the treatment for HCC and stored in −80 °C. Liver tissue from patients who underwent resection was collected, rapidly frozen and stored in −80 °C. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committee.

Antiviral therapy

Forty-seven patients received 100 mg LAM daily, and drug-resistant YMDD mutants developed in 26 (55%) of these patients, accompanied by an increase in HBV DNA ≥1log copies/ml. Seventeen of the 26 patients received 10 mg ADV in addition to LAM (100 mg) daily. The remaining nine continued to receive LAM monotherapy because of the lack of approval for ADV administration in Japan at the time, but received ADV with LAM after approval was obtained during the HCC posttreatment period. Eight NA-naïve patients received 0.5 mg ETV daily. These antiviral therapies were continued after the resection or percutaneous ablation.

Follow-up and HCC recurrence

The patients were followed for liver function and virological markers of HBV infection monthly, as well as blood counts and tumour makers including α-fetoprotein and des-γ-carboxylprothrombin. They also underwent ultrasonography or helical dynamic computed tomography every 3 months. Cirrhosis was diagnosed by laparoscopy or liver biopsy or by the clinical data, imaging modalities and portal hypertension. The median observation period after HCC treatment for the entire cohort was 2.7 years (range, 0.3–8.4 years). HCC recurrence was diagnosed by the typical hypervascular characteristics on angiography and/or histological examination with fine needle biopsy specimens, in addition to certain features on computed tomography and ultrasonography.

Markers of HBV infection

HBcAg was determined by enzyme-linked immunosorbent assay using a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantitated using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan) with a dynamic range over 2.6–7.6log copies/ml or COBAS TaqMan HBV v.2.0 (Roche Diagnostics) with a dynamic range over 2.1–9.0log copies/ml. Serum HBV DNA levels were measured using the Amplicor assay at both the start of NA therapy and the diagnosis of HCC and using the TaqMan assay at the diagnosis of HCC. For statistical analysis, the value of that HBV DNA was tentatively set at 2.1 if HBV DNA levels were under 2.1log copies/ml. HBV genotypes were determined serologically by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G), using a commercial kit (HBV Genotype EIA; Institute of Immunology). YMDD mutants were determined by polymerase chain reaction-based enzyme-linked minisequence assay using a commercial kit (Genome Science Laboratories, Tokyo, Japan).

HBcrAg measurement

Serum HBcrAg levels were measured using a CLEIA HBcrAg assay kit (Fujirebio Inc., Tokyo, Japan) with a fully automated analyser system (Lumipulse System; Fujirebio Inc.) as described previously (21). In brief, 150 μl of serum was incubated with 150 μl of pretreatment solution containing 15% sodium dodecyl sulphate at 60 °C for 30 min. After heat treatment, 120 μl of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with monoclonal antibody mixture (HB44, HB61 and HB114) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After 10 min of incubation at 37 °C and washing, further incubation was carried out for 10 min at 37 °C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After washing, 200 μl of substrate solution [3-(2′-spiroada-mantan)-4-methoxy-4-(3′-phosphoryloxy)phenyl-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA, USA) was added to the test cartridge, which was then incubated for 5 min at 37 °C. The relative chemiluminescence intensity was measured, and the HBcrAg concentration was calculated by a standard curve generated using a recombinant pro-HBeAg (amino acids −10 to 183 of the precore/core gene product). The HBcrAg concentration was expressed in U/ml, which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, the HBcrAg values were expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analyses, HBcrAg-negative cases were calculated as 3.0log U/ml.

Intrahepatic cccDNA measurement

Intrahepatic cccDNA levels were analysed as described previously (21). In brief, liver specimens surrounding the tumour tissue were obtained and stored at −80 °C before DNA extraction. HBV DNA was extracted using a QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on the absorbance at 260 nm. For this study, two oligonucleotide primers cccF2 (5′-cgtctgtgccttctcatctga-3′, nucleotides 1424–1444) and cccR4 (5′-gcacagcttggaggcttgaa-3′, nucleotides 1755–1737) and probe cccP2 (5′-VIC-accaatttat gcctacag-MGB-3′, nucleotides 1672–1655) were designed using PRIMER EXPRESS software (Applied Biosystems, Foster City, CA, USA) to flank the direct repeat region between the hepatitis B core and the polymerase gene. The use of cccF2 and cccR4, oligonucleotide primers spanning the direct repeat region of the HBV genome, allows the polymerase chain reaction of native viral DNA in the

Dane particle to block the amplification of products, because the partially double-stranded HBV DNA is disrupted in the direct repeat region. Twenty-five microlitres of extracted DNA (0.5 μg) was detected with the sequence detector system (ABI 7900HT; Applied Biosystems) in 50 μl of a PCR mixture containing TaqMan universal PCR Master Mix (Applied Biosystems), 300 nmol of each primer and 250 nmol of the probe. After initial activation of uracil-*N*-glycosylase at 50 °C for 2 min, AmpliTaq Gold (Applied Biosystems) was activated at 95 °C for 10 min. The subsequent PCR conditions consisted of 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 90 s per cycle (SRL Inc., Tokyo, Japan).

Statistical analyses

Standard statistical measures and procedures were used. Correlations between two variables were tested using Pearson's correlation analysis. Cox regression analysis was used to assess significant associations of the risk factors with tumour recurrence after HCC treatment. All factors found to be at least associated with recurrence ($P < 0.05$) were tested by multivariate analysis. Independent factors, associated with HCC recurrence, were calculated using stepwise Cox regression analysis. The cumulative recurrence-free survival rates after HCC treatment were analysed using the Kaplan–Meier method, and differences in the curves were tested using the log-rank test. A P value of < 0.05 in a two-tailed test was considered significant. Data analysis was performed with SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics at the start of NA therapy and HCC incidence

Table 1 presents a comparison of the patient characteristics at the start of NA therapy and the time of HCC diagnosis. Almost all the patients (93%) enrolled in this study had HBV genotype C. One patient had genotype B, and the genotypes of three patients could not be determined. The rate of HBV DNA disappearance from serum in all the patients was 64% (35/55; Amplicor monitor assay, < 2.6log copies/ml) and 51% (28/55; TaqMan assay, < 2.1log copies/ml), that of aspartate aminotransferase (AST) normalization (< 32 IU/L) was 56% (31/55) and that of ALT normalization (< 42 IU/L) was 71% (39/55) at the incidence of HCC. YMDD mutants were detected in 30 of 47 patients at the beginning of LAM monotherapy, and virological breakthrough (VBT), accompanied by an increase in HBV DNA (≥ 1log copies/ml), occurred in 26 patients with YMDD mutants by the diagnosis of HCC. Seventeen of these patients received ADV with LAM. No resistant mutation to ADV (rtA181T/S, rtN236T) occurred in patients receiving the combination therapy. Further, no drug-resistant mutant

Table 1. Patient characteristics at the start of nucleot(s)ide analogue therapy and the incidence of hepatocellular carcinoma

Characteristics	Start of NA therapy	Time of HCC Dx
Age (years)	51 (32–73)	54 (35–75)
Gender (male:female)	45:10	45:10
AST level (IU/L)	69 (27–195)	31 (16–207)
ALT level (IU/L)	78 (23–368)	29 (10–267)
Platelet count (10^5/mm^3)	11.4 (3.1–31.3)	12.9 (3.6–30.1)
Serum albumin level (g/dl)		3.8 (3.1–4.4)
Serum bilirubin level (mg/dl)		0.9 (0.4–2.4)
Prothrombin time (%)		90.8 (59–112)
Indocyanine green retention rate at 15 min (%)		14.5 (4–53)
Child–Pugh (A:B)		49:6
HBV genotype		
C	51 (93%)	51 (93%)
Others	4	4
HBeAg (+)	29 (53%)	23 (42%)
HBV DNA (log copies/ml)	7.1 (< 2.6 to > 7.6)	< 2.1 (< 2.1 to 8.5)
HBcrAg level (log U/ml)	6.6 (3.3 to > 6.8)	5.0 (< 3.0 to > 6.8)
Antiviral agents (LAM:LAM+ADV:ETV)	47:0:8	30:17:8
Duration of NA therapy before the incidence of HCC (years)		2.2 (0.2–7.4)
α-fetoprotein level (ng/dl)	6 (2–263)	4 (1–282)
Des-γ-carboxylprothrombin level (mAU/ml)		22 (< 10–933)
Tumour diameter (mm)		22 (7–60)
Tumour number (solitary:multiple)		50:5
Portal vein invasion (positive:negative)		49:6
TNM stage (I:II:III:IV)		25: 24: 5: 1
HCC treatment (resection:ablation)		37:18

Values are expressed as the median and range (parenthetically) or the number and percentage (parenthetically).
ADV, adefovir dipivoxil; ETV, entecavir; HBV DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; LAM, lamivudine; NA, nucleot(s)ide analogues.

was detected in the NA-naïve patients receiving ETV monotherapy.

Correlation between serum HBcrAg and serum HBV DNA levels at the incidence of HCC

The median serum HBcrAg value was 6.6log U/ml (range, 3.3 to > 6.8) at the start of NA therapy and 5.0log U/ml (range, < 3.0 to > 6.8) at the time of HCC diagnosis. We observed a positive correlation ($P < 0.001$; $r = 0.610$) between the levels of HBcrAg and HBV DNA in serum at the time of HCC diagnosis (Fig. 2A).

HBcrAg was detectable in 23 (82%) of 28 patients with undetectable HBV DNA levels using TaqMan assay and was > 4.8log U/ml in eight (29%) of 28 patients. In contrast, serum HBV DNA was detectable in spite of undetected HBcrAg in only two patients. Then, we examined the correlation between the serum HBcrAg levels at the time of HCC diagnosis and the antiviral effect. The median duration of on-treatment undetected serum HBV DNA was 1.1 years (range, 0.1–4.8) before the first diagnosis of HCC. As shown in Figure 2B, we observed a significant negative correlation between the levels of HBcrAg in serum at the time of HCC diagnosis and the duration of undetected HBV DNA in serum just before the first diagnosis of HCC ($P < 0.001$; $r = -0.568$).

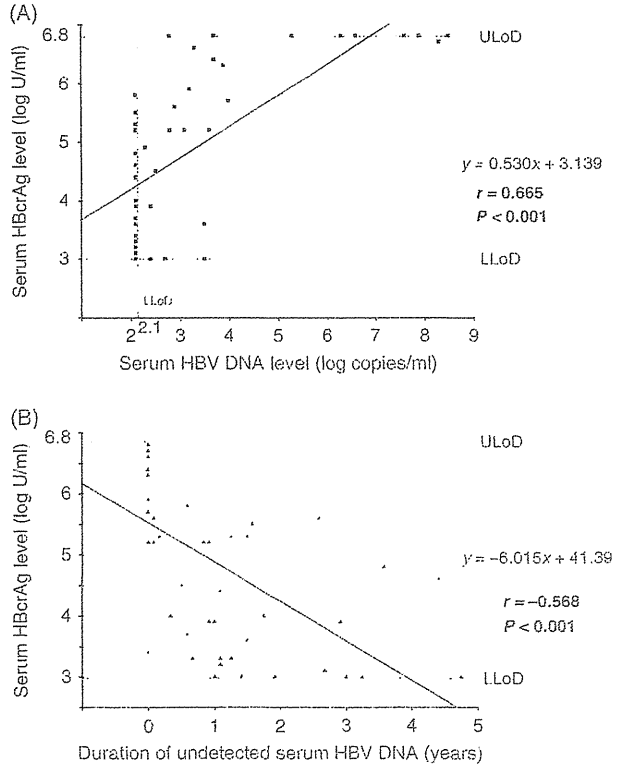

Fig. 2. (A) Correlation between serum HBcrAg and hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient. (B) Correlation between serum HBcrAg levels at the time of HCC diagnosis and the duration of undetected serum HBV DNA (< 2.6log copies/ml).

Factors associated with HCC recurrence

Hepatocellular carcinoma recurred in 21 (38%) of the 55 patients, 17 (46%) of 37 patients who had undergone resection and four (22%) of 18 patients who had undergone ablation. Because a proportion of patients who had undergone resection with TNM Stage II or over (24 of 37 patients) was greater than ablation (six of 18), there were more patients who had HCC recurrence after resection than ablation. Eight factors were associated with the recurrence in univariate analysis: HBeAg positivity at the start of NA therapy, HBV DNA ≥ 2.1log copies/ml, HBcrAg level ≥ 4.8log U/ml, AST level ≥ 50 IU/L, ALT level ≥ 40 IU/L, tumour multiplicity, portal vein invasion at the time of HCC diagnosis and HCC treatment. In the multivariate analysis, HBcrAg level ≥ 4.8log U/ml and portal vein invasion were independent risk factors for the recurrence of HCC (Table 2). The cumulative recurrence-free survival rates in patients with ≥ 4.8log U/ml HBcrAg levels at the time of HCC diagnosis were 70% at 1 year, 35% at 3 years and 28% at 5 years. In contrast, the rates in patients with < 4.8log U/ml HBcrAg levels were 96% at 1 year, 89% at 3 years and 89% at 5 years. The recurrence-free survival rates of the high HBcrAg group (≥ 4.8log U/ml) were significantly lower than those of the low HBcrAg group (< 4.8log U/ml; $P < 0.001$), as shown in Figure 3A. Then, the cumulative recurrence-free survival rates in patients with ≥ 2.1log copies/ml HBV DNA levels at the time of HCC diagnosis were 70% at 1 year, 44% at 3 years and 39% at 5 years. In contrast, the rates in patients with < 2.1log copies/ml HBV DNA levels were 93% at 1 year, 76% at 3 years and 76% at 5 years. The recurrence-free survival rates of the positive HBV DNA group (≥ 2.1log copies/ml) were significantly lower than those of the negative HBV DNA group (< 2.1log copies/ml; $P = 0.007$), as shown in Figure 3B. The cumulative recurrence-free survival rates were 33% at 1 year and 33% at 2 years with portal vein invasion, and 87% at 1 year, 73% at 2 years and 64% at 3 years without invasion. Three of the six patients with portal vein invasion died of recurrent HCC.

Correlation between intrahepatic cccDNA and serum HBV DNA levels at the incidence of HCC

We measured intrahepatic cccDNA using liver specimens from 22 of 37 patients who underwent resection. The median intrahepatic cccDNA value was 4.2log copies/μg (range, 3.0–5.0). As shown in Figure 4A and B, we observed significant positive correlations between the levels of intrahepatic cccDNA and HBV DNA in serum ($P = 0.019$; $r = 0.486$) and between the levels of intrahepatic cccDNA and HBcrAg in serum at the time of HCC diagnosis ($P = 0.028$; $r = 0.479$). Twenty-eight patients who underwent resection had early- or intermediate-stage

Table 2. Risk factors for hepatocellular carcinoma recurrence

	Univariate analysis		Multivariate analysis	
Factors	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Start of NA therapy				
Age (≥50 years)	1.79 (0.65–4.91)	0.257		
Gender (female)	0.98 (0.32–2.97)	0.981		
HBeAg(+)	2.85 (1.03–7.88)	**0.044**		
HBV DNA (≥6.0log copies/ml)	1.75 (0.50–6.07)	0.378		
AST level (≥50 IU/L)	1.09 (0.42–2.85)	0.862		
ALT level (≥70 IU/L)	1.09 (0.42–2.85)	0.862		
Platelet count (< 1.2×10^5 cells/mm^3)	2.56 (0.96–6.85)	0.061		
α-fetoprotein level (≥100 ng/ml)	0.99 (0.13–7.66)	0.996		
Time of HCC diagnosis				
Duration of NA therapy (≥2 years)	1.19 (0.49–2.88)	0.698		
HBeAg(+)	1.53 (0.63–3.70)	0.343		
HBV DNA (≥2.1log copies/ml)	3.36 (1.32–8.55)	**0.011**		
HBcrAg level (≥4.8log U/ml)	10.6 (2.45–46.1)	**0.002**	**8.96 (1.94–41.4)**	**0.005**
YMDD mutants (present:absent)	0.84 (0.35–2.03)	0.838		
AST level (≥50 IU/L)	2.44 (1.01–5.89)	**0.047**		
ALT level (≥40 IU/L)	2.44 (1.01–5.87)	**0.047**		
Platelet count (< 10^5 cells/mm^3)	2.20 (0.81–6.02)	0.123		
Serum albumin level (< 3.5 g/dl)	1.39 (0.53–3.63)	0.505		
Serum bilirubin level (≥1.5 mg/dl)	1.11 (0.62–2.00)	0.713		
Prothrombin time (< 80%)	2.23 (0.51–9.82)	0.286		
Child–Pugh (B)	0.70 (0.16–3.04)	0.634		
Indocyanine green retention rate at 15 min (≥30%)	0.58 (0.17–1.99)	0.389		
α-fetoprotein level (≥100 ng/ml)	1.81 (0.74–4.44)	0.194		
Des-γ-carboxylprothrombin level (≥100 mAU/ml)	2.09 (0.81–5.39)	0.129		
Tumour size (≥21 mm)	2.02 (0.81–5.07)	0.133		
Tumour number (multiple)	3.94 (1.29–12.1)	**0.016**		
Portal vein invasion	5.39 (1.69–17.2)	**0.004**	**3.94 (1.25–12.4)**	**0.019**
TNM stage (≥II)	2.08 (0.85–5.10)	0.110		
HCC treatment (resection)	3.10 (1.05–9.09)	**0.041**		

The bolded numbers: statically significant.

ALT, alanine transaminase; AST, aspartate aminotransferase; CI, confidence interval; HBV DNA, hepatitis B virus DNA; NA, nucleot(s)ide analogues; YMDD, thyrosine–methionine–aspartic acid–aspartic acid.

HCC (tumour diameter < 50 mm, absence of vascular invasion and well/moderately differentiated). In 17 of these patients, the intrahepatic cccDNA levels were measured using the resected specimens. The recurrence-free survival rates of the high cccDNA group (≥4.3log copies/μg) were significantly lower than those of the low cccDNA group (< 4.3log copies/μg; $P = 0.0438$), as shown in Figure 4C.

Comparison of the serum HBcrAg levels and the patient characteristics

We examined whether the serum HBcrAg levels at the time of HCC diagnosis were correlated with the baseline parameters before antiviral therapy. The HBcrAg levels were compared with the baseline HBeAg-positive and HBeAg-negative status and with the baseline HBV DNA levels ≥6.0log and < 6.0log copies/ml (Fig. 5). The HBcrAg levels were significantly higher in patients who were positive for HBeAg (median value: 5.6 vs. 3.6log U/ml; $P = 0.001$) and the baseline HBV DNA levels ≥6.0log copies/ml (median value: 5.2 vs. 3.3log U/ml; $P = 0.012$). There was no correlation between the other baseline parameters at the start of NA therapy and the serum HBcrAg levels at the time of HCC diagnosis. Then, we examined whether the serum HBcrAg levels at the time of HCC diagnosis were associated with on-treatment drug resistance during antiviral therapy. Figure 6 shows the comparison of the serum HBcrAg levels at the time of HCC diagnosis with or without the emergence of YMDD mutants and VBT before the development of HCC. The HBcrAg levels were marginally higher in patients with emergent YMDD mutants (median value: 5.2 vs. 3.8log U/ml; $P = 0.051$) and significantly higher in those with VBT (median value: 5.2 vs. 3.9log U/ml; $P = 0.006$). There was no correlation between serum HBcrAg at the time of HCC diagnosis and age of patients or tumour factors.

Discussion

In this study, we examined whether the intrahepatic cccDNA and HBcrAg levels as substitutes for cccDNA are associated with HCC recurrence in patients who

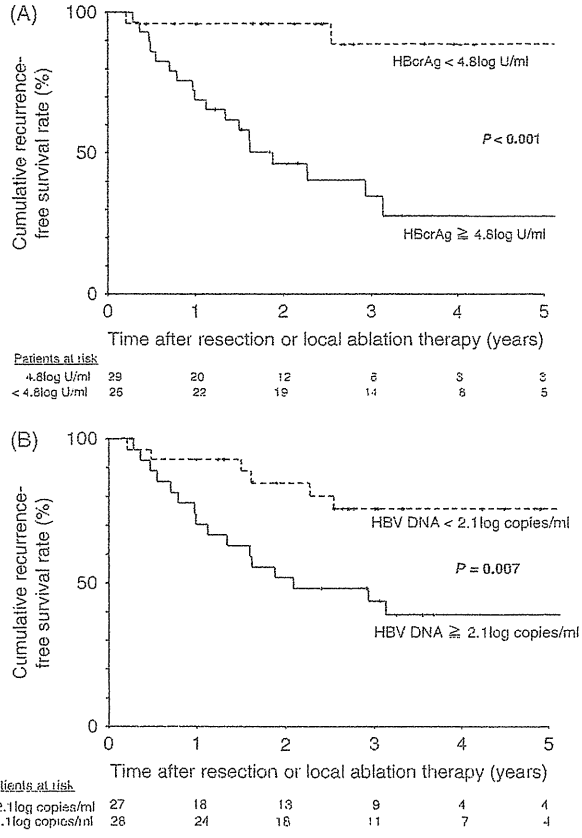

Fig. 3. (A) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum HBcrAg levels and comparison by the log-rank test. (B) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular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

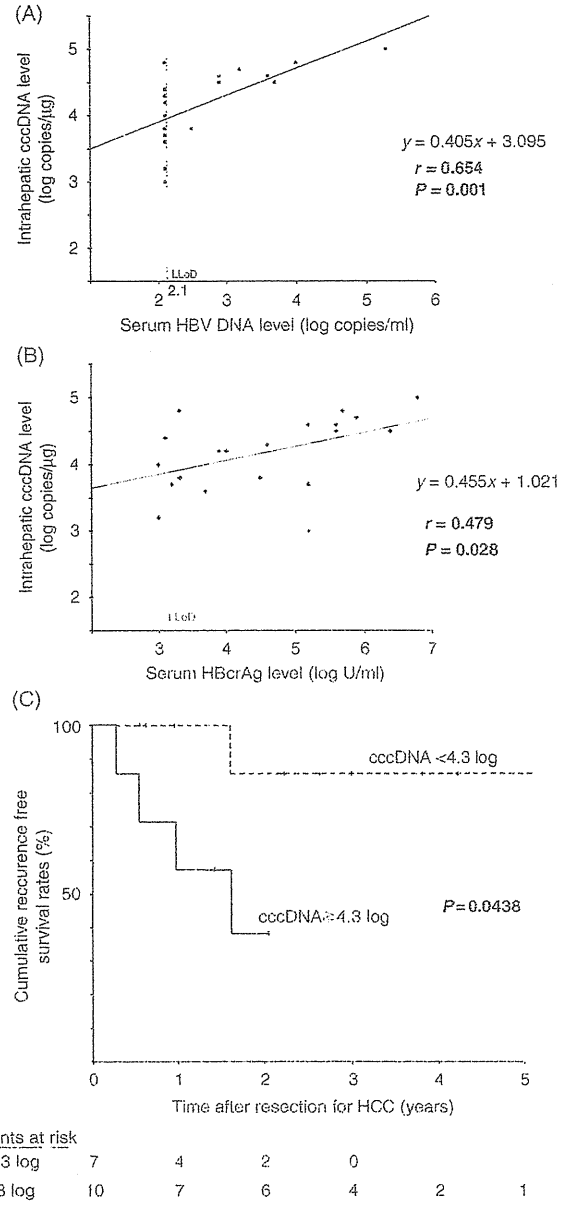

Fig. 4. (A) Correlation between intrahepatic covalently closed circular DNA (cccDNA) and serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient who underwent resection ($n = 22$). (B) Correlation between intrahepatic cccDNA and serum HBcrAg 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 ($n = 17$).

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 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

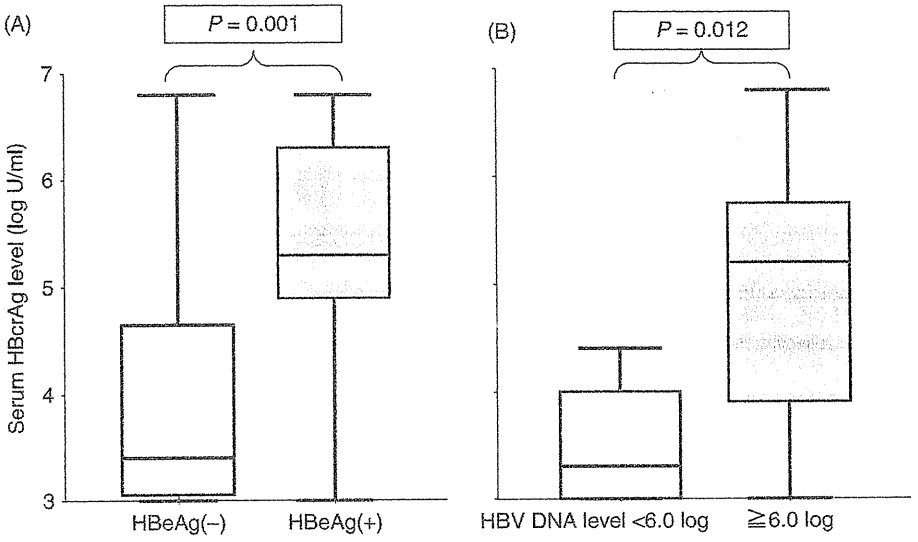

Fig. 5. Comparison of serum HBcrAg levels at the time of hepatocellular carcinoma diagnosis by the characteristics at the start of nucleot(s)ide analogue therapy (A) in patients with or without HBeAg and (B) in those with hepatitis B virus DNA (HBV DNA) levels < 6.0log or ≥6.0log copies/ml.

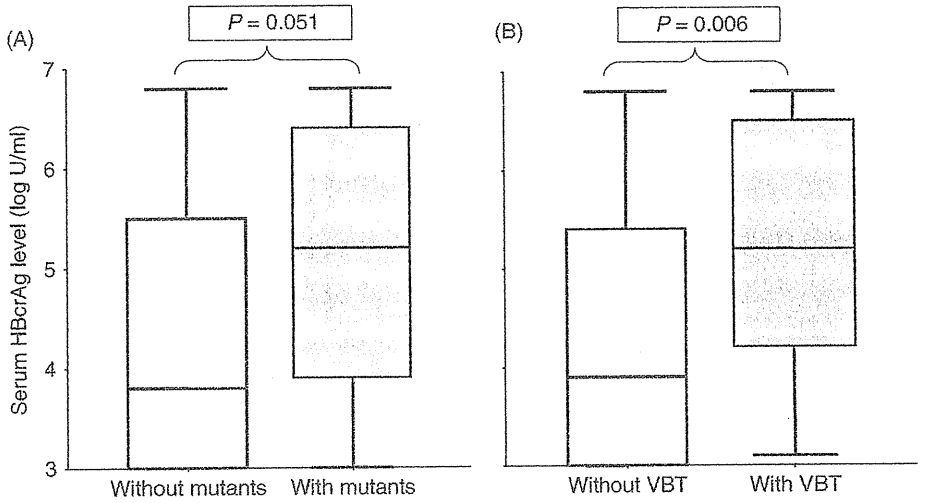

Fig. 6. Comparison of serum HBcrAg levels at the time of hepatocellular carcinoma (HCC) diagnosis (A) with or without thyrosine–methionine–aspartic acid–aspartic acid mutants and (B) virological breakthrough (VBT) before the development of HCC.

different results between past studies and the current study. Although serum HBV DNA was undetectable using TaqMan assay at the time of HCC diagnosis in 51% of the patients, who received NAs, serum HBcrAg was undetectable in only 18% of these patients. The other reason is that it was easy to identify the viral risk factors (e.g. HBeAg positivity) by measuring the serum HBcrAg level because the detection of HBcrAg enables the detection of HBcAg, HBeAg and the 22 kDa precore protein coded with the precore/core gene. The high recurrence rate of HCC after curative resection and ablation is attributable to two principal characteristics: intrahepatic metastasis and *de novo* multicentric carcinogenesis (32). It is assumed that a high viral load increases the risk of multicentric recurrence in the liver remnant in patients without optimal viral suppression by NA therapy. Recently, it was reported that the HBV load is associated with late recurrence over 2 years (30). On examining our cohort as per the recent report, high HBcrAg levels were found to be associated with late recurrence (data not shown). Consequently, we consider that HBcrAg is a more useful marker of HBV-related HCC recurrence than HBV DNA during NA therapy.

Nucleot(s)ide analogues are potent inhibitors of HBV replication, and can induce a rapid and drastic reduction in peripheral HBV DNA, seroclearance of HBeAg and remission of hepatic inflammation. Because of the stability of cccDNA in infected cells, the decline of

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 0.02log IU/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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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, ultrasonography [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

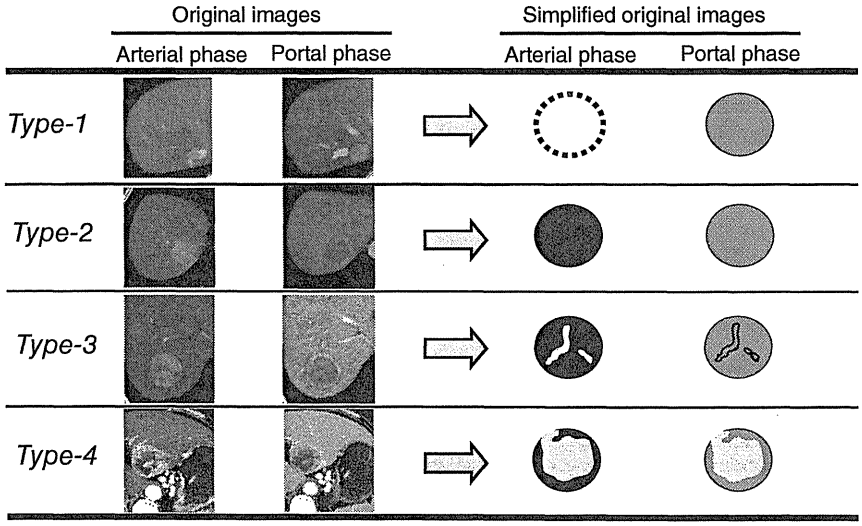

Figure 1 Sample of original dynamic computed tomography images and simplified images for each enhancement pattern.

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.

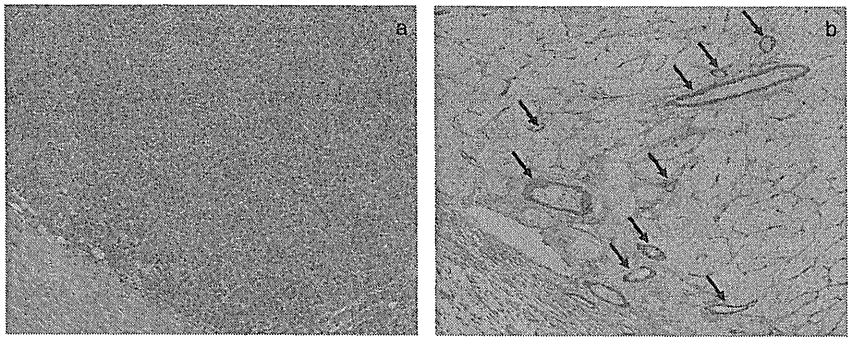

Figure 2 (a) Hematoxylin–eosin staining of hepatocellular carcinoma tissue. (b) Immunohistochemical staining for α-smooth muscle actin (α-SMA) of the same slice shown in (a). Solid arrows: typical Type II vessels. (Original magnifications: [a] ×100; [b] ×100.)

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 ($\times 10^4/\mu L$)	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 (μg/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 μg/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 mm/≥35 mm, $P = 0.005$), serum AST level (<40 IU/≥40 IU, $P = 0.069$) and serum DCP level (<30 AU/L/≥30 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 (≥35 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. Univari-

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	
	2: ≥35	14.72 (1.15–188.10)	0.039
Type of enhancement pattern	1: Type-1 and -2	1	
	2: Type-3	1.19 (0.17–8.43)	0.87
	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	
	2: Type-3	3.82 (1.28–11.44)	0.017
	3: Type-4	2.43 (0.60–9.86)	0.213
Age (years)	1: ≥65	1	
	2: <65	3.57 (1.29–9.90)	0.014

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

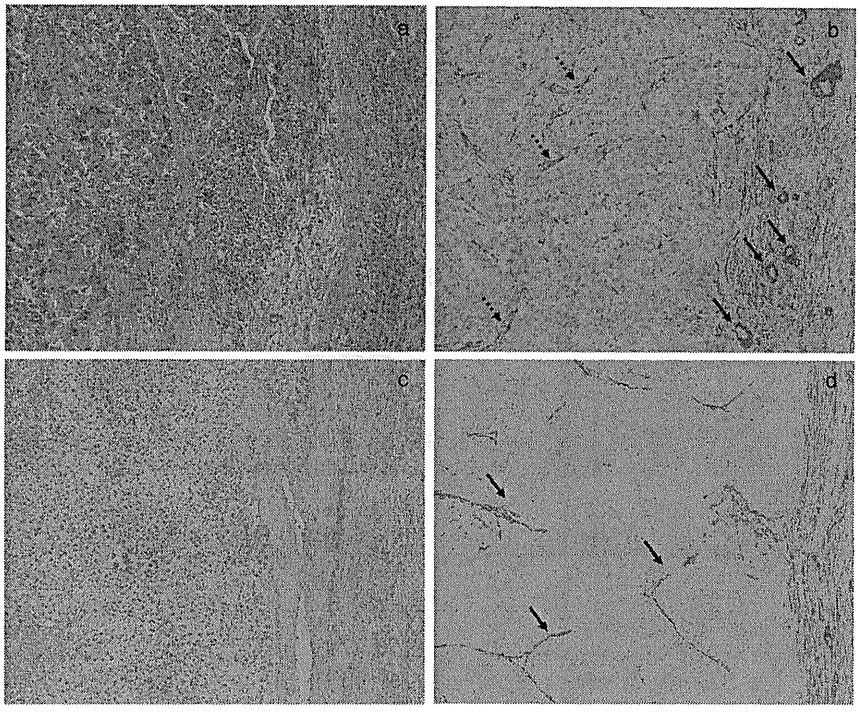

Figure 3 (a) Hematoxylin–eosin staining of hepatocellular carcinoma (HCC) tissue. (b) Immunohistochemical staining for α-smooth muscle actin (α-SMA) of the same slice shown in (a). Solid arrows: typical Type II vessels. Dotted arrows: α-SMA-positive vessels, which are atypical for Type II vessels. Note 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] ×100; [b] ×100; [c] ×100; [d] ×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

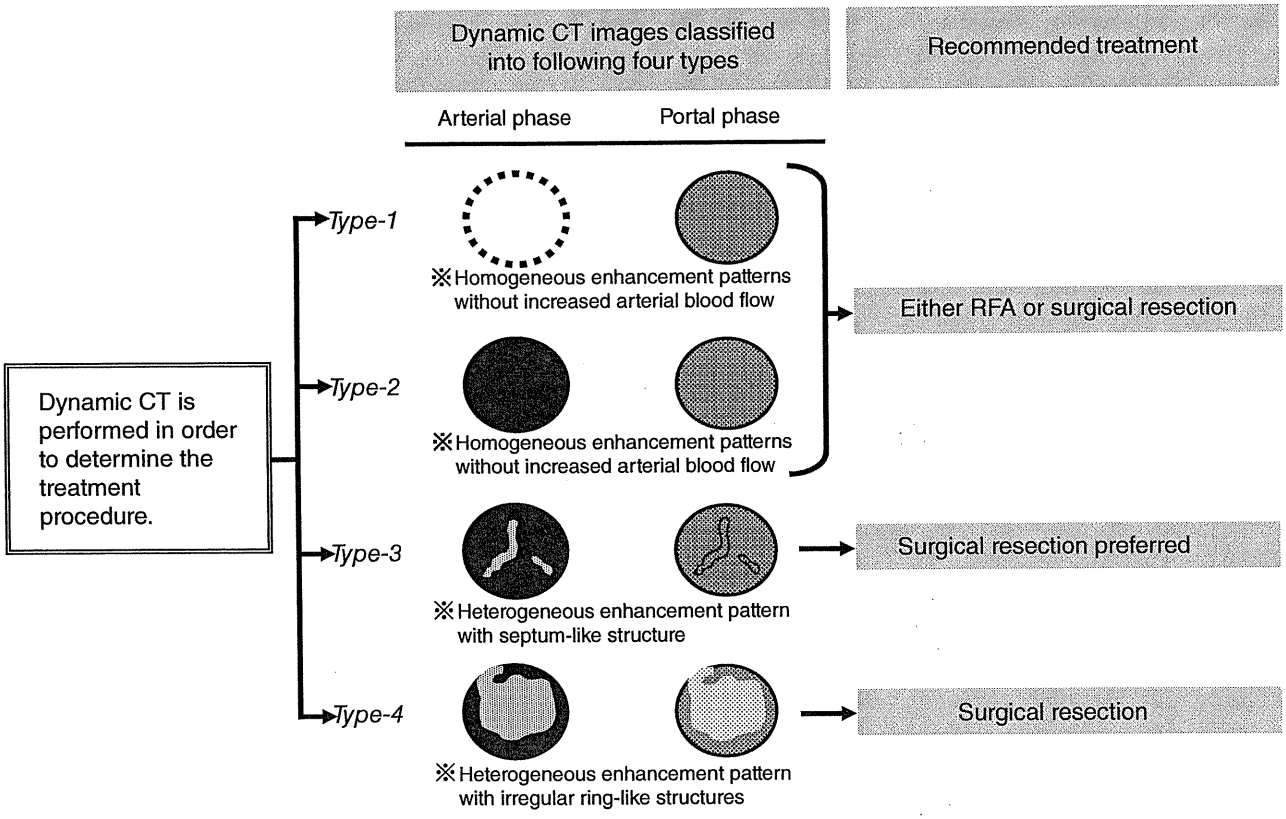

Figure 4 Treatment strategy for hepatocellular carcinoma according to a new classification of dynamic computed tomography (CT) images. RFA, radiofrequency ablation.

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.

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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 importance 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 HCC[6–12]. 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 investigators[14–17] 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 antiviral 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 arterioportography (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%)	
Laboraory 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. Alfa-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 intrahepatic 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.