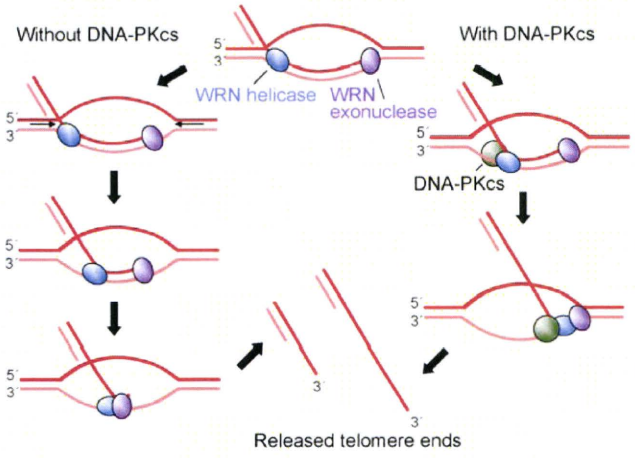

Figure 7. A model for protection of G-tails by DNA-PKcs. See text for detailed description of the model.

SIRT6 is required for the stable association of both WRN and DNA-PKcs with telomeric chromatin, suggesting the possibility of a role for SIRT6 to control the interaction between WRN and DNA-PKcs at telomeres [30, 31]. Additional studies are needed to determine whether and how Ku70/80 influences the interaction between DNA-PKcs and WRN, especially because Ku70/80 binds tightly to WRN and stimulates its exonuclease activity [32].

Mouse model studies indicate that both DNA-PKcs deficiency and WRN deficiency synergize with telomerase loss and shortened telomeres to accelerate the onset of aging related phenotypes. Mice deficient in both WRN and telomerase recapitulate most of the premature aging WS phenotypes in the later generation cohorts that experienced telomere shortening [26, 33]. Similarly, mice rendered doubly deficient in DNA-PKcs and telomerase exhibited accelerated aging-related degenerative phenotypes including tissue atrophy, compared to singly null mice, and this was further exacerbated in later generations [34]. The loss of either WRN or DNA-PKcs in telomerase deficient mice was associated with accelerated telomere shortening and chromosome fusions [26, 34]. Preservation of the telomeric tail is essential for preventing telomere dysfunction and chromosome fusions [35], and our biochemical data suggest that WRN and DNA-PKcs cooperate to prevent telomere tail shortening during processing at telomeric ends in telomerase deficient cells.

METHODS

Cells. U-2 OS cells stably transfected with a vector expressing an shRNA plasmid against control or WRN were grown in monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, 0.2 mg/ml hygromycin B (Invitrogen), 50 μg/ml streptomycin, and 50 U/ml penicillin as previously reported [36]. The U-2 OS cells stably expressing untargeted shRNA was transfected with siRNA against DNA-PKcs (sc-35200, Santa Cruz Biotechnology) or control siRNA (sc-37007, Santa Cruz Biotechnology), with or without pEYFP-WRN or pEYFP-WRN (E84A) vector, using lipofectamine 2000 Reagent (Invitrogen). Cells were incubated for 2 days and harvested.

Plasmids. DNA fragment encoding wild type WRN and WRN (E84A) was excised from pBK-WRN and pBK-WRN (E84A) with *Sal*I and *Ssp*I, and ligated into the *Sal*I and *Sma*I site of pEYFP-C1 vector (Clontech) to produce pEYFP-WRN and pEYFP-WRN (E84A) vectors, respectively.

Proteins. His-tagged WRN wild type (WT), WRN (E84A) and human Ku 70/86 were overexpressed in and purified from Sf9 insect cells using a baculovirus expression system as previously described [37, 38]. Recombinant human RPA was purified from *E. coli* BL21(DE3) pLysS transformed with p11d-tRPA as described previously [39]. DNA-PKcs was purified

from placenta as described previously [40]. His-tagged BLM was prepared from yeast strain JEL-1 [41].

G-tail telomere HPA assay. The G-tail length was quantified by the HPA using genomic DNA as described previously [44]. Signal intensity for each sample was normalized by DNA concentration using NANO drop.

DNA substrate. BB, BT and 5'-radiolabeled INV were annealed to form a telomeric D-loop [12]. BBmx, BT and 5'-radiolabeled INVmx were annealed to form a non-telomeric D-loop [13]. 5'-Radiolabeled and unlabeled 37-mer oligonucleotides were annealed to form a 22-bp forked duplex [42], an oligonucleotide 6 and a 5'-radiorabeled oligonucleotide 5 were annealed to form a 34-bp forked duplex [23], and X12-2, X12-3, X12-4 and 5'-radiolabeled X12-1 were annealed to form a Holliday junction [43]. The G-tailed substrate consisting of a 36-bp duplex with 14-bp of unique sequence followed by 22-bp of $(TTAGGG)_3TTAG$ sequence and a 20 nt 3'-ssDNA tail of the sequence $GG(TTAGGG)_3$ was constructed as previously described [24]. Briefly the substrate was formed by annealing the 5'-end labeled Tel Tail duplex oligonucleotide [24] into a hairpin to promote correct alignment of the telomeric repeats. An annealing reaction was at 95°C for 5 min, cooled stepwise (1.2 C°/min) to 60°C, incubated for 1 hr, and then cooled stepwise (1.2 C°/min) to 25°C. The hairpin was digested with *Eco*RV (New England BioLabs) to generate a blunt end, and the substrate was purified by PAGE.

Helicase and exonuclease assays. D-loop unwinding reactions were performed as described previously [12]. Briefly, the indicated amount of WRN or BLM was preincubated with DNA-PKcs, RPA or Ku on ice prior to addition of DNA. Assays contained 0.5 nM [^{32}P] 5' end-labeled D-loop substrate in 30 µl of standard reaction buffer [40 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA and 2 mM ATP]. Aliquots of 20 and 5 µl were electrophoresed in non-denaturing 8% polyacrylamide gels containing 0.1% SDS and 14% denaturing gels, respectively. Reaction products were quantified using a PhosphorImager and ImageQuant software (Molecular Dynamics). Reactions using forked duplex and G-tailed telomere substrates were performed in 20 µl standard reaction buffer [23, 24]. Reactions using Holliday junction substrates were performed in 20 µl HJ reaction buffer [40 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA and 2 mM ATP] [43].

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CONFLICT OF INTERESTS STATEMENT

The authors of this manuscript have no conflict of interests to declare.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Predictive value of tumor markers for hepatocarcinogenesis in patients with hepatitis C virus

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Abstract
Background Increases in tumor markers are sometimes seen in patients with chronic liver disease without hepatocellular carcinoma (HCC). The aim of this study was to determine the relationship between the levels of three tumor markers [alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%), and des-γ-carboxy prothrombin (DCP)] and hepatic carcinogenesis to identify hepatitis C virus (HCV) carriers at high risk for cancer development.
Methods A total of 623 consecutive HCV carriers with follow-up periods of >3 years were included. The average integration values were calculated from biochemical tests, and tumor markers, including AFP, AFP-L3%, and DCP, and factors associated with the cumulative incidence of HCC were analyzed.
Results HCC developed in 120 (19.3%) of the 623 patients. Age >65 years [adjusted relative risk, 2.303 (95% confidence interval, 1.551–3.418), $P < 0.001$], low platelet count [3.086 (1.997–4.768), $P < 0.001$], high aspartate aminotransferase value [3.001 (1.373–6.562), $P < 0.001$], high AFP level [≥10, <20 ng/mL: 2.814 (1.686–4.697), $P < 0.001$; ≥20 ng/mL: 3.405 (2.087–5.557), $P < 0.001$] compared to <10 ng/mL, and high AFP-L3% level [≥5, <10%: 2.494 (1.291–4.816), $P = 0.007$; ≥10%: 3.555 (1.609–7.858), $P < 0.001$] compared to <5% were significantly associated with an increased incidence of HCC on multivariate analysis.
Conclusions Increased AFP or AFP-L3% levels were significantly associated with an increased incidence of HCC. Among HCV carriers, patients with ≥10 ng/mL AFP or patients with ≥5% AFP-L3% are at very high risk for the development of HCC even if AFP is less than 20 ng/mL or AFP-L3% is less than 10%, which are the most commonly reported cutoff values.

Keywords Alpha-fetoprotein (AFP) · *Lens culinaris* agglutinin-reactive fraction of AFP · Hepatic regeneration · Necroinflammatory activity · Hepatocarcinogenesis

Introduction

Serum alpha-fetoprotein (AFP) is a widely used marker for hepatocellular carcinoma (HCC) [1]. However, serum AFP levels are increased in patients with liver diseases other than HCC, including viral hepatitis [2–4], with a prevalence of 10–42% [2, 5–7]. Increases in AFP are a marker of hepatic regeneration following hepatocyte destruction in viral hepatitis [8]. However, the pathogenesis and clinical significance of this phenomenon remain unclear.

The *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%) and des-γ-carboxy prothrombin (DCP) are also markers for HCC [9–12]. Available data suggest that these tumor markers are more highly specific for HCC than AFP alone [9]. However, there are no reports examining the prognostic value of these markers in hepatocarcinogenesis.

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Results of biochemical tests, including tumor markers, can fluctuate for a given patient and can vary between different patients, and repeated measurements over time may provide a more accurate picture of disease development or progression. The arithmetic mean value is often used to assess biochemical parameters over time, but this value can be greatly affected by the interval between measurements such that a short period of very high values can inappropriately skew the mean. We have previously argued that the average integration value is more meaningful than the arithmetic mean value for the purposes of monitoring disease progression [13, 14].

The aim of this study was to determine the relationship between three tumor markers (AFP, AFP-L3%, and DCP) to better identify hepatitis C virus (HCV) carriers at high risk for the development of HCC. Of note, we used the average integration values of these parameters in our analysis.

Patients, materials, and methods

Patient selection

A total of 1623 consecutive patients positive for anti-HCV antibody visiting the Department of Gastroenterology at Ogaki Municipal Hospital during the period January 1995 to December 1997 were considered for enrollment. The present study cohort included the following criteria for enrollment: (1) positive for anti-HCV antibody by second- or third-generation enzyme-linked immunosorbent assay and detectable HCV RNA for at least 6 months; (2) no evidence of positivity for hepatitis B surface antigen; (3) exclusion of other causes of chronic liver disease (i.e., alcohol consumption lower than 80 g/day, no history of hepatotoxic drug use, and negative tests for autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease); (4) follow-up period greater than 3 years; (5) measurement of AFP, AFP-L3%, and DCP at least every 6 months; (6) no evidence of HCC for at least 3 years from the start of the observation periods; and (7) interferon (IFN) therapy completed greater than 3 years before the detection of HCC in patients who received IFN therapy. A total of 623 patients fulfilled these criteria (Fig. 1).

Fibrosis was histologically evaluated in 187 of the 623 patients and staged according to Desmet et al. [15] as follows: F0, no fibrosis; F1, mild fibrosis; F2, moderate fibrosis; F3, severe fibrosis; and F4, cirrhosis. The remaining 436 patients were evaluated by ultrasound (US) findings and biochemical tests. The diagnosis of cirrhosis was made according to typical US findings, e.g., superficial nodularity, a coarse parenchymal echo pattern, and signs of portal hypertension (splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [16–18]. In this study patients who did not satisfy these criteria were classified as having chronic hepatitis. Four hundred and sixty-three patients were diagnosed with chronic hepatitis and 160 patients with cirrhosis.

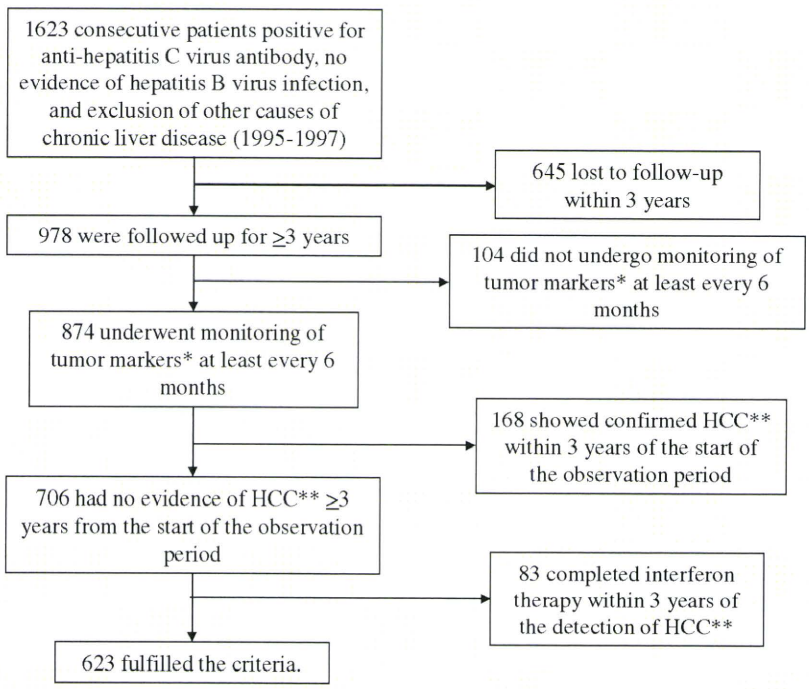

Fig. 1 Schematic flowchart of enrolled patients. *Serum alpha-fetoprotein (AFP), *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3%), and des-γ-carboxy prothrombin (DCP). **Hepatocellular carcinoma (*HCC*)

All patients were followed up at our hospital at least twice a year. During each follow-up examination, platelet count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transpeptidase (γ-GTP), total bilirubin, cholinesterase, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), albumin, total cholesterol, AFP, AFP-L3%, and DCP were measured. Platelet count and ALT, AST, γ-GTP, total bilirubin, cholinesterase, ALP, LDH, albumin, total cholesterol, AFP, AFP-L3%, and DCP values were expressed as average integration values [13, 14]. Briefly, using ALT as an example, the area of a trapezoid is calculated by multiplying the sum of two ALT values by one-half of the interval between the measurements. This value is then divided by the observation period to obtain the average integration value, and this technique provides a better representation of values over time when there are extremes of high and low values [14, 16]. In patients who developed HCC during the observation period, AFP, AFP-L3%, and DCP values obtained at least 1 year before the diagnosis of HCC were assessed. Serum AFP concentration was determined with a commercially available kit. AFP-L3% was measured by lectin-affinity electrophoresis and antibody-affinity blotting with the AFP Differentiation Kit L (Wako Pure Chemical Industries, Osaka, Japan) [10]. DCP was measured with a DCP reagent (Picolumi PIVKA-II; Eizai, Tokyo, Japan) [11]. Cutoff levels for AFP, AFP-L3%, and DCP were set at 20 ng/mL, 10%, and 40 mAU/mL, respectively, according to previous reports [10–12]. HCV genotype and quantification of HCV RNA (Amplicor 2; Roche Diagnostics, Tokyo, Japan) were determined in 513 cases. All patients underwent imaging modalities (US, computed tomography [CT], or magnetic resonance imaging [MRI]), every 3 months in patients with cirrhosis and every 6 months in patients with chronic hepatitis.

The diagnoses of HCC were confirmed by histologic examination of resected hepatic tumors or US-guided needle biopsy specimens. When biopsy of the tumor was contraindicated, the HCC diagnosis was made using clinical criteria and imaging findings obtained from B-mode US, CT angiography, or MRI [19, 20]. HCC was histologically diagnosed in 46 patients, and in the remaining 74 patients, the diagnosis was made based on clinical criteria [19, 20]. All tumors were 3 cm or less in maximum diameter, and there were 3 nodules or less on diagnosis.

One hundred eighty-nine patients received IFN therapy. Patients were classified into three groups according to the type of response to IFN therapy: sustained virologic response (SVR), defined as the absence of serum HCV RNA at 6 months after IFN therapy; the non-SVR group, defined as the presence of serum HCV RNA at 6 months after IFN therapy; and the no IFN therapy group.

Patients were classified into three groups for each of the tumor markers according to the average integration values of AFP, AFP-L3%, and DCP: A1, <10 ng/mL ($n = 452$); A2, ≥10, <20 ng/mL ($n = 80$); and A3, ≥20 ng/L ($n = 91$); L1, <5% ($n = 588$); L2, ≥5, <10% ($n = 18$); and L3, ≥10% ($n = 17$); and D1, <20 mAU/mL ($n = 379$); D2, ≥20, <40 mAU/mL ($n = 170$); and D3, ≥40 mAU/mL ($n = 51$), respectively.

The present study ended on 31 December 2008 or the date of identification of HCC occurrence. The median follow-up period was 9.0 years (range 3.0–13.0 years). The total number of blood examinations was 25.721, and the median number of blood examinations was 23 (range 6–105) per subject.

Statistical analysis

Statistical analysis was performed with the Statistical Program for Social Science (SPSS ver.17.0 for Windows; SPSS Japan, Tokyo, Japan). Continuous variables are shown as medians (ranges). The Mann–Whitney U-test was used for continuous variables, and Fisher's exact test was used for categorical variables. Actuarial analysis of the cumulative incidence of hepatocarcinogenesis was performed by the Kaplan–Meier method, and differences were tested by the log-rank test. The Bonferroni correction was performed for multiple comparisons. The Cox proportional hazards model and forward selection method were used to estimate the relative risk of HCC development associated with age (≤65 or >65 years), sex (female or male), body mass index (BMI ≤25.0 or >25.0 kg/m^2), HCV genotype (type 1 or type 2), viral concentration (≤100 or >100 KIU/mL), platelet count (<12.0 × 10^4/mm^3 or ≥12.0 × 10^4/mm^3), ALT (≤35 or >35 IU/mL), AST (≤40 or >40 IU/mL), total bilirubin (≤1.2 or >1.2 mg/dL), γ-GTP (≤56 or >56 IU/mL), ALP (≤338 or >338 IU/mL), cholinesterase (<431 or ≥431 IU/mL), LDH (≤250 or >250 IU/mL), albumin (<3.5 or ≥3.5 g/dL), total cholesterol (<130 or ≥130 mg/dL), cirrhosis (presence or absence), and IFN treatment (no therapy, non-SVR, or SVR) for univariate and multivariate analyses. We used the lower or upper limit of the reference values at our institute as cutoff values for platelet count, ALT, AST, total bilirubin, γ-GTP, ALP, cholinesterase, LDH, albumin, and total cholesterol levels. Statistical significance was set at $P < 0.05$.

The study protocol was approved by the Ethics Committee at Ogaki Municipal Hospital in January 2009 and the study was performed in compliance with the Helsinki Declaration. Informed consent was obtained from each patient for analyzing patient records and images.

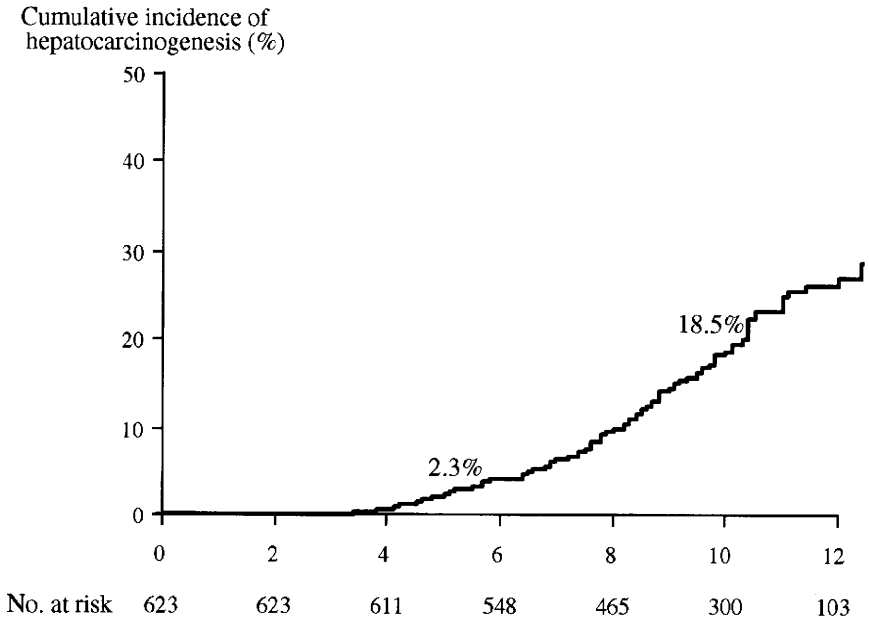

Fig. 2 Overall cumulative incidence rate of HCC

Table 1 Patient characteristics

Age (years)	61 (26–84)
Sex (F/M)	265/358
BMI (kg/m^2)	22.5 (12.0–34.9)
HCV genotype (type 1/type 2)	356/157
Viral concentration (KIU/mL)	270 (0.5–6300)
AFP (ng/mL)	4.8 (0.8–341.5)
AFP-L3 (%)	0.1 (0.0–32.5)
DCP (mAU/mL)	18.1 (8.5–99.6)
Platelets ($\times 10^4$/mm^3)	14.8 (3.0–33.9)
ALT (IU/L)	46.4 (10.1–340.4)
AST (IU/L)	48.5 (13.3–168.9)
γ-GTP (IU/L)	37.6 (9.9–2207)
Total bilirubin (mg/dL)	0.6 (0.2–2.7)
ALP (IU/L)	276.4 (86.8–845.5)
Cholinesterase (IU/L)	242.9 (38.8–545.30)
LDH (IU/L)	196.4 (118.4–650.1)
Albumin (g/dL)	4.0 (2.4–4.9)
Total cholesterol (mg/dL)	155.8 (77.9–264.1)
Fibrosis (F0/F1/F2/F3/F4)[a]	32/73/56/24/2
Cirrhosis (present/absent)	160/463
IFN therapy (none/non-SVR/SVR)	434/146/43

Continuous variables are quoted as medians (ranges)

BMI body mass index, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *AFP-L3Lens culinaris* agglutinin-reactive fraction of AFP, *DCP* des-γ-carboxy prothrombin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *GTP* gamma glutamyl transpeptidase, *ALP* alkaline phosphatase, *LDH* lactate dehydrogenase, *IFN* interferon, *SVR* sustained virologic response

[a] Staging of chronic hepatitis according to Desmet et al. [15]

Results

HCC developed in 120 (19.3%) of the 623 patients. The 5- and 10-year cumulative incidences of HCC were 2.3 and 18.5%, respectively (Fig. 2). Demographic and medical data for the 623 patients are summarized in Table 1.

Factors associated with the incidence of hepatic carcinogenesis on univariate analysis

Factors associated with the incidence of HCC are listed in Table 2. Age ≥65 years, high AFP level, high AFP-L3% level, high DCP level, low platelet count, high ALT level, high AST level, high LDH level, high ALP level, low cholinesterase level, low albumin level, presence of cirrhosis, and response to IFN therapy were significantly associated with the development of HCC on univariate analysis.

The 5-, 7-, and 10-year cumulative incidences of HCC were 1.1, 2.1, and 7.5% in group A1; 2.6, 9.6, and 42.1% in group A2; and 6.6, 18.3, and 50.0% in group A3, respectively, and the cumulative incidence of HCC differed significantly between groups A1 and A2 and groups A1 and A3 (Fig. 3). The 5-, 7-, and 10-year cumulative incidences of HCC were 1.4, 4.6, and 15.6% in group L1; 19.6, 39.7, and 73.6% in group L2; and 12.5, 25.0, and 56.7% in group L3, respectively, and the cumulative incidence of HCC differed significantly between groups L1 and L2 and groups L1 and L3 (Fig. 4). The 5-, 7-, and 10-year cumulative incidences of HCC were 0.5, 4.6, and

Table 2 Factors associated with hepatocarcinogenesis (univariate analysis)

	Crude hazard ratio (95% CI)	*P*
Age (years)		
≤65	1	
>65	2.318 (1.580–3.400)	<0.001
AFP (ng/mL)		
A1; <10	1	
A2; ≥10, <20	6.061 (3.768–9.750)	<0.001
A3; ≥20	8.985 (5.874–13.744)	<0.001
AFP-L3 (%)		
L1; <5	1	
L2; ≥5, <10	8.032 (4.388–14.700)	<0.001
L3; ≥10	3.781 (1.838–7.778)	<0.001
DCP (mAU/mL)		
D1; <20	1	
D2; ≥20, <40	1.209 (0.788–1.855)	0.385
D3; ≥40	4.535 (2.840–7.241)	<0.001
Platelets ($\times 10^4$/mm^3)		
≥12.0	1	
<12.0	5.887 (3.982–8.702)	<0.001
ALT (IU/L)		
≤35	1	
>35	2.632 (1.574–4.400)	<0.001
AST (IU/L)		
≤40	1	
>40	8.120 (4.115–16.024)	<0.001
LDH (IU/L)		
≤250	1	
>250	1.970 (1.249–3.106)	<0.001
ALP (IU/L)		
≤338	1	
>338	2.509 (1.724–3.650)	<0.001
Cholinesterase (IU/L)		
>431	1	
≤431	3.288 (2.209–4.893)	<0.001
Albumin (g/dL)		
≥3.5	1	
<3.5	3.948 (2.635–5.917)	<0.001
Cirrhosis		
Absent	1	
Present	3.474 (2.413–5.002)	<0.001
IFN therapy		
No therapy	1	
Non-SVR	0.312 (0.180–0.539)	<0.001
SVR	0.215 (0.075–0.620)	0.004

Continuous variables are quoted as medians (ranges)

CI confidence interval, *AFP* alpha-fetoprotein, *AFP-L3Lens culinaris* agglutinin-reactive fraction of AFP, *DCP* des-γ-carboxy prothrombin, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *LDH* lactate dehydrogenase, *ALP* alkaline phosphatase, *IFN* interferon, *SVR* sustained virologic response

14.8% in group D1; 1.8, 4.3, and 16.3% in group D2; and 10.0, 25.0, and 48.2% in group D3, respectively, and the cumulative incidence of HCC differed significantly between groups D1 and D3 and groups D2 and D3 (Fig. 5).

Factors associated with the incidence of hepatic carcinogenesis on multivariate analysis

Factors associated with the incidence of HCC as analyzed by the Cox proportional hazards model and the forward selection method are listed in Table 3. Age >65 years, low platelet count, high AST level, high AFP level, and high AFP-L3% level were significantly associated with the incidence of HCC. Factors associated with the incidence of HCC were analyzed in patients with chronic hepatitis and cirrhosis (Table 4). High age, low platelet count, high AST level, and high AFP level were significantly associated with the incidence of HCC in chronic hepatitis, and male sex, high age, low platelet count, high AFP level, and high AFP-L3% level were significantly associated with the incidence of HCC in cirrhosis. Factors associated with the incidence of HCC were analyzed in patients with and without IFN treatment (Table 5). Male sex, low platelet count, low cholinesterase level, and high AFP level were significantly associated with the incidence of HCC in patients with IFN therapy and male sex, high age, low platelet count, high AFP level, and high AFP-L3% level were significantly associated with the incidence of HCC in patients without IFN therapy.

Discussion

Advances in US, CT, and MRI have allowed for the more frequent and earlier detection of small HCC tumors less than 2 cm in diameter during the routine follow-up of patients with chronic liver disease [21–23]. However, the performance and resolution of the imaging device, the skills of individual operators, and the diagnostic acumen of the interpreting radiologist all affect the early detection of HCC. AFP, AFP-L3%, and DCP levels have been used as prognostic markers rather than diagnostic markers for HCC [9]. However, the detection rate of small HCC tumors with these markers is low; AFP-L3% and DCP have low sensitivity, and AFP has low specificity. Sassa et al. [12] reported detection rates of 22.6 and 48.4% for AFP-L3% and DCP, respectively, in patients with small HCC tumors. It is currently thought that serum markers are useful for follow-up after HCC therapy in patients with high tumor marker levels before treatment [24].

We have previously reported that the average integration value of ALT correlates with the cumulative incidence of hepatocarcinogenesis, even within the normal range [13, 14]. In the present study, the average integration value of AFP was not selected as a factor associated with the

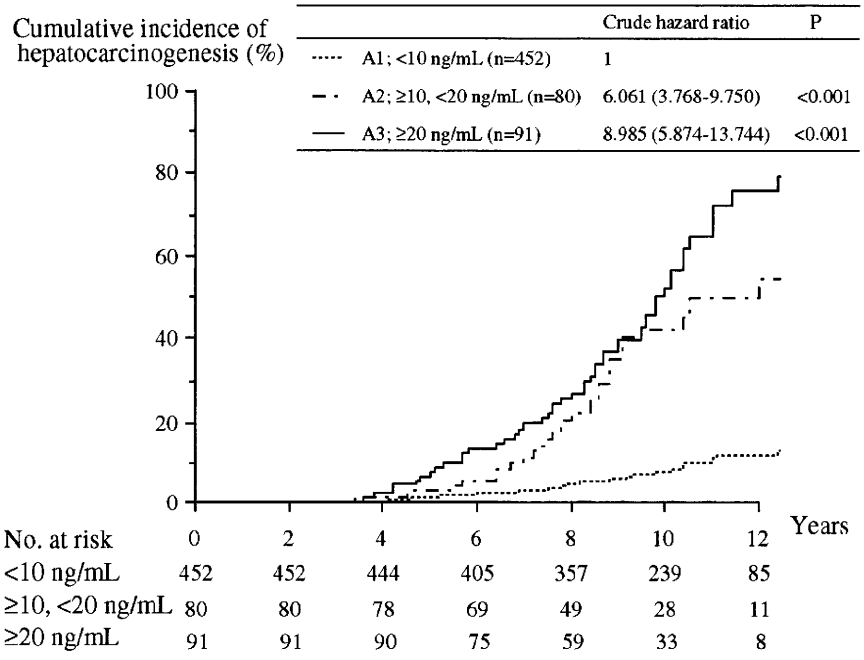

Fig. 3 Incidence of HCC according to the average integration value of AFP. The cumulative incidence of HCC differed significantly between groups A1 (<10 ng/mL) and A2 (≥10, <20 ng/mL) and groups A1 and A3 (≥20 ng/L)

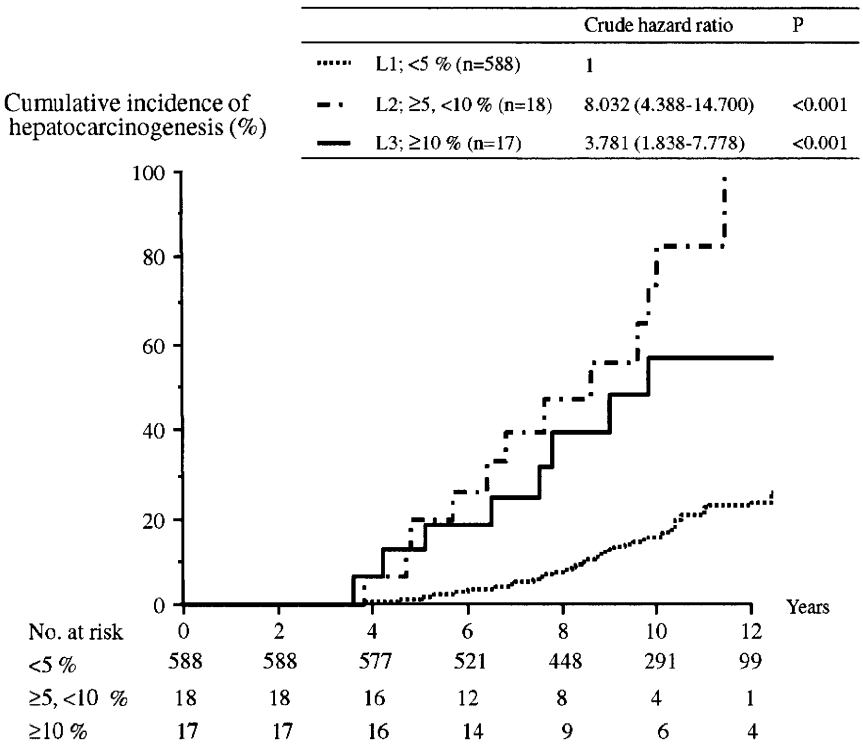

Fig. 4 Incidence of HCC according to the average integration value of AFP-L3%. The cumulative incidence of HCC differed significantly between groups L1 (<5%) and L2 (≥5, <10%) and groups L1 and L3 (≥10%)

incidence of HCC on multivariate analysis. AFP production is thought to be increased in response to injury, possibly due to increased hepatocyte turnover, in patients with HCV who do not have HCC [25]. In contrast, increased ALT levels are correlated with hepatocellular necrosis but not with hepatocyte proliferation. This difference may at least partially explain the absence of correlation between ALT and AFP levels.

The multivariate analysis in our series was carried out to minimize the influence of confounding factors, and 5 factors were selected by the forward selection method. Age >65 years, low platelet count, high AST value, high AFP

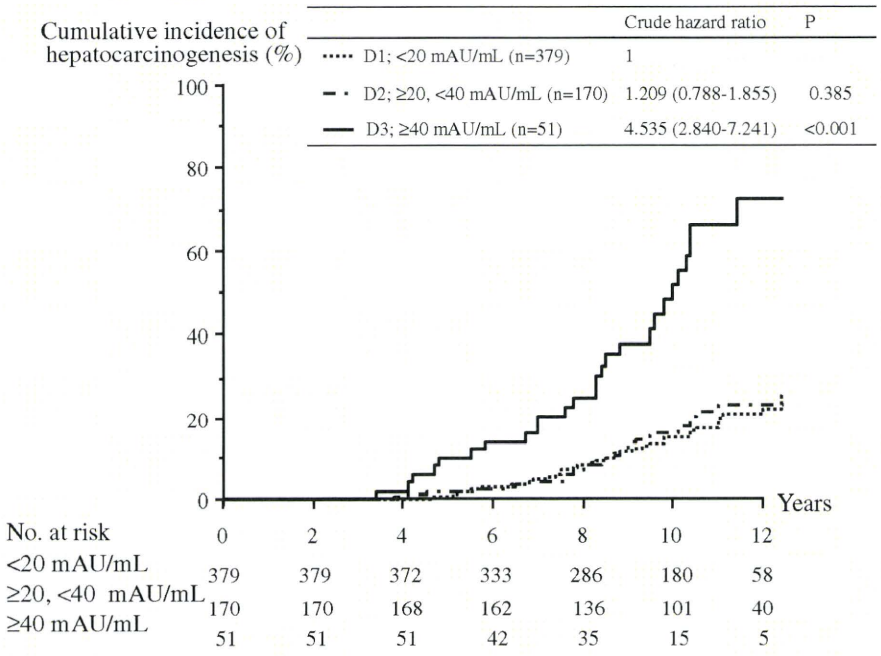

Fig. 5 Incidence of HCC according to the average integration value of DCP. The cumulative incidence of HCC differed significantly between groups D1 (<20 mAU/mL) and D3 (≥40 mAU/mL) and groups D2 (≥20, <40 mAU/mL) and D3

Table 3 Factors associated with hepatocarcinogenesis (multivariate analysis)

	Adjusted hazard ratio (95% CI)	*P*
Age (years)		
≤65	1	
>65	2.303 (1.551–3.418)	<0.001
Platelets ($\times 10^4$/mm^3)		
≥12.0	1	
<12.0	3.086 (1.997–4.768)	<0.001
AST (IU/L)		
≤40	1	
>40	3.001 (1.373–6.562)	0.006
AFP (ng/mL)		
A1; <10	1	
A2; ≥10, <20	2.814 (1.686–4.697)	<0.001
A3; ≥20	3.405 (2.087–5.557)	<0.001
AFP-L3 (%)		
L1; <5	1	
L2; ≥5, <10	2.494 (1.291–4.816)	0.007
L3; ≥10	3.555 (1.609–7.858)	0.002

AST aspartate aminotransferase, *AFP* alpha-fetoprotein, *AFP-L3 Lens culinaris* agglutinin-reactive fraction of AFP

level, and high AFP-L3% level were significantly associated with hepatic carcinogenesis in our multivariate analysis, but serum ALT level was not a risk factor for developing HCC. Ikeda et al. [26] reported that the cumulative incidence of HCC increased significantly in cirrhotic patients with an AFP level ≥10 ng/mL compared to those with an AFP level <10 ng/mL, and the adjusted risk ratio was 15.788 in HCV patients. They speculated that AFP is a marker of disease activity or severity and cellular regeneration, and it acts as a better predictor of HCC with viral etiology of cirrhosis. As an index of hepatic regeneration, the AFP level better represents the risk of hepatic carcinogenesis than an index of liver injury (e.g., ALT level). In addition to AFP, AFP-L3% was identified as a factor predicting the development of HCC, and this is a specific marker for the existence of HCC. Therefore, elevations in AFP-L3% may reflect an occult cancer that is undetectable with current imaging modalities. More intensive surveillance is needed for patients such as those who fulfill the criteria of groups L2 and L3 in our series, although these groups were very small in size. However, similar to other laboratory values, as high AFP-L3% values may be associated with severe liver damage, it is necessary to interpret these values carefully. DCP is well known to be also a specific marker of HCC. DCP is more closely related to tumor size than AFP and AFP-L3% [27]. Therefore, it is thought that these were the reasons that DCP was not selected as a predictive marker for HCC in our multivariate analysis.

Among the other risk factors we identified for the development of HCC, a low platelet count stands out. The platelet count is a useful marker for the diagnosis of cirrhosis [28], and cirrhosis is an established risk factor for HCC in HCV carriers [26, 28–30]. Taken together with our other findings, the low platelet count suggests that HCC develops in patients with progressive or advanced liver disease. We additionally used ultrasound (US) to distinguish cirrhotic patients from non-cirrhotic patients [16–18]. The presence of cirrhosis on US was strongly associated with an increased

Table 4 Factors associated with hepatocarcinogenesis on multivariate analysis in patients with chronic hepatitis and cirrhosis

	Chronic hepatitis ($n = 463$)	Cirrhosis ($n = 160$)
Age (years): ≤65 vs. >65	<0.001	0.008
Gender: female vs. male		<0.001
Platelets ($\times 10^4$/mm^3): ≥12.0 vs. <12	0.001	0.007
AST (IU/L): ≤40 vs. >40	0.043	
AFP (ng/mL): <10 vs. ≥10, <20 vs. ≥20	<0.001	0.003
AFP-L3 (%): <5 vs. ≥5, <10 vs. ≥10		0.017

AST aspartate aminotransferase, *AFP* alpha-fetoprotein, *AFP-L3 Lens culinaris* agglutinin-reactive fraction of AFP

Table 5 Factors associated with hepatocarcinogenesis on multivariate analysis in patients with and without IFN treatment

	With IFN ($n = 189$)	Without IFN ($n = 434$)
Age (years): ≤65 vs. >65		0.001
Gender: female vs. male	0.005	<0.001
Platelets ($\times 10^4$/mm^3): ≥12.0 vs. <12.0	0.047	<0.001
Cholinesterase (IU/L): ≥431 vs. <431	0.007	
AFP (ng/mL): <10 vs. ≥10, <20 vs. ≥20	<0.001	<0.001
AFP-L3 (%): <5 vs. ≥5, <10 vs. ≥10		<0.001

IFN interferon, *AFP* alpha-fetoprotein, *AFP-L3 Lens culinaris* agglutinin-reactive fraction of AFP

incidence of HCC on univariate analysis, but US-determined cirrhosis was not identified as a risk factor on multivariate analysis. Histologic assessment of fibrosis and cirrhosis was obtained in only 187 patients (30.0%), and patients with F4 fibrosis had a higher incidence of HCC in our univariate analysis. However, the population of patients with material available for histologic review was only one-third the size of the entire study population, and this small number may have negatively affected our ability to detect the predictive nature of fibrosis at all levels of severity. In contrast to serum ALT, serum AST levels were significantly associated with the incidence of HCC. AST levels are often abnormal in patients with cirrhosis when ALT values are in the normal range, and the AST/ALT ratio is frequently greater than 1 in cirrhotic patients [31]. Elevated AST activity is a surrogate marker for cirrhosis. Aging is associated with a number of events at the molecular, cellular, and physiological levels that influence carcinogenesis and subsequent cancer growth [32]. It has been hypothesized that an age-associated decrease in DNA repair [33] contributes to the development of HCC.

Recent reports have shown that AFP levels fall following the administration of IFN with or without ribavirin [34, 35]. IFN has been shown to have antiviral, anti-inflammatory, and anticancer activities [36]. One study demonstrated an anticancer effect of IFN when this agent was given following intrahepatic recurrence after HCC resection [37], and in our study, previous treatment with IFN was a factor associated with a reduced incidence of HCC on univariate analysis. The median ages of our patients with and without IFN treatment were 53 years (range 28–71) and 65 years (range 26–84), respectively; the age in those receiving IFN was significantly lower than the age in the group without IFN ($P < 0.0001$). It is thought that age and IFN therapy are confounding factors because IFN therapy has better results in younger patients. Although IFN was not identified as a predictive factor on multivariate analysis, the possibility cannot be denied that IFN may play an important role in modulating AFP levels prior to the onset of HCC.

In conclusion, increased AFP or AFP-L3% levels were significantly associated with an increased incidence of HCC. Among HCV carriers, patients with ≥10 ng/mL AFP or patients with ≥5% AFP-L3% are at very high risk for the development of HCC even if AFP is less than 20 ng/mL or AFP-L3% is less than 10%, which are the most commonly reported cutoff values. Intensive imaging modalities including US, CT, and MRI are recommended every 3–6 months for these patients.

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Conflict of interest There is no conflict of interest to disclose.

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Original Article

Transcatheter chemoembolization for unresectable hepatocellular carcinoma and comparison of five staging systems

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Aim: We compared the ability of five staging system to predict survival in patients with hepatocellular carcinoma (HCC) treated with chemoembolization.

Methods: The study subjects were 214 patients with HCC treated with repeated chemoembolization alone using cisplatin and lipiodol. Predictors of survival were assessed by multivariate analysis. Before chemoembolization was carried out, the modified Japan Integrated Staging (m-JIS), Japan Integrated Staging (JIS score), Barcelona (BCLC) stage, Liver Cancer Study Group of Japan/Tumor–Node–Metastasis (LCSGJ/TNM) and Italian score (CLIP score) were checked. To validate the prognostic value of these staging systems, the survival curve was obtained and analyzed by the Kaplan–Meier method. Discriminatory ability and predictive power were compared using Akaike's information criterion (AIC) score and the likelihood ratio (LR) χ^2.

Results: Overall survival was 1 year in 82.9%, 3 years in 39.9% and 5 years in 15.1%. Multivariate analysis identified more than 90% lipiodol accumulation (grade I) after the first chemoembolization ($P = 0.001$), absence of portal vein tumor thrombosis (PVTT) ($P < 0.001$) and liver damage A ($P = 0.012$) as independent determinants of survival. AIC score and the LR χ^2 showed superior predictive power of the m-JIS system in 95 patients with grade I accumulation of lipiodol after first chemoembolization.

Conclusion: The discriminate ability of the m-JIS score is substantially better than those of other staging systems and has better prognostic predictive power in patients with grade I accumulation of lipiodol after first chemoembolization.

Key words: hepatocellular carcinoma, chemoembolization, staging systems, cisplatin, Akaike's information criterion.

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common malignant tumors worldwide.[1–5] Recent advances in imaging and treatment modalities have resulted in a number of improvements in the prognosis of patients with HCC. Patients with small-size HCC, for example, are commonly treated by surgical resection and locoregional therapy such as percutaneous ethanol injection (PEI), microwave coagulation therapy (MCT), laser photocoagulation and radiofrequency ablation (RFA), and these treatments are often associated with satisfactory long-term prognosis.[6–10] These locoregional therapies are not suitable in all patients, however, mainly due to the presence of large tumor size, multiple HCC tumors or a serious underlying chronic liver disorder. Aging can also prevent patients being treated by surgical resection or

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liver transplantation, although senile patients have been found to have good underlying liver function.[11,12] Though it is ideal if the senile patients can adjust to RFA, image screening might be necessary for that.[13]

Transcatheter arterial embolization was applied to most inoperable HCC using gelatin sponge particles and anticancer agents.[14] Then, lipiodol (Lipiodol Ultrafluide; Laboratoire Guerbet, Aulnay-Sous-Bois, France) was introduced to enhance the therapeutic effect mainly in Japan, named transarterial chemoembolization.[15–19] However, chemoembolization irrespective of presence of anticancer agent and lipiodol has been controversial in the prognosis in the 1990s.[20,21] After 2000, chemoembolization produced survival benefits in two randomized controlled trials and meta-analyses.[16,19,22,23] Chemoembolization is currently the mainstay of treatment for unresectable HCC worldwide.

As the prognostic prediction before treatment is important, various staging systems have been reported.[24–28] While staging systems on resection and RFA were reported, only a few studies have compared staging systems in patients treated with repeated transarterial chemoembolization alone for HCC.[29–34] We have been reporting on the results of chemoembolization that uses cisplatin.[15,16] The aim of the present study was to identify which staging system shows superior predictive ability for outcome in a cohort of patients who underwent repeated transarterial chemoembolization using cisplatin and lipiodol. No study has compared staging systems in patients repeatedly treated with the same anticancer drug in transarterial chemoembolization.

METHODS

Patients

A TOTAL OF 214 patients with HCC treated with repeated chemoembolization at Hiroshima University Hospital from June 1983 to December 2008 were enrolled.

They were not treated with surgical resection, local ethanol injection, microwave coagulation or systemic chemotherapy. Treatment of all patients was followed by chemoembolization using cisplatin and lipiodol suspension throughout the study period. The study group consisted of 147 men and 67 women ranging in age from 42–92 years (median, 69 years). Of these, 172 patients (80%) were positive for hepatitis C virus and 15 (7%) for hepatitis B virus. One hundred and three patients (48%) were classified as having Child–Pugh class A disease and 108 (51%) as Child–Pugh class B disease. The median total bilirubin level was 1.0 mg/dL, and median serum albumin was 3.6 g/dL. The median value of the maximum diameter of the main tumor was 33 mm (range, 6–130). Eighty-nine patients (41%) had a solitary tumor, 74 (35%) had two to three tumors and 51 (24%) had four or more tumors.

Table 1 Characteristics of 214 patients who underwent repeated transcatheter chemoembolization using cisplatin and lipiodol suspension for unresectable hepatocellular carcinoma

Age (years)†	69 (42–92)
Sex (M/F)	147/67
Etiology (HCV/HBV/HBV + HCV/others)	172/15/3/24
Child–Pugh class (A/B/C)	103/108/3
Liver damage (A/B/C)	88/107/19
Total bilirubin (mg/dL)†	1.0 (0.2–3.8)
Albumin (g/dL)†	3.6 (2.4–4.7)
Prothrombin time activity (%)	76 (30–130)
Indocyanine green retention rate	25.5 (3.6–72.3)
Tumor size (mm)†	33 (6–130)
Total number of tumors 1/2–3/>3	89/74/51
Portal vein tumor thrombus (presence/absence)	9/205
PS (0/1)	211/3
Tumor occupancy rates (<50, ≥50)	210/4
α-Fetoprotein (ng/mL)†	43.0 (5–35 610)
Des-γ-carboxy prothrombin (mAU/mL)†	167 (10–37 900)
Embolization (with/without)	96/118
Period of follow up (months)†	19 (1–158)

†Data are median (range).
HBV, hepatitis B virus; HCV, hepatitis C virus; PS, performance status.

All subjects were analyzed by the modified Japan Integrated Staging (m-JIS),[28] Japan Integrated Staging (JIS score),[25] Barcelona (BCLC) stage,[27] Liver Cancer Study Group of Japan/Tumor–Node–Metastasis (LCSGJ/TNM)[24] and Italian score (CLIP score)[26] before chemoembolization.

Clinical characteristics of the study group are summarized in Table 1. The study was conducted in accordance with the Declaration of Helsinki and written informed consent was obtained from all participating patients.

Methods

Preparation of chemotherapeutic agents

We used cisplatin (Randa; Nippon Kayaku, Tokyo, Japan) mixed with lipiodol from 1983 to December 2004. Cisplatin powder for clinical use was not available in Japan during this period. Because cisplatin solution, which was available, has poor affinity for lipiodol, we prepared cisplatin powder from the commercially avail-

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able cisplatin solution to obtain an effective chemoembolization agent, as previously reported.[35–37]

After cisplatin powder became available from December 2004 to December 2008, we mixed it with lipiodol (IA-call; Nippon Kayaku). Lipiodol was mixed at a ratio of 1 mL per 10 mg cisplatin.[15]

Imaging and confirmation of diagnosis

Pretreatment imaging studies included abdominal ultrasonography (US), contrast-enhanced dynamic computed tomography (CT), dynamic magnetic resonance imaging (MRI), digital subtraction angiography (DSA), angiography combined with CT during arterial portography (CTAP) and hepatic arteriography (CTHA). All tumors were diagnosed by distinctive findings on US; dynamic CT, dynamic MRI, or both; DSA or CTAP; and CTHA. Diagnosis was confirmed by early enhancement in the arterial phase and hypo-attenuation in the portal venous or equilibrium phase on contrast-enhanced dynamic CT or dynamic MRI or by hypo-attenuation on CTAP and hyper-attenuation on CTHA. In addition, changes in serum tumor markers (α-fetoprotein [AFP] or des-γ-carboxy prothrombin [DCP]) were used to support the imaging-based diagnosis.

Transcatheter chemoembolization

Chemoembolization was performed through the femoral artery with use of the Seldinger technique with local anesthesia. Arteriography of the celiac trunk and superior mesenteric artery was performed to visualize the arterial vascularization of the liver and to evaluate portal vein patency. An angiographic catheter was inserted into the hepatic artery where the target tumor was located. Chemoembolization agents were injected through the hepatic artery. Gelatin sponge particles were used after chemoembolization in patients with a membrane-covered lesion and a segmental lesion in the periphery, as these patients had relatively little liver damage.

From 1983 to June 2000, chemoembolization was performed under DSA. From June 2000 to December 2008, chemoembolization was performed under CTAP and CTHA.

Evaluation of therapeutic effect of chemoembolization

The efficacy of chemoembolization was evaluated by CT at 3 months after treatment as follows: when lipiodol was seen in more than 90% of the tumor, efficacy was considered grade I; in 50–90% of the tumor, grade II; and in less than 50% of the tumor, grade III.[15] Grading for lipiodol retention was based on quantitative measurement of tumor diameter in all tumors, based on the assumption that the tumor portion with retained lipiodol was necrotic tissue.[38]

Follow-up protocol

Concentrations of serum tumor markers, including AFP and DCP, were measured once a month after chemoembolization; follow-up US was performed every 3 months; and CT or MRI was performed every 6 months. Patients showing an increase in tumor markers, diminution of lipiodol accumulation or new nodules remote from the treated nodules were readmitted for an additional round of chemoembolization using the same procedure. On follow up, patients treated with chemoembolization who did not show complete uptake of lipiodol (i.e. those classified as grade I) but did show the presence of a viable tumor, namely by arterial phase enhancement on CT/MRI, were retreated with chemoembolization within 3–6 months of the first treatment. Patients with tumor progression, appearance of portal vein tumor thrombosis (PVTT) and liver failure were excluded from further treatment.

Statistical analysis

Cumulative survival rate was calculated from the initial date of chemoembolization and assessed by the Kaplan–Meier life-table method, with differences evaluated by the log–rank test. Univariate analysis of predictors of survival was assessed by the Kaplan–Meier method and differences were evaluated by the log–rank test. Multivariate analysis of predictors of survival was assessed by a Cox proportional hazards model. Statistical significance was defined as a $P < 0.05$. We also calculated hazard ratios and 95% confidence intervals (95% CI). All *P*-values less than 0.05 on two-tailed tests were considered significant. Variables that achieved statistical ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into a multiple Cox proportional hazards model to identify significant independent factors. Parameters used for the prediction of survival were lipiodol accumulation, tumor number, PVTT (presence or absence), liver damage, AFP, DCP, age, sex, etiology, embolization (with or without), CT scan during hepatic arteriography and arterial portography (with vs without), and tumor size. To validate the prognostic value of these staging systems, the survival curve was obtained and analyzed by the Kaplan–Meier method; and to compare discriminatory ability and predictive power, the likelihood ratio (LR) χ^2 and Akaike's

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Table 2 Transcatheter chemoembolization using cisplatin and lipiodol suspension

No. of procedures	2 (1–9)
Mean dose of cisplatin per single session (mg)	35 (5–67.5)
Total dose of cisplatin per single case (mg)	60 (10–390)
Lipiodol accumulation after chemoembolization (grade I/II/III) (%)	55/33/12

information criterion (AIC) score were used in 95 patients with grade I accumulation. The AIC statistic was defined as AIC = −2 log maximum likelihood + 2 × the number of parameters in the model. A smaller AIC value indicated a more desirable model for predicting outcome. The Cox proportional hazards model was used to calculate the LR χ^2 to determine homogeneity (small differences in survival among patients at the same stage within each system). The model with the higher χ^2 by the LR test was considered the better model. All analyses were performed with SPSS software (ver. 16, SPSS, IL, USA).

RESULTS

Therapeutic effects of transcatheter chemoembolization using cisplatin and lipiodol suspension

THE MEDIAN NUMBER of chemoembolization procedures per patient was two (range, 1–9). The mean dose of cisplatin per single session of chemoembolization was 35 mg (range, 5.0–67.5), and the median total dose of cisplatin per patient was 60 mg (range, 10–390). Lipiodol accumulation was evaluated after first chemoembolization as grade I in 58 patients (55%), grade II in 36 (33%) and grade III in 13 (12%) (Table 2).

Survival rates

Cumulative survival curves of patients treated with chemoembolization using cisplatin and lipiodol suspension for unresectable HCC showed survival rates of 92% at 1 year, 40% at 3 years, 18% at 5 years and 12% at 7 years (Fig. 1).

We then investigated the relationship between survival after the initiation of chemoembolization and various clinicopathological variables by univariate analysis. Results showed that survival correlated significantly with grade I accumulation ($P = 0.003$), absence of PVTT ($P = 0.001$) and liver damage A ($P = 0.005$) (Table 3). Grade I accumulation, absence of PVTT, liver damage A and number of tumors = 1 were then entered into the multiple Cox proportional hazards model, which identified grade I accumulation ($P < 0.001$), absence of PVTT ($P < 0.001$) and liver damage A ($P = 0.026$) as significant and independent determinants of survival (Table 4).

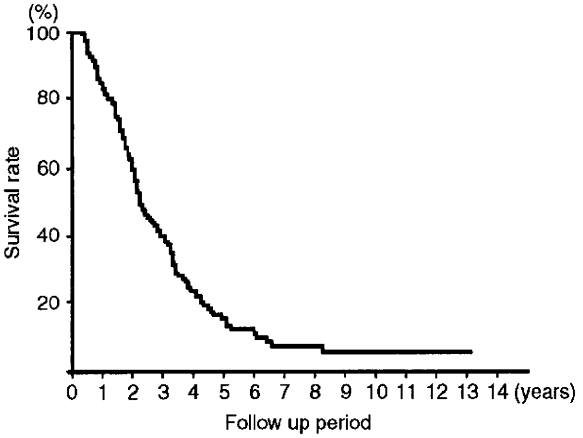

Figure 1 Cumulative survival curves of patients treated with chemoembolization using cisplatin and lipiodol suspension for unresectable hepatocellular carcinoma showed survival rates of 92% at 1 year, 40% at 3 years, 18% at 5 years and 12% at 7 years.

Table 3 Univariate analyses of predictors of survival by the log-rank test

Variable	*P*-value
Grade (I vs II and III) LDP accumulation	0.003
Portal vein tumor thrombus (absent vs present)	0.001
Liver damage (A vs B and C)	0.005
Child-Pugh (A vs B and C)	0.11
Total number of tumors (1 vs ≥2)	0.091
α-Fetoprotein (<200 vs ≥200)	0.365
Des-γ-carboxy prothrombin (<200 vs ≥200)	0.63
Age (<60 vs ≥60)	0.133
Sex (M vs F)	0.98
HBV/HCV/non-B, non-C (HBV vs HCV and non-B, non-C; HBV and HCV vs non-B, non-C; HBV and non-B, non-C vs HCV)	0.33
Embolization (with vs without)	0.108
CT scan during hepatic arteriography and arterial portography (with vs without)	0.71
Tumor size (<20 mm vs ≥20 mm)	0.817

CT, computed tomography; HBV, hepatitis B virus; HCV, hepatitis C virus.

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Table 4 Multivariate analyses of predictors of survival by Cox proportional hazards model

Factor	Category	Hazard ratio	95% CI	*P*-value
Grade LDP accumulation	1; I 2; II/III	0.467	0.314–0.697	<0.001
PVTT	1; Absence 2; present	0.200	0.09–0.461	<0.001
Liver damage	1; A 2; B/C	0.722	0.534–0.961	0.026
Total number of tumors	1; one =; multiple	0.723	0.493–1.059	0.096

CI, confidence interval; PVTT, portal vein tumor thrombus.

Patient distribution, survival according to staging system and discriminatory ability of each staging system in 95 patients with grade I accumulation

To improve the statistical power of our analysis, we compared predictive ability among the staging systems in a subgroup of 95 patients with grade I accumulation after first chemoembolization. Distribution of the 95 patients with grade I accumulation among the different classes for each staging system is described in Table 5. Distributions of patients in all staging systems showed similar results. Using the Kaplan–Meier method, all staging systems, except the LCSGJ/TNM system, correctly differentiated survival for patients in different stages.

The m-JIS system showed the best discrimination ability and monotonicity of gradient as confirmed by AIC score test (279.7). Using the Cox regression LR χ^2-test (6.53), we confirmed that the m-JIS system also had the best homogeneity ability (Table 6). AIC score was in the descending order of m-JIS, JIS score, BCLC stage, LCSGJ/TNM and CLIP score. Next, we omitted stage IV ($n = 5$) in LCSGJ/TNM, score 3 ($n = 2$) in CLIP score, stage C ($n = 1$) and D ($n = 4$) in BCLC stage, score 0 ($n = 3$) and score 4 ($n = 3$) in JIS score, score 0 ($n = 4$), score 4 ($n = 1$) and score 5 ($n = 2$) in m-JIS due to small sample and re-analyzed by AIC score test. As a result, AIC score was in the descending order of m-JIS (237.0), LCSGJ/TNM (248.3), JIS score (248.4), BCLC stage (255.5) and CLIP score (264.6). Therefore, we confirmed that the m-JIS system also had the best homogeneity ability.

Cumulative survival curves of patients treated with chemoembolization using cisplatin and lipiodol suspension for unresectable HCC showed survival rates according to the five staging systems in 95 patients with grade I accumulation (Figs 2–6).

DISCUSSION

CLINICAL STAGING SYSTEMS for HCC patients should provide guidance for patient assessment and appropriate therapy and are useful for decisions on when to treat patients aggressively while avoiding the overtreatment of patients who would not tolerate therapy or whose life expectancy rules out any chance of success. Current examples include the m-JIS,[28] JIS,[25] BCLC staging system,[27] LCSGJ/TNM[24] and CLIP score.[26] Clinical staging is also an essential tool for comparison between groups in therapeutic trials and between different studies. In this study, the m-JIS system also showed the best discrimination ability and monotonicity of gradient in a subgroup of 95 patients with grade I LDP accumulation after first chemoembolization.

These five different staging systems were developed in and depend on different groups of patients. The differences between them are strongly dependent on the particular characteristics of the group of patients they are used in. Moreover, different treatments have a marked influence on the prognosis of patients in these staging systems. It is possible that the different staging systems should be evaluated separately in specific groups of patients. Moreover, when comparing the performance of staging systems, consideration is likely necessary of both the type of treatment and its efficacy. In the present study we therefore evaluated the prognostic power of each of these staging systems in the same group of patients after chemoembolization. We first identified factors related to survival in univariate and multivariate analyses. Results showed that a significant and independent determinant of survival was grade I accumulation of lipiodol after first chemoembolization such as in our previous report.[16] We then therefore evaluated the prognostic power of each of these staging systems in these grade I patients after chemoembolization.

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Table 5 Survival of 95 patients with grade I accumulation after chemoembolization by different staging systems (Kaplan–Meier survival analysis; comparison by log-rank test)

Staging system	No. of patients	Median survival (months)	Range (months)	*P*-value of log-rank test
LCSGJ/TNM				0.084
I	13	36	(6–114)	
II	48	21	(1–99)	
III	29	16	(2–104)	
IV	5	10	(5–55)	
CLIP				0.004
0	22	18	(1–99)	
1	43	26	(1–144)	
2	28	13	(2–104)	
3	2	14	(7–21)	
BCLC				0.009
0	9	26	(6–37)	
A	48	17	(1–114)	
B	33	21	(2–104)	
C	1	56	56	
D	4	11	(9–27)	
JIS				<0.001
0	3	9	(6–79)	
1	36	26	(1–114)	
2	43	20	(1–104)	
3	10	16	(9–56)	
4	3	7	(5–10)	
m-JIS				<0.001
0	4	60	(6–114)	
1	29	26	(6–99)	
2	46	17	(1–104)	
3	13	19	(7–61)	
4	1	7	7	
5	2	8	(5–10)	

LCSGJ/TNM, Liver Cancer Study Group of Japan/Tumor-Node-Metastasis; CLIP, Italian score; BCLC, Barcelona stage; JIS, Japan Integrated Staging; m-JIS, modified Japan Integrated Staging.

Table 6 Performance evaluation of five scoring system in 95 patients with grade I

Staging system	Homogeneity LR χ^2-test (*P*)	AIC
m-JIS	6.53 (0.011)	279.70
JIS	5.83 (0.016)	280.63
BCLC	0.49 (0.480)	282.40
TNM	1.74 (0.189)	282.55
CLIP	2.99 (0.083)	283.55

m-JIS, modified Japan Integrated Staging; JIS, Japan Integrated Staging; BCLC, Barcelona stage; LCSGJ/TNM, Liver Cancer Study Group of Japan/Tumor-Node-Metastasis; CLIP, Italian score; AIC, Akaike's information criterion; LR, likelihood ratio.

The m-JIS and JIS showed good stratification of our patients among the different stages, with good discrimination. Our patients were classified with m-JIS and JIS primarily into the lower classes, suggesting that these staging systems were created primarily for intermediate to early HCC. The m-JIS and JIS differed with regard to liver damage and Child-Pugh class. A significant and independent determinant of survival in our study was liver damage. This finding may suggest that classification of liver damage is useful in the evaluation and prediction of outcome of patients with early-stage liver diseases.[39] Accordingly, we consider that the discriminant ability of the m-JIS score is substantially better than that of the JIS score. Our patients were classified by CLIP

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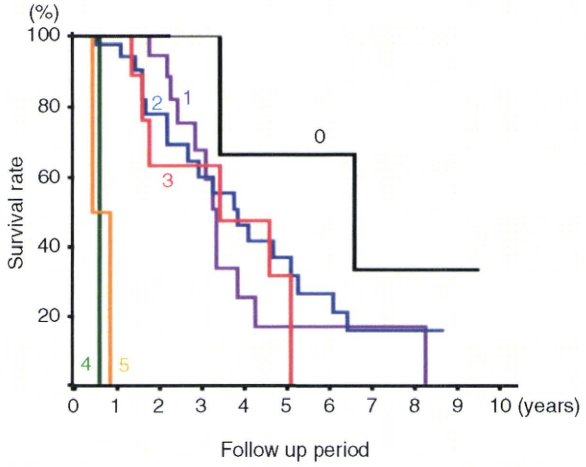

Figure 2 Overall survival according to modified Japan Integrated Staging (m-JIS) score in 95 patients with grade I accumulation after chemoembolization.

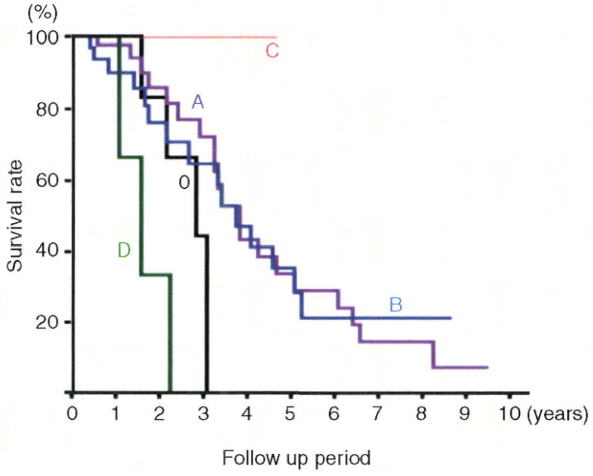

Figure 4 Overall survival according to Barcelona (BCLC) stage in 95 patients with grade I accumulation after chemoembolization.

primarily into the lower classes, suggesting that these staging systems have been created primarily for intermediate to advanced tumors. In our cohort, the LCSGJ/TNM system had no prognostic value. This observation confirms its major drawback, namely the absence of variables related to hepatic function, a variable associated with prognosis in most of the surgical and nonsurgical studies.[40] The BCLC was derived from the results of surgical treatment of early tumors and the natural history of untreated HCC.[27]

The discriminate ability of the m-JIS score is substantially better than that of the other staging systems and has better prognostic predictive power in patients undergoing chemoembolization with cisplatin and lipiodol. Therefore, when the m-JIS score is high, treatment methods for HCC may require reconsideration on account of the limit on repeated chemoembolization. Ineffective repeated chemoembolization may cause poorer hepatic reserve, and subsequently fail to further treatment. Because PVTT and liver damage A were not

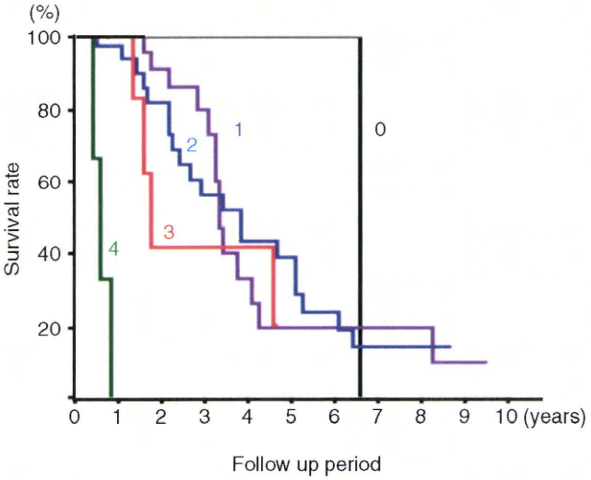

Figure 3 Overall survival according to Japan Integrated Staging (JIS) score in 95 patients with grade I accumulation after chemoembolization.

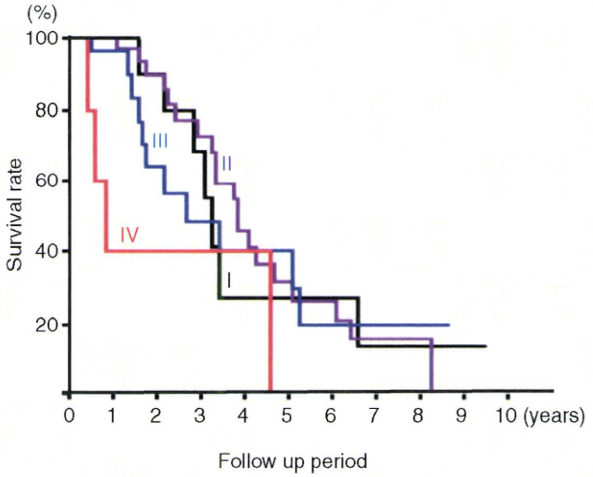

Figure 5 Overall survival according to Liver Cancer Study Group of Japan/Tumor–Node–Metastasis (TNM) in 95 patients with grade I accumulation after chemoembolization.

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