ and 19 AU/L, respectively.

Statistical analysis and ethical considerations

The factors associated with poorly-differentiated HCC, SNEG and CMN were analyzed by the χ^2 -test and Fisher's exact test. The independent factors associated with preoperative diagnosis of poorly-differentiated HCC were identified by multivariate logistic regression analysis. The potential predictive factors for poorly-differentiated HCC, SNEG and CMN were age, sex, hepatitis B surface antigen (HBsAg), HCV antibody, platelet count, aspartate transaminase (AST), albumin, bilirubin, AFP, DCP, prothrombin activity, indocyanine

green retention rate at 15 min (ICG-R15), and tumor size. Several variables were transformed into categorical data consisting of two or three simple ordinal numbers for univariate and multivariate analyses. All factors that were at least marginally associated with poorly differentiated HCC, SNEG and CMN ($P < 0.10$) in univariate analysis were entered into a multivariate logistic regression analysis. Significant variables were selected by the stepwise method. A two-tailed P -value less than 0.05 was considered significant. Data analysis was performed using the SPSS ver. 11.0 software.

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

RESULTS

Distribution of enhancement patterns and proportions of histopathological types

PREOPERATIVE IMAGE ANALYSIS of 86 HCC patients showed the following results: Type-1 in 10 (11%) patients; Type-2 in 41 (48%) patients; Type-3 in 24 (28%) patients; and Type-4 in 11 (13%) patients. Furthermore, the percentages of poorly-differentiated HCC according to the enhancement pattern were zero of 10 (0%) patients with Type-1, three of 41 (7%) with Type-2, three of 24 (13%) with Type-3, and eight of 11 (73%) with Type-4. The percentages of SNEG/CMN according to the enhancement pattern were 12 of 51 nodules (24%) of Type-1 and -2, 13 of 24 (54%) of Type-3, and five of 11 (45%) of Type-4 (Table 2).

Correlation between preoperative features and diagnosis of poorly-differentiated HCC

We also investigated the factors that correlated with preoperative diagnosis of poorly-differentiated HCC. Univariate analysis showed the type of enhancement pattern (Type-4, Type-3 or other enhancement pattern, $P < 0.001$), tumor size ($<35 \text{ mm}/\geq 35 \text{ mm}$, $P = 0.005$), serum AST level ($<40 \text{ IU}/\geq 40 \text{ IU}$, $P = 0.069$) and serum DCP level ($<30 \text{ AU/L}/\geq 30 \text{ AU/L}$, $P = 0.079$) to correlate with preoperative diagnosis of poorly-differentiated HCC. These parameters were entered into multivariate logistic regression analysis. The percentage of poorly-differentiated HCC was significantly higher for large-size HCC ($\geq 35 \text{ mm}$, risk ratio 14.72, 95% confidence interval [CI] 1.15–188.10) and Type-4 enhancement pattern (risk ratio 12.86, 95% CI 1.56–105.94) (Table 3).

Correlation between preoperative features and diagnosis of SNEG and CMN types

We also investigated the factors associated with the development of SNEG and CMN types of HCC. Univariate

Table 2 Distribution of enhancement patterns and frequency of each macroscopic type and poorly-differentiated HCC by histological examination

Enhancement pattern	No. of nodules	Poorly-differentiated HCC	Distribution of macroscopic type according to the enhancement pattern					
			SNIM	SN	SNEG	CMN	IF	
Type-1	10/86 (11%)	0/10 (0%)	5/10 (50%)	3/10 (30%)	1/10 (10%)	0/10 (0%)	1/10 (10%)	
Type-2	41/86 (48%)	3/41 (7%)	2/41 (5%)	27/41 (66%)	7/41 (17%)	4/41 (10%)	1/41 (2%)	
Type-3	24/86 (28%)	3/24 (13%)	0/24 (0%)	11/24 (46%)	10/24 (42%)	3/24 (13%)	0/24 (0%)	
Type-4	11/86 (13%)	8/11 (73%)	0/11 (0%)	6/11 (55%)	3/11 (27%)	2/11 (18%)	0/11 (0%)	

CMN, confluent multinodular type; HCC, hepatocellular carcinoma; IF, infiltration type; SN, simple nodular type; SNEG, simple nodular type with extranodular growth; SNIM, small nodular type with indistinct margin.

Table 3 Results of multivariate logistic regression analysis for predictive factors of poorly-differentiated hepatocellular carcinoma according to preoperative factors

Factors	Category	Hazard ratio (95% confidence interval)	P-value
Tumor size (mm)	1: <35	1	0.039
	2: ≥35	14.72 (1.15–188.10)	
Type of enhancement pattern	1: Type-1 and -2	1	0.87
	2: Type-3	1.19 (0.17–8.43)	
	3: Type-4	12.86 (1.56–105.94)	
		0.001	

ate analysis identified the following three factors that correlated with SNEG/CMN type: age (<65/≥65 years, $P = 0.030$), type of enhancement pattern (Type-4, Type-3 or other enhancement pattern, $P = 0.055$) and tumor size (<35 mm/≥35 mm, $P = 0.060$). These parameters were entered into multivariate logistic regression analysis. The percentage of SNEG/CMN types of HCC was significantly higher for Type-3 enhancement pattern (risk ratio 3.82, 95% CI 1.28–11.44) and age less than 65 years (risk ratio 3.57, 95% CI 1.29–9.90) (Table 4).

Cumulative recurrence rate according to each enhancement pattern

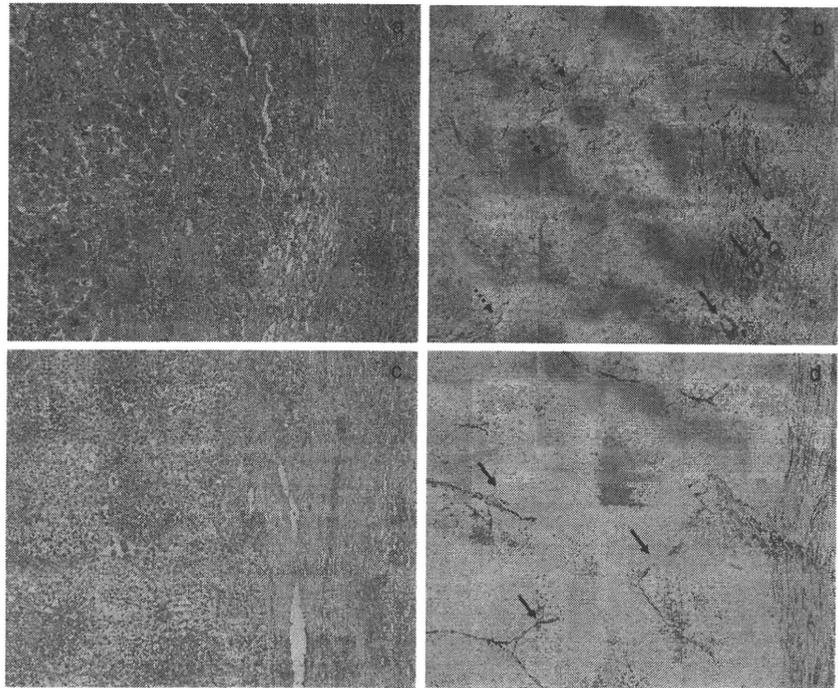
During a median observation period of 4.8 years, 46 (54%) out of 86 patients developed HCC recurrence in all patients. The cumulative recurrence rates after the surgical resection for HCC according to each enhancement pattern were 14% at the end of the first year, 44% at the third year and 54% at the fifth year for patients with Type-1 and -2 enhancement patterns; 13% at the first year, 28% at the third year and 38% at the fifth year

Table 4 Results of multivariate logistic regression analysis for predictive factors of SNEG and CMN types of HCC

Factors	Category	Hazard ratio (95% confidence interval)	P-value
Type of enhancement pattern	1: Type-1 and -2	1	0.017
	2: Type-3	3.82 (1.28–11.44)	
	3: Type-4	2.43 (0.60–9.86)	
Age (years)	1: ≥65	1	0.014
	2: <65	3.57 (1.29–9.90)	

CMN, confluent multinodular type; HCC, hepatocellular carcinoma; SNEG, simple nodular type with extranodular growth.

Figure 3 (a) Hematoxylin–eosin staining of hepatocellular carcinoma (HCC) tissue. (b) Immunohistochemical staining for α -smooth muscle actin (α -SMA) of the same slice shown in (a). Solid arrows: typical Type II vessels. Dotted arrows: α -SMA-positive vessels, which are atypical for Type II vessels. Note that the typical Type II vessels tended to be located in the peripheral parts of the HCC. (c) Hematoxylin–eosin staining of HCC tissue. (d) Immunohistochemical staining for α -SMA of the same slice shown in (c). Solid arrows: α -SMA-positive vessels, which are atypical for Type II vessels. HCC tissue exhibited thick trabecular pattern, and α -SMA-positive vessels were recognized in the gaps of the trabecular pattern. In this tumor, few typical Type II vessels were detected, and most α -SMA-positive vessels were atypical for Type II vessels. (Original magnifications: [a] $\times 100$; [b] $\times 100$; [c] $\times 100$; [d] $\times 100$.)



for patients with Type-3 enhancement pattern; and 9% at the first year, 52% at the third year and 84% at the fifth year for patients with Type-4 enhancement pattern. There was no difference with statistical significance among each enhancement pattern ($P = 0.163$).

Cumulative survival rate according to each enhancement pattern

The cumulative survival rates after the surgical resection for HCC according to each enhancement pattern were 100% at the end of the first year, 96% at the third year and 85% at the fifth year for patients with Type-1 and -2 enhancement patterns; 100% at the first year, 100% at the third year and 83% at the fifth year for patients with Type-3 enhancement pattern; and 100% at the first year, 100% at the third year and 86% at the fifth year for patients with Type-4 enhancement pattern. There was no statistical significance on survival rate among each enhancement pattern ($P = 0.758$).

Correlation between immunopathological findings and HCC enhancement patterns

Dynamic CT examination revealed ring-like enhancement pattern (Type-4) in 11 of 86 (13%) patients. We examined the distribution of Type II vessel in 11 nodules of Type-4. Seven of these nodules showed

heterogeneous distribution of typical Type II vessels (Fig. 3a,b). The tumor tissue in one of these nodules exhibited a thick trabecular pattern and typical Type II vessels were not present in the tumor (Fig. 3c,d). The other three nodules contained large necrotic areas in the tumor and a homogeneous distribution of Type II vessels.

DISCUSSION

A VARIETY OF potentially curative therapies is currently available for HCC. However, except for surgical resection, a potential risk of tumor dissemination always exists in patients who receive such therapies. Therefore, in order to use the most suitable therapy for the individual patient, it is important to predict the potential risk of HCC before treatment.

Previously reported, to predict poorly differentiated HCC and macroscopic type of SNEG and CMN is especially important for good therapeutic progress.^{17–19} In our study, Type-4 enhancement pattern was identified as an independent predictive factor of poorly-differentiated HCC. Patients who show Type-4 enhancement pattern in preoperative dynamic CT study are at higher risk of poorly-differentiated HCC (about 13-fold) than Type-1 and -2 enhancement patterns. On

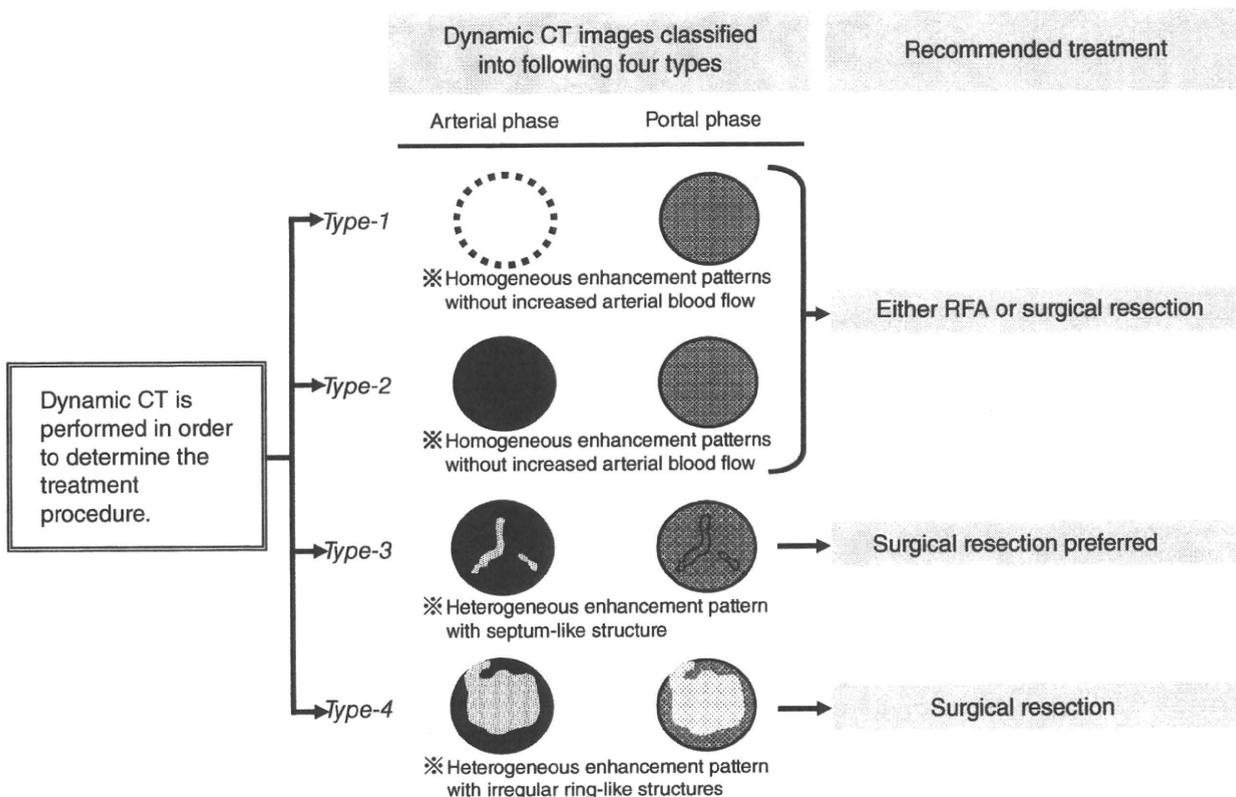


Figure 4 Treatment strategy for hepatocellular carcinoma according to a new classification of dynamic computed tomography (CT) images. RFA, radiofrequency ablation.

the other hand, Type-3 enhancement pattern was an independent predictor of SNEG and CMN, with higher risk (~fourfold) than Type-1 and -2 enhancement patterns. These results could be important for the selection of suitable therapy.

On the other hand, as previously reported, patients with SNEG and CMN have a high complication rate of poorly-differentiated HCC.²² However, in this study, there was no statistical relation between SNEG/CMN and Type-4 enhancement pattern that had statistical relation with poorly-differentiated HCC. One possible cause which brought this result is that the present study size is too small. Therefore, future studies are needed of larger size to reconsider this point.

Although the present study included only a small sample number of Type-4 enhancement pattern, the results showed that this pattern was associated with α -SMA staining pattern. Based on the results of the present study, at least three explanations were considered for the Type-4 enhancement pattern: (i) non-equal distribution of α -SMA-positive Type II vessels;

(ii) the thick trabecular pattern and lack of typical Type II vessels; and (iii) large necrotic tissue in the HCC nodule. It is clear from this examination that the cause of Type-4 enhancement pattern is not dependent on tissue necrosis only but other factors may be involved including Type II vessels. Admittedly, the present results do not fully explain the pathomechanism of Type-4 enhancement pattern. Further studies are needed to determine the main mechanism of this enhancement pattern.

With respect to prognosis after curative surgical resection based on each enhancement pattern, there are no significant differences among the four groups regarding recurrence and survival rate in this study. However, this study group mostly received curative therapy of "surgical resection" for HCC treatment. Therefore, we are currently investigating local recurrence and survival rate after RFA therapy based on this classification.

The present study included HCC only. However, heterogeneous enhancement resembling the Type-4 enhancement pattern is recognized in other hepatic

tumors (e.g. cholangiocellular carcinoma [CCC] and fibrolamellar HCC [F-HCC]). It is noteworthy, however, that these tumors are rare in chronic hepatitis or liver cirrhosis compared with HCC. CCC comprises 4.4% of primary liver cancers,^{23,24} while F-HCC forms only 0.68% of liver tumors in Japan. Thus, detection of heterogeneous enhancement pattern on dynamic CT images should be considered first to represent HCC with highly malignant potential.

Therefore, we indicate our treatment strategy for HCC according to a new classification of dynamic CT images (Fig. 4). The present results indicate that for patients with Type-4 or Type-3 enhancement patterns on dynamic CT images who have adequate liver reserve to allow any treatment including surgical resection, the above information could be used as an index to prioritize surgical resection. Especially, in patients with Type-4 enhancement pattern, we strongly recommend surgical resection.

In conclusion, the present study demonstrated a strong relationship between Type-4 and -3 enhancement pattern and malignant characteristics of HCC. The management of HCC with Type-4 and -3 enhancement pattern should include a thorough therapeutic approach including surgical resection.

ACKNOWLEDGMENT

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REFERENCES

- 1 El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 1999; 340: 745–50.
- 2 Bosch X, Ribes J, Borrás J. Epidemiology of primary liver cancer. *Semin Liver Dis* 1999; 19: 271–85.
- 3 Okuda K, Fujimoto I, Hanai A, Urano Y. Changing incidence of hepatocellular carcinoma in Japan. *Cancer Res* 1987; 47: 4967–72.
- 4 Johnson PJ, Williams R. Cirrhosis and the aetiology of hepatocellular carcinoma. *J Hepatol* 1987; 4: 140–7.
- 5 Ikeda K, Saitoh S, Koida I *et al.* A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* 1993; 18: 47–53.
- 6 Poon RT, Fan ST, Lo CM *et al.* Hepatocellular carcinoma in the elderly: results of surgical and nonsurgical management. *Am J Gastroenterol* 1999; 94: 2460–6.
- 7 Yamanaka N, Okamoto E, Toyosaka A *et al.* Prognostic factors after hepatectomy for hepatocellular carcinomas. A univariate and multivariate analysis. *Cancer* 1990; 65: 1104–10.
- 8 Kawasaki S, Makuuchi M, Miyagawa S *et al.* Results of hepatic resection for hepatocellular carcinoma. *World J Surg* 1995; 19: 31–4.
- 9 Shirabe K, Kanematsu T, Matsumata T, Adachi E, Akazawa K, Sugimachi K. Factors linked to early recurrence of small hepatocellular carcinoma after hepatectomy: univariate and multivariate analyses. *Hepatology* 1991; 14: 802–5.
- 10 Jwo SC, Chiu JH, Chau GY, Loong CC, Lui WY. Risk factors linked to tumor recurrence of human hepatocellular carcinoma after hepatic resection. *Hepatology* 1992; 16: 1367–71.
- 11 Nagasue N, Kohno H, Hayashi T *et al.* Lack of intratumoral heterogeneity in DNA ploidy pattern of hepatocellular carcinoma. *Gastroenterology* 1993; 105: 1449–54.
- 12 Izumi R, Shimizu K, Li T *et al.* Prognostic factors of hepatocellular carcinoma in patients undergoing hepatic resection. *Gastroenterology* 1994; 106: 720–7.
- 13 Otto G, Heuschen U, Hofmann WJ, Krumm G, Hinz U, Herfarth C. Survival and recurrence after liver transplantation versus liver resection for hepatocellular carcinoma: a retrospective analysis. *Ann Surg* 1998; 227: 424–32.
- 14 Takayama T, Makuuchi M, Hirohashi S *et al.* Early hepatocellular carcinoma as an entity with a high rate of surgical cure. *Hepatology* 1998; 28: 1241–6.
- 15 Zhang BH, Yang BH, Tang ZY. Randomized controlled trial of screening for hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2004; 130: 417–22.
- 16 Hong SN, Lee SY, Choi MS *et al.* Comparing the outcomes of radiofrequency ablation and surgery in patients with a single small hepatocellular carcinoma and well-preserved hepatic function. *J Clin Gastroenterol* 2005; 39: 247–52.
- 17 Llovet JM, Vilana R, Brú C *et al.* Increased risk of tumor seeding after percutaneous radiofrequency ablation for single hepatocellular carcinoma. *Hepatology* 2001; 33: 1124–9.
- 18 Yu HC, Cheng JS, Lai KH *et al.* Factors for early tumor recurrence of single small hepatocellular carcinoma after percutaneous radiofrequency ablation therapy. *World J Gastroenterol* 2005; 11: 1439–44.
- 19 Sumie S, Kuromatsu R, Okuda K *et al.* Microvascular invasion in patients with hepatocellular carcinoma and its predictable clinicopathological factors. *Ann Surg Oncol* 2008; 15: 1375–82.
- 20 Ikeda K, Saitoh S, Suzuki Y *et al.* Relationship of angiographic finding with neovascular structure detected by immunohistochemical staining of alpha-smooth muscle actin in small hepatocellular carcinoma. *J Gastroenterol Hepatol* 1998; 13: 1266–73.
- 21 Liver Cancer Study Group of Japan. *General Rules for the Clinical and Pathological Study of Primary Liver Cancer*. 2nd English edn. Tokyo: Kanehara, 2003.

- 22 Kanai T, Hirohashi S, Upton MP *et al.* Pathology of small hepatocellular carcinoma. A proposal for a new gross classification. *Cancer* 1987; 60: 810–19.
- 23 Liver Cancer Study Group of Japan. Survey and follow-up of primary liver cancer in Japan. Report 11. *Acta Hepatol Jpn* 1995; 36: 208–18.
- 24 Liver Cancer Study Group of Japan. Survey and follow-up of primary liver cancer in Japan. Report 12. *Acta Hepatol Jpn* 1997; 38: 317–30.

Original Article

Administration of interferon for two or more years decreases early stage hepatocellular carcinoma recurrence rate after radical ablation: A retrospective study of hepatitis C virus-related liver cancer

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Background: Since hepatocellular carcinoma often recurs after surgical resection or radiofrequency ablation, we analyzed a retrospective large cohort of patients with small hepatocellular carcinoma caused by hepatitis C virus (HCV).

Methods: Among 379 patients with HCV RNA-positive small hepatocellular carcinoma (multiple up to three nodules, 3 cm or less each), 77 received interferon-alpha injection and 302 received no anti-viral therapy.

Results: Four patients (5.2%) attained sustained virological response (SVR). Cumulative recurrence rates in the treated and untreated groups were 41.1% and 57.5% at the end of the third year, and 63.0% and 74.5% at the fifth year, respectively ($P = 0.013$). Fifth year-recurrence rates in treated group were 25.0% in SVR, 85.7% in biochemical response, 71.1% in no response, and 46.7% in patients with continuous administration. When four patients with SVR were excluded, recurrence

rates in short-term interferon therapy (<2 years) and long-term therapy (≥ 2 years) were 46.2% and 39.3% at the third year, and 66.2% and 57.4% at the fifth year, respectively ($P = 0.012$). Multivariate analysis showed that long-term interferon therapy significantly decreased recurrence rate (hazard ratio for interferon <2 years 0.80, interferon ≥ 2 years 0.60, $P = 0.044$), after adjustment with background covariates including indocyanine green retention rate ($P = 0.018$), alpha-fetoprotein ($P = 0.051$), and tumor treatment ($P = 0.066$).

Conclusion: A long-term administration of low-dose interferon significantly decreased recurrence of hepatocellular carcinoma after surgical resection or radiofrequency ablation.

Key words: hepatitis C, hepatocellular carcinoma, Interferon, prevention, recurrence

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) remains one of the most common cancers, and cause of cancer death, worldwide. Since the recurrence rate of HCC is high even after potentially curative therapies with surgical resection or radiofrequency ablation (RFA) therapy, suppression of recurrence is of great impor-

tance for prolonging the life of patients with hepatitis C virus (HCV)-related liver disease. This high recurrence rate, after curative therapy, was explained by occult intra-hepatic metastasis of HCC or by multi-centric carcinogenesis in the setting of chronic viral hepatitis or liver cirrhosis.^{1,2}

Interferon (IFN) is effective in reducing hepatocellular carcinogenesis rate through suppression of necro-inflammatory process and in eliminating HCV in some patients with chronic hepatitis C and cirrhosis. Although IFN proves to be valuable in suppression of the risk of carcinogenesis in many literatures,³⁻⁵ only several reports mentioned the efficacy of IFN in the suppression of tumor recurrence or in prolongation of survival period after ablation of HCC⁶⁻¹². We once

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demonstrated the preventive activity of HCC recurrence by IFN-beta in a randomized controlled trial,⁵ but intravenous type of IFN-beta was not universally available outside Japan in spite of the superiority of tumor suppressive activity to IFN-alpha.¹³⁻¹⁷ Some investigators¹⁴⁻¹⁷ showed that IFN acted as an anti-cancer agent in the treatment of HCC *in vivo* and *in vitro*. However, the actual efficacy of IFN in preventing recurrence of HCV-associated HCC in optimally treated patients remains unclear. Since some prospective study failed to demonstrate a beneficial effect of IFN-alpha in cumulative recurrence rate,¹¹ we analyzed a large cohort of patients for a long period up to 18 years.

To what extent IFN suppresses the recurrence rate of early stage of HCC, we analyzed a large retrospective cohort with and without a long-term administration of IFN-alpha in patients with HCC. The purposes of this study were (i) to evaluate the influence of IFN-alpha on HCC recurrence rate after treatment of an early stage of HCV-related HCC, and (ii) to explore effective ways of IFN administration, if any.

PATIENTS AND METHODS

Study population

A TOTAL OF 729 patients were diagnosed as having HCC associated with HCV-related chronic liver disease from 1990 to 2006 in our hospital. Among them, 379 patients underwent surgical resection or sufficient medical ablation therapy for small HCC (multiple up to three nodules, 3 cm or less each). All were positive for anti-hepatitis C antibody and negative for hepatitis B surface antigen. The consecutive patients were analyzed, who met inclusion criteria of (1) initial diagnosis of HCC (2) early stage of HCC (multiple up to three nodules, 3 cm or less each) (3) potentially curative manner of resection or radiofrequency ablation for HCC, and (4) positive HCV RNA. Exclusion criteria of this study were (1) positive portal vein invasion on imaging of computerized tomography or ultrasonography (2) residual HCC on imaging diagnosis after surgical or medical therapy (3) Child-Pugh score C (4) other etiology of liver disease (hepatitis B, alcoholic, non-alcoholic liver disease, etc.) (5) use of other anti-viral agents including interferon-beta (6) use of retinoid derivatives, and (7) concomitant malignant tumor in addition to HCC.

The diagnosis of HCC was established by integrated imagings of ultrasonography, dynamic computerized tomography (CT), magnetic resonance imaging (MRI).

To exclude additional small HCC nodules in the liver, computerized tomographic hepatic arteriography (CT-HA) and computerized tomographic arteriography (CT-AP) were also performed in 356 patients (93.9%). Among the consecutive 379 patients with surgical resection or sufficient radiofrequency ablation for HCC, 77 (20.3%) patients received intermittent IFN-alpha injection two or three times a week for 6 months or longer, mainly after the year of 1995 when this medication became available for use in Japan: Two (3.4%) of 59 patients received IFN therapy during 1990-1994, 21 (21.2%) of 99 patients during 1995-2000, and 54 (24.2%) of 223 patients during 2001-2006, respectively. The other 302 patients did not receive IFN therapy or other anti-viral therapy. None of the patients received any other anti-viral or anti-carcinogenic treatment including nucleoside analogues. We therefore, performed this analytical study as a retrospective cohort study.

Clinical background and laboratory data

Table 1 summarizes the profiles and laboratory data of the IFN group (group A) and the untreated group (group B) at the time of diagnosis of HCC. The median age in the IFN group was lower than that of the untreated group by 3 years, but the other features were not different between the two groups regarding demography, liver function, state of HCC, and treatment of HCC.

Interferon treatment and judgment of the effect

Seventy-seven patients underwent IFN therapy after treatment of HCC. IFN therapy was usually initiated within several months after ablation of HCC, and a median period from HCC treatment to initiation of IFN was 5.6 months.

All the patients received IFN-alpha (natural or recombinant): Seven received interferon plus ribavirin combination therapy, and 68 underwent interferon monotherapy. Ten patients (13.0%) underwent interferon therapy for 6 months or less, 15 patients (19.5%) for 7 to 12 months, 13 patients (16.8%) for 13 to 24 months, 28 (36.4%) for 25 to 60 months, and the remaining 11 (14.3%) for a prolonged period of 61 months or longer. As a whole, a median dose of 242 million units was administered during the median period of 24.2 months. A total of 50.6% of all the patients received IFN for 2 years or longer.

Judgment of IFN effect was classified according to elimination of HCV RNA and alanine aminotransferase (ALT) value at a time of 6 months after the end of the

Table 1 Profiles and laboratory tests of the patients with and without interferon

Groups/characteristic	Group A (interferon)	Group B (none)	P*
Patients characteristics			
N	77	302	
Age (year) (median, range)	63 (43–77)	66 (39–87)	0.003
Sex (Male/Female)	46/31	191/111	0.57
Positive HBs antigen	0	0	NS
Positive HCV antibody	77 (100%)	302 (100%)	NS
Positive HCV-RNA	77 (100%)	302 (100%)	NS
Cancer characteristics before treatment			
Number of nodules			0.89
Solitary	63	260	
Two	11	33	
Three	3	9	
Size of maximal tumor (median, range)	18 (5–30)	18 (8–30)	0.50
Vascular invasion on imaging	0	0	NS
Cancer therapy			
Surgery	35 (45.5%)	146 (48.3%)	0.65
Radiofrequency ablation	42 (54.5%)	156 (51.7%)	
Laboratory findings (median, range)			
Albumin (g/dl)	3.6 (2.4–4.3)	3.6 (2.4–4.5)	0.80
Bilirubin (mg/dl)	1.0 (0.3–2.5)	1.0 (0.2–3.3)	0.96
Aspartic transaminase (IU)	54 (16–311)	54.5 (13–191)	0.94
Alanine transaminase (IU)	57 (12–273)	54 (11–230)	0.89
Platelet (×1000/cmm)	100 (20–272)	110 (20–256)	0.85
ICG R15 (%)	25 (1–75)	27 (2–78)	0.58
Alpha-fetoprotein (mg/L)	22 (3–1411)	22 (1–4950)	0.28
DCP (AU/L)	19 (11–635)	17 (0–1470)	0.50

*Non-parametric test (χ^2 test or Mann-Whitney *U*-test). DCP, des-gamma-carboxyprothrombin; ICG R15, indocyanine green retention test at 15 minutes.

treatment. Sustained virological response (SVR) was defined as persistent disappearance of HCV RNA after therapy, biochemical response (BR) as normal ALT values (40 IU/L or less) without elimination of HCV RNA for at least 6 months after therapy, and no response (NR) as persistently abnormal or only transient normalization of ALT for less than 6 months.

Follow-up and diagnosis of HCC

Physicians examined the patients every 4 weeks after entry to the study. Liver function tests and hematologic and virologic tests were conducted every month. To diagnose recurrent HCC nodules at an early stage, imaging studies were performed every 3 months, using ultrasonography and computerized tomography. Alpha-fetoprotein and des-gamma-carboxyprothrombin were also assayed bimonthly. When angiography demonstrated a characteristic hypervascular nodule, it was usually a specific finding for HCC in these follow-up patients, and histological confirmation was usually not

required in the majority of these HCC patients. Most of the “angiographically-diagnosed HCC” showed intra-hepatic multiplicity and pathognomonic findings of capsule formation or nodule-in-nodule appearance, or even portal vein invasion. If angiography did not show any hypervascular stain in a small hepatic nodule, histological study was always performed.

A total of 8 patients could not continue the IFN treatment due to side effects, following studies of tumor recurrence and survival were analyzed on an intention-to-treat basis.

Eight patients were lost to follow-up: 2 in IFN group and 6 in untreated group. Treated and untreated patients were followed at intervals of one month for a median observation period of 4.6 years, ranged from 0.1 to 18.4 years: 5.6 years in interferon group and 4.2 years in untreated group. The date of the last follow-up for this study was 30th August, 2009.

The end point of the study was tumor recurrence after treatment.

Statistical methods

The obtained clinical data were analyzed on an intention-to-treat basis. Standard statistical measures and procedures were used in the analysis. The chi-square, Fisher's exact test, and Mann-Whitney's *U*-tests were used to analyze the differences of background features and biochemical data between the two groups. HCC recurrence rate was calculated from the day of HCC treatment in both groups, using the Kaplan-Meier technique. The differences in recurrence curves were tested using the log-rank test. Cox proportional hazard analysis was performed to evaluate independent predictors of tumor recurrence after treatment. A *P*-value of less than 0.05 with two-tailed analysis was considered significant. Data analysis was performed using the computer program SPSS version 11 (SPSS Inc. Chicago, IL).¹⁸

RESULTS

Effects and toxicity of interferon

SVR WERE FOUND in 4 (5.2%) of 77 patients in IFN-treated group and none in untreated group. BR were found in 7 (9.1%), NR in 36 (46.8%), and undetermined judgment due to continuous administration currently in 30 (39.0%).

Almost all of the patients given IFN therapy showed varied degrees of fever, chills, myalgias, headache, and general malaise after the first injection of IFN. Most of patients revealed a various degree of leukocytopenia and thrombocytopenia. A total of 8 patients (10.4%) withdrew from IFN therapy before development of tumor recurrence. Three patients with depression or psychosis ceased the IFN therapy. The other 5 patients also stopped IFN administration because of varied degree of adverse effects: thrombocytopenia, insomnia, slight degree of hepatic encephalopathy, minor episode of cerebrovascular accident, and generalized fatigue with significant weight loss.

Recurrence rates of hepatocellular carcinoma

During the median observation period of 4.6 years, HCC recurred in 264 patients (69.7%); 45 patients belonged to the IFN group, and the other 219 patients to the untreated group. The cumulative recurrence rate in all patients was 16.2% at the end of the first year following the surgical treatment of HCC, 39.6% at the second year, 54.5% at the third year, 73.0% at the fifth year, 82.8% at the seventh year, and 85.5% at the 10th year. Crude recurrence rates in the IFN group and

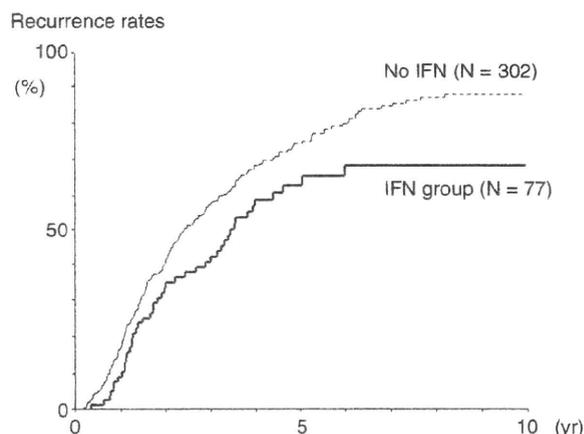


Figure 1 Cumulative recurrence rates of hepatocellular carcinoma in patients with and without interferon therapy.

untreated group were 9.1% and 18.8% at the end of the first year, 33.3% and 42.1% at the second year, 41.1% and 58.1% at the third year, 63.0% and 76.6% at the 5th year, 68.5% and 86.2% at the seventh year, and 68.5% and 93.2% at the 10th year, respectively (Fig. 1). The recurrence rate in the IFN group was significantly lower than that of the untreated group (log-rank test: $P = 0.013$).

In univariate analysis, factors associated with tumor recurrence were explored in all of the 379 patients *en masse*. HCC recurrence was associated with high indocyanine green retention rate at 15 minutes (ICG R15) ($P = 0.004$), low albumin concentration ($P = 0.005$), no IFN therapy ($P = 0.010$), prolonged prothrombin time ($P = 0.041$), and RFA as treatment for HCC ($P = 0.046$).

Multivariate analysis disclosed that recurrence of HCC was independently associated with IFN therapy (hazard ratio 0.66, $P = 0.020$), a high ICG R15 of 20% or more (hazard ratio 1.43, $P = 0.008$), and RFA therapy (hazard ratio 1.32, $P = 0.041$). IFN treatment proved to prevent tumor recurrence after ablation of HCC in those patients with an early stage of HCC (Table 2).

Recurrence rates according to interferon effect

Tumor recurrence rates were evaluated according to judgment of IFN effect in the treated group: SVR ($n = 4$), BR ($n = 7$), NR ($n = 36$), continued IFN administration ($N = 30$), and untreated group.

Table 2 Independent factors affecting the recurrence of hepatocellular carcinoma after curative treatment

Factors	Category	Hazard ratio (95% CI)	P
Interferon therapy	1: No	1	0.020
	2: Yes	0.66 (0.46–0.94)	
ICG R15	1: <20%	1	0.008
	2: ≥20%	1.43 (1.10–1.85)	
Cancer treatment	1: Surgical resection	1	0.041
	2: PRFA	1.32 (1.01–1.72)	

ICG R15, indocyanine green retention rate at 15 minutes; PRFA, percutaneous radiofrequency ablation therapy.

Recurrence rates in the subgroup of SVR, BR, NR, continued administration, and untreated patients were 0%, 0%, 6.7%, 12.5%, and 18.8% at the end of the first year, 25.0%, 28.6%, 37.0%, 25.3%, and 42.1% at the second year, 25.0%, 42.9%, 52.0%, 32.6%, and 58.1% at the third year, 25.0%, 85.7%, 71.1%, 46.7%, and 76.6% at the fifth year, and 25.0%, 100%, 79.3%, 54.3%, and 86.2% at the seventh year, respectively (Fig. 2a). The recurrence rates in a combined group of SVR and continued IFN administration were significantly lower than those in a combined cohort of the other groups (log-rank test, $P = 0.0005$) (Fig. 2b). The recurrence rates of the former and the latter groups were 30.6% and 56.7% at the end of the third year, 43.3% and 75.0% at the fifth year, and 43.3% and 84.7% at the seventh year, respectively.

Recurrence rates according to length of interferon administration

Since HCV RNA eradication (SVR) was found in only four patients, significance of prolonged administration of IFN was assessed in those patients with positive HCV RNA during therapy ($n = 73$).

Recurrence rates in the subgroup with a long IFN therapy of 2 years or more ($n = 39$), a short IFN therapy of less than 2 years ($n = 34$), and in the untreated patients ($n = 302$) were 8.7%, 7.1%, and 18.8% at the end of the first year, 23.9%, 46.2%, and 58.1% at the second year, 39.3%, 46.2%, and 58.1% at the third year, 57.4%, 66.2%, 76.6% at the fifth year, and 66.0%, 77.5%, and 86.2% at the seventh year, and 66.0%, 77.5%, and 93.2%, respectively (Fig. 3). The recurrence rates in the long IFN-therapy group was significantly lower than those with a short therapy group and untreated group (log-rank test, $P = 0.012$).

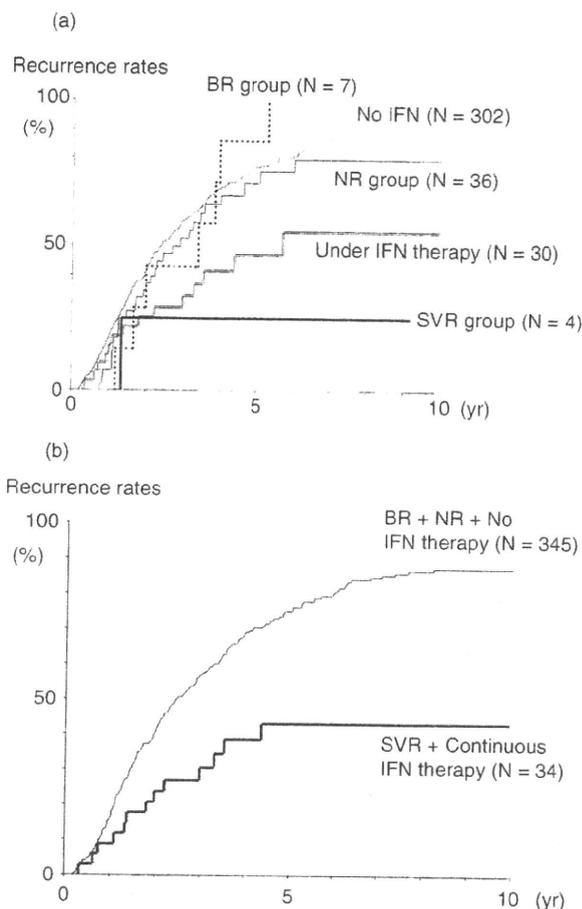


Figure 2 (a) Cumulative recurrence rates of hepatocellular carcinoma according to the effect of interferon. (b) Cumulative recurrence rates of hepatocellular carcinoma in a combined group of sustained virological response and continuous interferon administration and those in a combined group of biochemical response, no response, and no interferon therapy.

To elucidate the impact of a long-term administration of IFN in the prevention of HCC recurrence, multivariate hazard analysis was introduced in the IFN-treated patients without SVR effect ($n = 73$) and the untreated patients ($n = 302$). Multivariate analysis showed that a long-term IFN therapy significantly lowered the recurrence rate in patients with HCV-related HCC: hazard ratios of short-term therapy less than two years and long-term therapy for two years or longer of 2 years or more were 0.80 and 0.60, respectively ($P = 0.044$). The other covariates for recurrence rate included high ICGR15, high AFP value, and initial treatment modality (Table 3).

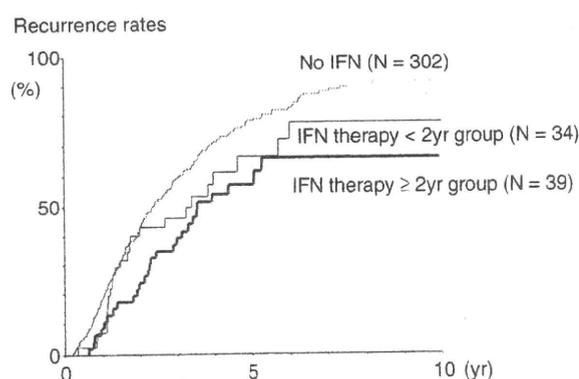


Figure 3 Cumulative recurrence rates of hepatocellular carcinoma in patients without sustained virological response. Recurrence rates were assessed according to the length of interferon administration.

Overall survival rates

A total of 159 patients died during the observation period: 23 (29.9%) in the IFN-treated group and 136 (45.0%) in the untreated group. Crude survival rates of patients after potentially curative therapy for HCC in the IFN-treated and untreated patients were 90.7% and 88.5% at the end of the third year, 85.6% and 68.8% at the fifth year, 76.5% and 50.9% at the seventh year, and 47.0% and 34.7% at the tenth year, respectively (Fig. 4). The survival rates of IFN-treated group were significantly higher than that of those of untreated group (log-rank test, $P = 0.0044$).

Table 3 Independent factors affecting the recurrence of hepatocellular carcinoma after curative treatment, according to the length of interferon administration†

Factors	Category	Hazard ratio (95% CI)	<i>P</i>
Interferon therapy	1: None	1	0.044
	2: <2 years	0.80 (0.51–1.24)	
	3: ≥2 years	0.60 (0.40–0.91)	
ICG R15	1: <20%	1	0.018
	2: ≥20%	1.37 (1.06–1.77)	
Alpha-fetoprotein	1: <40 mg/L	1	0.051
	2: ≥40 mg/L	1.31 (1.00–1.71)	
Cancer treatment	1: Surgical resection	1	0.066
	2: PRFA	1.28 (0.98–1.65)	

†Four patients with sustained virological response were excluded in the analysis. ICG R15, indocyanine green retention rate at 15 minutes; PRFA, percutaneous radiofrequency ablation therapy.

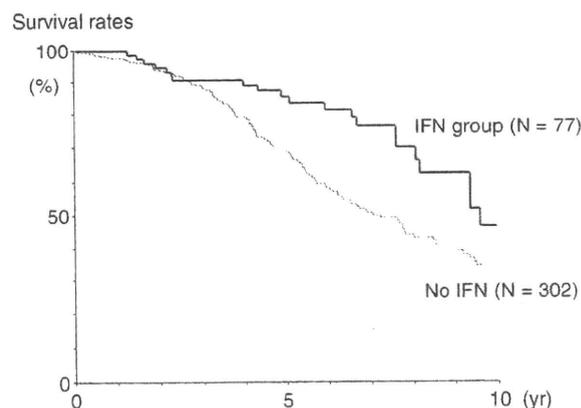


Figure 4 Overall survival rates of patients with or without interferon therapy after potentially curative therapy for hepatocellular carcinoma.

Multivariate analysis showed overall survival rates were significantly affected by interferon therapy ($P = 0.014$), albumin concentration ($P = 0.015$), platelet count ($P = 0.014$), and ICG R15 ($P = 0.0068$) (Table 4). Hazard ratio for death in those patients with IFN therapy was 0.55 (95% confidence interval 0.34–0.88).

DISCUSSION

ALTHOUGH THIS STUDY was not a prospective, randomized one, there was no significant difference in the background features and laboratory tests except for age, between the treated and untreated groups. This study was based on a long-term observation for a median of 4.6 years, and the number of patient was sufficiently large for sensitivity and reliabil-

Table 4 Independent factors affecting the survival rates of patients with hepatocellular carcinoma after curative treatment

Factors	Category	Hazard ratio (95% C.I.)	<i>P</i>
Interferon therapy	1: None	1	0.014
	2: Yes	0.55 (0.39–0.88)	
ICG R15	1: <20%	1	0.0068
	2: ≥20%	1.65 (1.15–2.37)	
Albumin	1: <3.5 g/dl	1	0.015
	2: ≥3.5 g/dl	0.64 (0.44–0.92)	
Platelet count	1: <100,000/mm ³	1	0.014
	2: ≥100,000/mm ³	0.64 (0.45–0.91)	

ICG R15, indocyanine green retention rate at 15 minutes.

ity for the data regarding recurrence and survival. We also analyzed only those patients with "an early stage" of HCC to minimize the influence of tumor recurrence due to small and undetectable metastatic tumors often found in patients with large or multiple tumors. In the establishment of the diagnosis of early stage of HCC, more than 93% of the patients underwent intensive imaging investigation with CT-HA and CT-AP, together with dynamic CT and dynamic MRI study. Therefore, the diagnosis of a few numbers with small-sized tumor was sufficiently reliable in the study.

This cohort study indicated IFN suppressed the recurrence rate after potentially curative treatment of HCC caused by HCV. Indeed SVR effect after IFN therapy did decrease recurrence rate, majority of patients were not tolerable for a large amount of IFN administration with or without ribavirin because of an old age or advanced liver disease with significant cytopenia. This study demonstrated interferon significantly decreased tumor recurrence rate, irrespective of "anti-viral interferon effect". This study also revealed relatively "rapid" anti-carcinogenic effect compared with the results of a study performed by Mazzaferro *et al.*¹¹ Most cases of late-phase recurrence are thought to be due to metachronous multicentric, or *de novo*, carcinogenesis. This is quite understandable, because the remaining liver, often cirrhotic, is still at high risk of carcinogenesis.

Our study also emphasizes that long-term, low-dose, intermittent administration of IFN was useful in prevention of tumor recurrence in patients without SVR, with a hazard ratio of 0.60 compared to those with no IFN administration.

The reason why IFN administration suppresses the recurrence rate in HCV-related liver disease remains uncertain. One reason may be anti-tumor activity in the early stage of HCC and another antiviral or anti-necroinflammatory effect for hepatitis. Our data did not disclose the relationship between ALT normalization and prevention of cancer recurrence, since the number of BR group was small (N = 7), and since many patients were currently continuing IFN therapy with normal ALT. Human lymphoblastoid IFN alpha has a powerful anti-proliferative effect on human hepatoma cell line PLC/PRF/5, both *in vitro* and *in vivo*, after implantation in nude mice.¹⁹ Lai *et al.*²⁰ showed IFN induced objective tumor regression in a significant number of patients with inoperable hepatocellular carcinoma in a randomized controlled trial. Considering the short period to recurrence in our study, IFN may have a direct anti-tumor effect on clinically undetectable HCC. Wang *et al.*²¹ showed

anti-angiogenesis activity of IFN, and Wu *et al.*²² demonstrated suppression of vascular endothelial growth factor and inhibition of tumor signaling pathways. Moreno *et al.*²³ reported that IFN induced remission of liver fibrosis irrespective of anti-viral effect. Control of necro-inflammatory process may therefore induce a suppression of the growth process of HCC. Tarao *et al.*²⁴ reported that high aminotransferase activity resulted in an increased HCC recurrence rate. A randomized controlled trial of IFN for patients with cirrhosis showed that IFN therapy decreased the HCC appearance rate in association with disappearance of HCV-RNA.³ We also demonstrated IFN suppressed the carcinogenesis rate in patients with chronic hepatitis type C.⁵ Taking into account that hepatocellular carcinogenesis in HCV-related chronic liver disease is accelerated by a prolonged period of necro-inflammation of hepatocytes, IFN is hypothesized to diminish the HCC appearance rate through suppression of excessive replication and turnover of hepatocytes. Since the entire process of hepatocellular carcinogenesis from initial transformation of a hepatocyte to detectable growth is considered to take at least several years, the influence of IFN on the carcinogenesis rate or recurrence rate might not be evaluated in as short period of three years or less. Aside from the exact mechanism of the prevention of HCC recurrence, our study demonstrated an encouraging result in the medical management of HCC.

Since these results were not generated from a prospective randomized study, we tried to adjust background biases using multivariate analysis between the treated and untreated group, if any. We should realize the significance of the decrease in recurrence rate by IFN therapy with a hazard ratio by 0.66. Cost-effectiveness and individual and social expenses should be evaluated in detail between those patients with reduction of recurrence rate and those with high recurrence rate with additional tumor ablation therapy. Considering that a long-term prospective trial with and without IFN arm seemed very difficult to perform ethically and economically, we should further accumulate these comparative studies and consider the efficacy of weekly injections of pegylated IFN and adequate dose and length of IFN therapy. Identification of suitable cases for IFN therapy and exact mechanisms of suppression of tumor recurrence are of paramount importance for increasing number of patients with HCC.

In conclusion, long-term intermittent IFN therapy reduced HCC recurrence rate in patients with HCV-related HCC.

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REFERENCES

- Arii S, Tanaka J, Yamazoe Y *et al.* Predictive factors for intrahepatic recurrence of hepatocellular carcinoma after partial hepatectomy. *Cancer* 1992; 69: 913–19.
- Ikeda K, Arase Y, Kobayashi M *et al.* Significance of multicentric cancer recurrence after potentially curative ablation of hepatocellular carcinoma: a longterm cohort study of 892 patients with viral cirrhosis. *J Gastroenterol* 2003; 38: 865–76.
- Nishiguchi S, Kuroki T, Nakatani S *et al.* Randomized trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet* 1995; 346: 1051–5.
- Kasahara A, Hayashi N, Mochizuki K *et al.* Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. *Hepatology* 1998; 27: 1394–402.
- Ikeda K, Saitoh S, Arase Y *et al.* Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C – a long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. *Hepatology* 1999; 29: 1124–30.
- Ikeda K, Arase Y, Saitoh S *et al.* Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor – a prospective randomized study of hepatitis C virus-related liver cancer. *Hepatology* 2000; 32: 228–32.
- Kubo S, Nishiguchi S, Hirohashi K *et al.* Effects of long-term postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. *Ann Intern Med* 2001; 15: 963–7.
- Shiratori Y, Shiina S, Teratani T *et al.* Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. *Ann Intern Med* 2003; 138: 299–306.
- Sakaguchi Y, Kudo M, Fukunaga T *et al.* Low-dose, long-term, intermittent interferon-alpha-2b therapy after radical treatment by radiofrequency ablation delays clinical recurrence in patients with hepatitis C virus-related hepatocellular carcinoma. *Intervirology* 2005; 48: 64–70.
- Hung CH, Lee CM, Wang JH *et al.* Antiviral therapy after non-surgical tumor ablation in patients with hepatocellular carcinoma associated with hepatitis C virus. *J Gastroenterol Hepatol* 2005; 20: 1553–9.
- Mazzaferro V, Romito R, Schiavo M *et al.* Prevention of hepatocellular carcinoma recurrence with alpha-interferon after liver resection in HCV cirrhosis. *Hepatology* 2006; 44: 1543–54.
- Jeong SC, Aikata H, Katamura Y *et al.* Effects of a 24-week course of interferon-alpha therapy after curative treatment of hepatitis C virus-associated hepatocellular carcinoma. *World J Gastroenterol* 2007; 13: 5343–50.
- Kashiwagi K, Furusyo N, Kubo N *et al.* A prospective comparison of the effect of interferon-alpha and interferon-beta treatment in patients with chronic hepatitis C on the incidence of hepatocellular carcinoma development. *J Infect Chemother* 2003; 9: 333–40.
- Damdinsuren B, Nagano H, Sakon M *et al.* Interferon-beta is more potent than interferon-alpha in inhibition of human hepatocellular carcinoma cell growth when used alone and in combination with anticancer drugs. *Ann Surg Oncol* 2003; 10: 1184–90.
- Matsumoto K, Okano J, Murawaki Y. Differential effects of interferon alpha-2b and beta on the signaling pathways in human liver cancer cells. *J Gastroenterol* 2005; 40: 722–32.
- Murata M, Nabeshima S, Kikuchi K *et al.* A comparison of the antitumor effects of interferon-alpha and beta on human hepatocellular carcinoma cell lines. *Cytokine* 2006; 33: 121–8.
- Damdinsuren B, Nagano H, Wada H *et al.* Stronger growth-inhibitory effect of interferon (IFN)-beta compared to IFN-alpha is mediated by IFN signaling pathway in hepatocellular carcinoma cells. *Int J Oncol* 2007; 30: 201–8.
- SPSS *SPSS for Windows Version 11.0 Manual*. SPSS, Chicago, IL, 2001.
- Dunk AA, Ikeda T, Pignatelli M, Thomas HC. Human lymphoblastoid interferon: in vitro and in vivo studies in hepatocellular carcinoma. *J Hepatol* 1986; 2: 419–29.
- Lai CL, Lau JY, Wu PC *et al.* Recombinant interferon-alpha in inoperable hepatocellular carcinoma: a randomized controlled trial. *Hepatology* 1993; 17: 389–94.
- Wang L, Wu WZ, Sun HC *et al.* Mechanism of interferon alpha on inhibition of metastasis and angiogenesis of hepatocellular carcinoma after curative resection in nude mice. *J Gastrointest Surg* 2003; 7: 587–94.
- Wu WZ, Sun HC, Shen YF *et al.* Interferon alpha 2a down-regulates VEGF expression through PI3 kinase and MAP kinase signaling pathways. *J Cancer Res Clin Oncol* 2005; 131: 169–78.
- Moreno MG, Muriel P. Remission of liver fibrosis by interferon-alpha 2b. *Biochem Pharmacol* 1995; 50: 515–20.
- Tarao K, Takemiya S, Tamai S *et al.* Relationship between the recurrence of hepatocellular carcinoma (HCC) and serum alanine aminotransferase levels in hepatectomized patients with hepatitis C virus-associated cirrhosis and HCC. *Cancer* 1997; 79: 688–94.