of diagnosis of recurrence or the latest follow up. All patients were followed until death or 31 May 2008.

Statistical analysis

Differences between groups were analyzed by the Mann–Whitney *U*-test for continuous variables and the χ^2-test for categorical variables. The overall and recurrence-free survival curves were generated by the Kaplan–Meyer method and compared with the log-rank test. Paired continuous data was evaluated by Wilcoxon signed-rank test. A *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Pre- and postoperative characteristics and pathology findings

PATIENTS AND HISTOLOGICAL characteristics are summarized in Table 1. Preoperatively, AFP and DCP were measured in all cases and AFP-L3 was measured in 94. AFP (>10 ng/mL) and DCP (>40 mAU/mL) were positive in 61 cases (61%) and 40 cases (40%), respectively. AFP-L3 was positive (≥10%) in 21 cases (22%). After liver transplantation, serum AFP and DCP values promptly decreased. A detailed analysis of the serum tumor markers is described later.

After histopathological evaluation of the explanted liver, 61 remained within a size and number that fulfilled the Milan criteria, and 53 of them showed no evidence of vascular invasion, whereas eight had microvascular invasions. The other 39 had HCC with pathological findings that exceeded the Milan criteria. The histological grade of HCC was well-differentiated in 25, moderately differentiated in 60, poorly differentiated in six and necrotic tissue in nine.

Postoperative course and HCC recurrence

During the median follow up of 44 months (0.8–120), 19 subjects died. Overall, survival at 1, 3 and 5 years was 89%, 80% and 77%. The causes of death were recurrence of HCC in seven, and other in 12. Recurrence of HCC was diagnosed in nine patients. Recurrence-free survival at 1 and 3 years was 95% and 89%, respectively. By the time of HCC recurrence, seven were maintained on tacrolimus and steroid, and the other two were switched to cyclosporine and steroid.

Characteristics of HCC recurrence

Details of the patients with HCC recurrence and the tumor characteristics are shown in Tables 2 and 3. Recurrent HCC was first suspected based on increases in the tumor marker levels in all cases, and pain was

Table 1 Patient characteristics and histological findings

Factors	All patients ($n = 100$)	Recurrent (+) ($n = 9$)	Recurrent (−) ($n = 91$)	*P*
Preoperative findings				
Age (years)	56 (40–67)	54 (44–62)	56 (40–67)	0.48
Sex (M : F)	82:18	8:1	74:17	0.57
AFP (ng/mL)	20 (1–11999)	253 (9–6721)	17 (1–11999)	0.005
AFP-L3 (%)†	0 (0–77.8)	24 (0–77.8)	0 (0–56)	0.01
DCP (mAU/mL)	26 (10–10592)	134 (10–10592)	23 (10–876)	0.01
Number of tumor (single/multiple)	45/50	2/7	43/43	0.16
Max tumor size (cm)	2.5 ± 1.4	3.0 ± 1.5	2.3 ± 1.4	0.50
Milan criteria met	82	6	76	0.54
Pathological findings				
Number of tumor (single/multiple)	34/66	2/7	32/59	0.71
Max tumor size (cm)	2.6 ± 1.5	3.1 ± 1.9	2.5 ± 1.5	0.20
Vascular invasion positive	20	4	16	0.08
Histological grade				0.04
Well differentiated	25	0	26	
Moderately differentiated	60	7	52	
Poorly differentiated	6	2	4	
Necrosis	9	0	9	
Milan criteria met	61	4	57	0.31

†AFP-L3 was measured in 94 recipients.
AFP, α-fetoprotein; AFP-L3, L3 fraction of AFP; DCP, des-γ carboxy prothrombin; HCC, hepatocellular carcinoma.

Table 2 Characteristics of original hepatocellular carcinoma (HCC) in nine recurrent HCC cases

Case	Age/sex	Milan criteria†	Tumor marker before LT			Histological findings			Histological grade (vascular invasion)
			AFP (ng/mL)	AFP-L3 (%)	DCP (mAU/mL)	Max. tumor size (cm)	Number	Bilobar spread	
1	57/M	Met	55	13	22	5.0	1	No	Mod‡ (−)
2	54/M	Not met	96	<1	1994	7.0	14	No	Poor§ (+)
3	54/M	Not met	2526	3	10	3.7	16	Yes	Mod‡ (−)
4	53/M	Met	323	48	58	1.7	4	Yes	Mod‡ (−)
5	51/M	Met	9	<1	225	3.0	7	Yes	Mod‡ (−)
6	52/M	Met	253	ND	740	2.0	3	No	Mod‡ (+)
7	62/F	Not met	4517	52	161	1.0	19	Yes	Mod‡ (+)
8	44/M	Met	390	35	21	1.3	2	No	Mod‡ (−)
9	57/M	Met	2086	78	10 592	4.0	1	No	Poor§ (+)

†Based on preoperative imaging studies.
‡Mod, moderately differentiated.
§Poor, poorly differentiated.
AFP, α-fetoprotein; AFP-L3, L3 fraction of AFP; DCP, des-γ carboxy prothrombin; ND, not done; HCC, hepatocellular carcinoma; LT, liver transplantation.

Table 3 The mode and the timing of diagnosis of hepatocellular carcinoma (HCC) recurrence in nine recurrent HCC cases

Case	The first sign of recurrence (A)	Imaging test with negative result	Confirmatory test (B)	AFP/AFP-L3/DCP†	Recurrent site	Days from A to B	Days from LT to B	Treatment	Outcome (days from LT)
1	DCP	CT ×2, BS ×1	CT	112/87/243 800	Liver	40	95	C, TACE	Dead (229)
2	AFP	CT ×3, BS ×1	CT	47/21/38	Lymph node	134	399	Ope, C, RTx	Dead (1017)
3	AFP	CT ×3, BS ×3, MRI ×1	PET, MRI	8830/<1/12	Bone	179	312	Ope, C, RTx	Dead (1329)
4	DCP, Pain	None	CT, BS	1/<1/342	Bone	17	308	RTx, C	Dead (371)
5	DCP	BS ×1	CT	3/<1/200	Lung	60	752	VATS	Alive (1963)
6	AFP, DCP	CT ×5, head CT ×1, AG ×1, PET ×1, BS ×1	CT	26 448/37/5354	Liver	153	574	Ope, C	Dead (766)
7	AFP	CT ×3, MRI ×1	CT	1311/68/297	Bone	208	229	None	Dead (312)
8	AFP	BS ×1	CT	16/<1/13	Lung	99	890	VATS	Alive (1431)
9	AFP, DCP	CT ×3, BS ×1, head CT ×1, head MRI ×1, PET ×1	CT	3602/71/2416	Liver	112	203	Ope, C	Dead (357)

†Tumor marker results obtained at the time of confirmatory tests.
AFP, α-fetoprotein; AFP-L3, L3 fraction of AFP; AG, angiography; BS, bone scintigraphy; C, chemotherapy; CT, computed tomography; DCP, des-γ carboxy prothrombin; HCC, hepatocellular carcinoma; LT, liver transplantation; MRI, magnetic resonance imaging; Ope, operation; PET, positron emission tomography scan; RTx, radiation therapy; TACE, trans-arterial chemoembolization; VATS, video-assisted thoracoscopic surgery.

associated with one case (case 4). In two cases (cases 1 and 4), the tumor marker increase at the time of tumor recurrence was different from that prior to LDLT. Once HCC recurrence was suspected, intensive radiographic examinations were performed. Most commonly, CT scans of the chest and abdomen and bone scintigraphy were included in the first set of imaging studies. This set of images successfully led to a diagnosis of HCC recurrence in three cases (cases 4, 5 and 8). In the other six cases with recurrence, however, repeated radiographic evaluations were necessary. In case 3, bone lesions were revealed by positron emission tomography scan, followed by magnetic resonance imaging 179 days after the first increase in AFP. In case 9, a sinus lesion detected by head CT scan was diagnosed as a postoperative fungal infection. The true recurrence site in this case was the graft, based on dynamic CT scan 112 days after the first increase in the serum tumor markers. The median (range) time from first sign of HCC recurrence to radiographic confirmation was 112 days (17–208). The median (range) number of patient visits for imaging studies was five times (2–10).

Hepatocellular carcinoma recurrence was diagnosed within 1 year in five recipients, between 1–2 years in two, and after 2 years in two. The median (95% confidence interval) survival after the diagnosis of HCC recurrence was 6.3 months (3.7–8.9). All but one subject were treated with following modalities; surgical tumor reduction in six, transarterial chemoembolization in one, systemic chemotherapy in six or regional irradiation in three. One (case 7) received symptom-relieving drugs. Two cases with lung lesion (cases 5 and 8) were surgically treated with video-assisted thoracoscopic surgery and both remain alive at 18 and 39 months after surgery and are free from other recurrence. Another two (cases 2 and 3) survived more than 1 year after surgery for recurrent HCC.

Sequential changes in AFP

The median (range) AFP value 2 months after LDLT was significantly lower than that pre-transplantation (2 ng/mL [1–182]; $P < 0.001$). Sequential changes in serum AFP levels among those without HCC recurrence are shown in Figure 1(a). AFP levels decreased immediately post-transplantation. In all cases without HCC recurrence, AFP levels decreased to lower than 20 ng/mL within 2 months post-transplantation. Serum AFP values at 1 year after LDLT were 2.8 ± 2.3 ng/mL, with

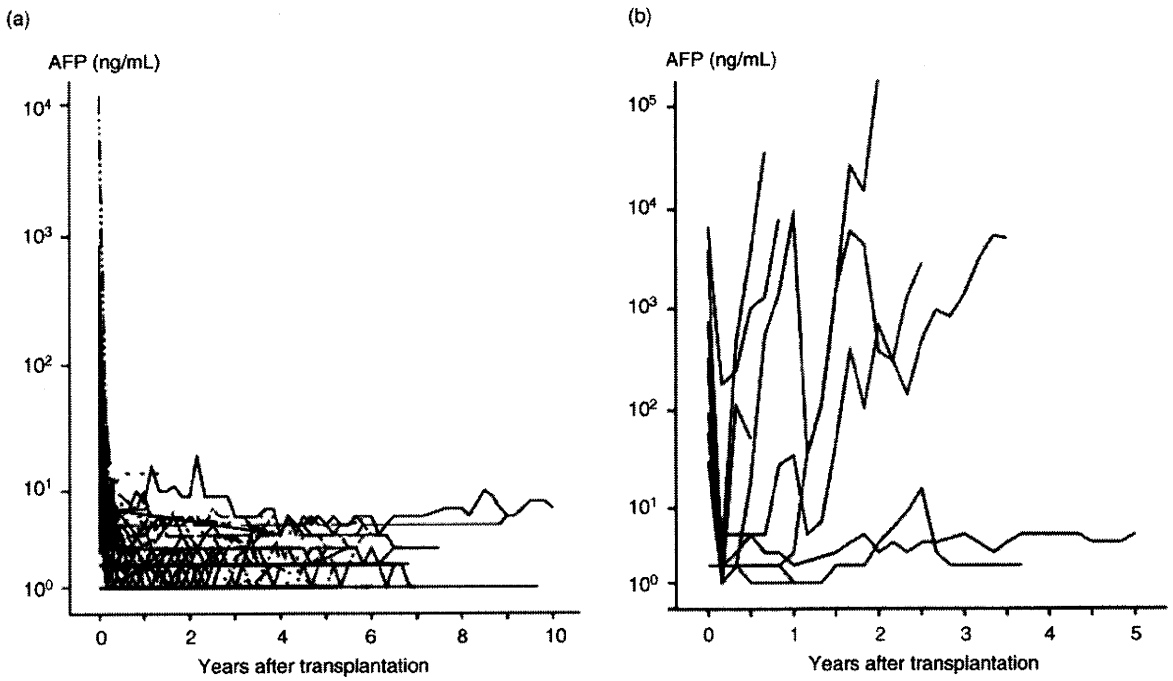

Figure 1 Sequential changes in serum α-fetoprotein (AFP) levels are shown. (a) Ninety-one recipients without hepatocellular carcinoma (HCC) recurrence. AFP levels decreased and remained below 20 ng/mL in call cases. (b) Nine cases with HCC recurrence. AFP levels increased to over 20 ng/mL in six cases and continuously increased thereafter.

the 90th percentile at less than 5 ng/mL. Figure 1(b) shows the changes in AFP levels among subjects with HCC recurrence. AFP levels increased to over 20 ng/mL in six cases and continuously increased thereafter. AFP-L3 was over 10% in five of six cases. An increase in serum AFP levels between 11 and 20 ng/mL was observed in four cases. In one case, HCC recurrence was diagnosed when the AFP values rose from 2 to 16 ng/mL (case 8). In the other two cases, AFP values fluctuated between 5 and 19 ng/mL without evidence of recurrent HCC. When cut-off levels of 10 and 20 ng/mL were used, the sensitivity and specificity for HCC recurrence after liver transplantation were 7/9 (78%) and 89/91 (98%), and 6/9 (67%) and 91/91 (100%), respectively.

Sequential changes in DCP

The median (range) DCP value 2 months after LDLT dropped significantly from the pre-transplantation value to 14 mAU/mL (10–93) ($P < 0.001$). Figure 2 shows the sequential changes in serum DCP levels among those with and without HCC recurrence. Figure 2(a) shows the decrease in the DCP values after surgery. The mean serum DCP value at 1 year was 27 ± 51 mAU/mL, with the 90th percentile at 27 mAU/mL. Unlike AFP, a transient increase in DCP levels without evidence of recurrence was observed postoperatively in five cases. The causes of a DCP increase to over 40 mAU/mL included the following: biliary duct obstructions with or without biliary lithiasis ($n = 3$); oral administration of warfarin for pulmonary embolism ($n = 1$) and unknown ($n = 1$) (Table 4). The DCP value of the latter case remained between 40 and 75 mAU/mL for 2 years until the end of the follow-up period. The DCP values in the earlier four cases promptly normalized after resolution of the medical conditions. Figure 2(b) shows the change in the DCP values among those with HCC recurrence. An increase in the DCP values to over 40 mAU/mL was observed in six patients with HCC recurrence, which was different from the five above-mentioned cases. In one case whose lung metastasis was surgically removed, DCP promptly normalized. In the other seven cases, the DCP values

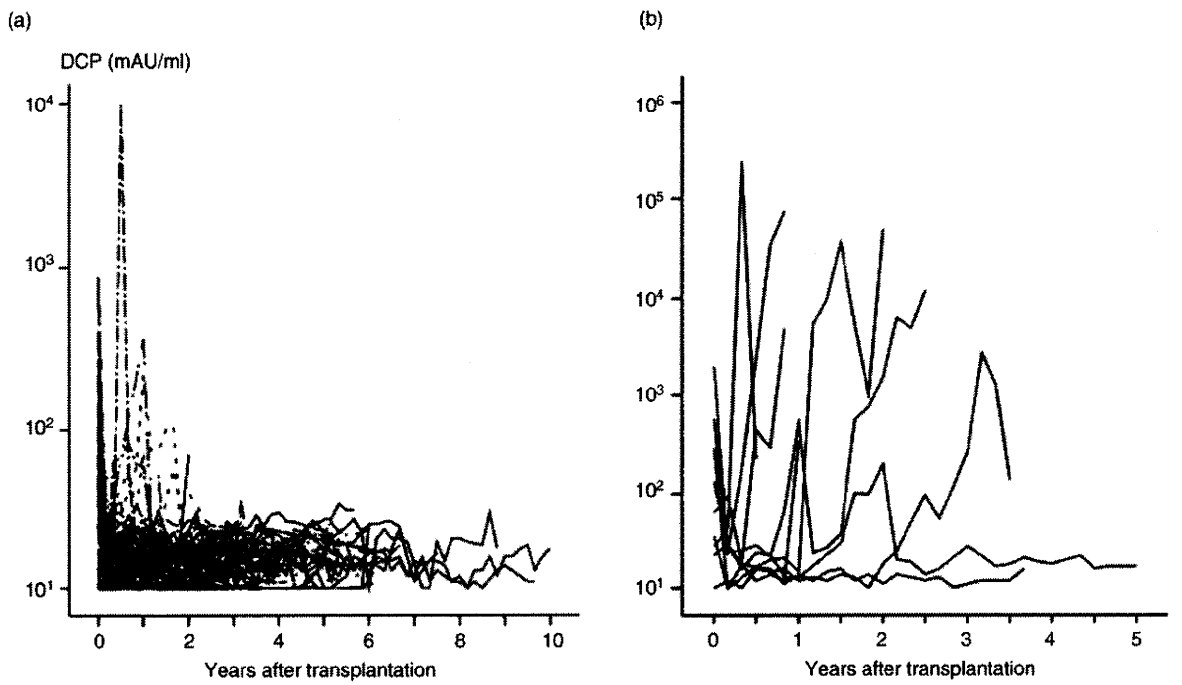

Figure 2 Sequential changes in serum des-γ carboxy prothrombin (DCP) levels are shown. (a) Ninety-one recipients without hepatocellular carcinoma (HCC) recurrence. DCP levels decreased to lower than 40 mAU/mL in most of the cases. A transient increase in DCP levels without evidence of recurrence was observed postoperatively in five cases. (b) Nine cases with HCC recurrence. An increase in the DCP values to over 40 mAU/mL was observed in six of the nine patients.

Table 4 Elevation of DCP value and outcome

Age/sex	Time from LT	Imaging study	Diagnosis	Duration of DCP >40 mAU/mL	Max. DCP value
67/M	1 year	US	Biliary stricture	3 months	56
56/M	10 months	US, CT, BS	Biliary stricture	3 months	361
63/M	4 months	None	Warfarin	3 months	9884
56/M	4 months	US, CT, BS	Unknown	2 years	75
60/M	8 months	CT	Biliary stricture	10 months	108

BS, bone scintigraphy; CT, computed tomography; DCP, des-γ carboxy prothrombin; LT, liver transplantation; US, ultrasonography.

never returned to normal. Using a cut-off level of 40 mAU/mL, sensitivity was 6/11 (54.5%) and specificity 86/89 (96.6%).

DISCUSSION

WE FOLLOWED 100 LDLT recipients with HCC for a median of 3.6 years, and diagnosed nine recurrent cases. Patients were monitored with frequent tumor marker measurements and annual dynamic CT scan of the liver, but chest CT scan was not routinely performed. Annual to biannual abdominal CT scans and monitoring of AFP levels seem to be standard at many centers,[5–8,13–15] but there is no established surveillance protocol for recurrent HCC following liver transplantation.

Regalia *et al.*[13] reported that 132 orthotopic liver transplantation recipients underwent post-transplant surveillance using serum AFP levels every 3 months and imaging studies (ultrasonography, CT, chest XP, bone scan) twice annually. In 21 patients, HCC recurred, 71% within 18 months. Survival after HCC recurrence was better among patients treated surgically than among those without surgery (2-year survival 57% vs 14%). The study from the Mount Sinai group of 57 HCC recurrent cases[14] indicated that median survival from recurrence was 8.9 months. Their HCC recurrence surveillance included CT chest and liver, and serum AFP levels were measured frequently. Recurrence was detected in 80% within 2 years, and 47/57 had multiple recurrences. Todo *et al.*[2] reported the results of LDLT for HCC patients in Japan. In his report, 40 of 316 patients had recurrent HCC. The recurrence sites in these three reports were similar: 15% to 19% in the graft; 42% to 53% in other organs, including lungs and bones; and 31% to 43% in multiple organs. In these reports, however, the mode of diagnosis and detection of the tumor as a result of periodical surveillance were not reported.

The Mount Sinai group[20] suggests the use of a different surveillance schedule for HCC recurrence in those at low or high risk. High-risk patients are those with HCC in which tumors exceed the Milan criteria, are poorly-differentiated or exhibit vascular invasion.[20] Others also report that it is more cost-effective when surveillance is performed only for patients with risk factors for HCC recurrence.[21,22] The risk factors determined by Chen include a tumor larger than 4.5 cm, tumors in both lobes of the liver, and macroinvasion. If patients have no risk, the odds for HCC recurrence are low, and the patient does not require postoperative surveillance for recurrence.[22] These cost-effectiveness analyses, however, were performed in a setting in which frequent imaging studies were considered mandatory. Because nearly half of the recurrences occur in multiple organs after liver transplantation, surveillance with imaging studies may not be sufficient.

In the present study, although the number of patients was small, the tumor marker levels were the first sign of recurrent HCC in all cases. When we focused on the two long-term survivors, recurrent HCC was found more than 2 years post-transplantation, and was detected as a solitary lung metastasis by chest CT scan. On the other hand, seven other patients with recurrent HCC died. To confirm the sites of recurrence in those cases, repeated imaging studies were required. As previously reported,[2,13,14] many cases experienced recurrence in multiple organs, and the tumors were often not amenable to resection. As a result, three cases received only palliative or symptom-relieving therapy. However, it would also be notable that two cases whose recurrence lesions were treated surgically survived more than 1 year. These results suggested that the surgical treatment for recurrent HCC, regardless of additional chemotherapy, may yield longer survival for patients with HCC recurrence.

α-Fetoprotein levels are reported to be elevated in cases with active hepatitis and thus the cut-off level to

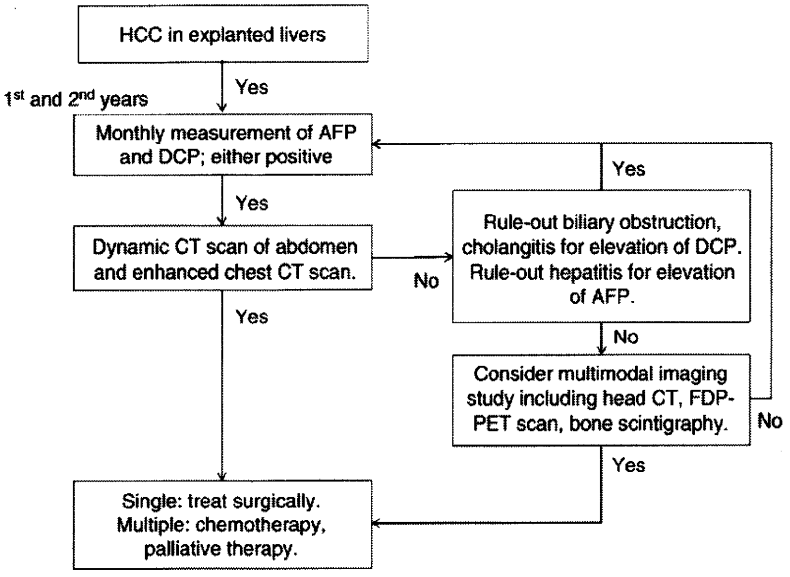

Figure 3 Flowchart outlining our surveillance system for the diagnosis of recurrent hepatocellular carcinoma (HCC) after liver transplantation. AFP, α-fetoprotein; CT, computed tomography; DCP, des-γ carboxy prothrombin; FDP-PET, ^{18}F-fluorodeoxyglucose positron emission tomography.

detect HCC among patients with chronic hepatitis or cirrhosis has been discussed.[23,24] To increase the specificity, the use of different cut-off levels for AFP (20, 100 or 200 ng/mL) are used. Measurement of the AFP-L3 fraction is useful for distinguishing the cause of the AFP elevation.[25] In the present study, AFP values among non-recurrent HCC recipients remained equal to or lower than 10 ng/mL. Further, we did not observe an AFP increase over 20 ng/mL among patients without HCC recurrence. Our findings indicated that an AFP increase to over 10 ng/mL may be an appropriate indicator for further evaluation for HCC recurrence. It should be noted, however, that recurrent hepatitis was not observed among our studied population and, accordingly, an increase in AFP levels due to chronic hepatitis was not observed.

In contrast, increased serum DCP levels were observed in five patients without recurrence. DCP levels are influenced by vitamin K metabolism, and thus by biliary obstruction.[26] Such a condition must be ruled out by taking a careful history or by performing liver imaging studies. Although the specificity of DCP in this study was lower than that of AFP, the combination of both markers may increase the sensitivity. When the cut-off values were 10 ng/mL for AFP and 40 mAU/mL for DCP, the sensitivity of the combination increased to 9/9 (100%) and specificity was 84/91 (91%).

Serum tumor marker measurement is easy to perform, and can be repeated for a long time. Based on the present study, we propose a surveillance protocol for recurrent HCC based on tumor marker measurement (Fig. 3). Although we reported the usefulness of monthly measurements of AFP and DCP, the optimal frequency is not clear. Because most of the recurrent cases were diagnosed within 2 years in our study, monthly measurement would be useful for the first 1–2 years after transplantation. For those surviving longer than 2 years, two to four times per year may be adequate for long-term follow up. Once recurrent HCC is suspected based on tumor marker levels or symptoms, then confirmatory imaging studies can be performed. If the recurrence comprises a solitary nodule, surgical resection should be planned. Because very late recurrence is also reported,[27] long-term measurement of serum tumor markers is justified in all HCC cases after liver transplantation.

In summary, the present findings indicate that tumor marker measurements were useful for early detection of recurrent HCC among 100 LDLT recipients with HCC. Tumor marker increases were followed by thorough imaging studies, and led to surgical treatment in six cases. Approximately 30–40% of recurrent HCC was detected in multiple sites, however, and thereby indication for surgery was limited. Alternative approaches must be considered for those with multiple site recurrence. Tumor marker increases may not lead to curative treatment in those cases, but may indicate a requirement for adjuvant chemotherapy or immunosuppressive

therapy modifications to inhibit tumor growth. Further studies are necessary to determine the usefulness of monitoring AFP and DCP levels for HCC recurrence.

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ORIGINAL ARTICLE – HEPATOBILIARY TUMORS

Significance of Alpha-Fetoprotein and Des-γ-Carboxy Prothrombin in Patients with Hepatocellular Carcinoma Undergoing Hepatectomy

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ABSTRACT

Background. Alpha-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) are well-known tumor markers of hepatocellular carcinoma (HCC). The aims of this study are to calculate the sensitivity/specificity of AFP and DCP measurement for the diagnosis of HCC, measure response rates of the markers following curative-intent resections, determine the correlations between the marker levels and clinicopathological prognostic variables, and determine the correlations between the marker levels before hepatectomy and those at diagnosis of recurrence.

Methods. A retrospective cohort study of 714 consecutive patients with HCC undergoing hepatectomy was carried out.

Results. The areas under the receiver operating characteristic curves were 0.79 versus 0.91 for AFP and DCP, respectively ($P < 0.001$). Positive AFP and DCP status became negative at 6 months post surgery in 184/229 (80.3%) and 245/246 (99.6%) patients, respectively (cutoff values being 20 ng/ml for AFP and 40 mAU/ml for DCP; $P < 0.0001$). No correlation was found between marker levels ($rs = 0.23$). The level of DCP, but not that of AFP, showed a close correlation with tumor size ($rs = 0.51$ and 0.19, respectively). They were associated with indices of tumor invasiveness without showing any specific associations. AFP and DCP levels in patients showing recurrence in ≤6 months correlated with the levels measured before surgery ($rs = 0.78$ and 0.49, respectively) but not in those showing recurrence after 2 years ($rs = 0.31$ and 0.30, respectively).

Conclusions. DCP is a more accurate, albeit complementary, HCC marker than AFP. While the levels of both markers increased with advancing tumor growth, no specific associations were found. The marker values at recurrence indicated the type of recurrence.

Early diagnosis remains the key to effective therapy in cases of hepatocellular carcinoma (HCC).[1] Although serum alpha-fetoprotein (AFP), a biological tumor marker of HCC, has long been used as a tool for HCC surveillance, it is not an ideal screening test due to its low sensitivity/specificity.[2–5] Liebman et al. first reported, in 1984, an increase in the plasma levels of des-γ-carboxy prothrombin (DCP), which is an abnormal prothrombin and also otherwise known as protein induced by vitamin K deficiency or antagonist-II (PIVKA-II), in patients with HCC.[6] Since then, the significance of DCP has been examined by many investigators and it was introduced as a routine laboratory test for HCC during the early 1990s in Japan.[7–9] In addition, a two-step enzyme immunoassay method was developed and has been in use since 1997; it shows a tenfold higher sensitivity for detection as compared with the conventional enzyme immunoassay method.[10] Consensus appears to have been reached on both DCP and AFP being independent tumor markers in HCC.[8,11–17] However, it still remains controversial whether or not DCP is superior to AFP as a single marker.[12,16–22]

The second role of tumor markers is in the monitoring of response to therapy. Ideally, the levels of tumor markers should fall to within normal range after effective treatment. This aspect is especially important in the case of

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transcatheter arterial embolization, because radiological findings do not necessarily reflect the degree of biological remission achieved by necrosis or fibrosis.[23] Comparisons of AFP and DCP in this regard have not been conducted.

Thirdly, elevation of tumor marker levels reportedly represents specific clinicopathological variables identified as prognostic factors.[14,21,22,24–26] Although high plasma levels of DCP reportedly indicate the presence of portal venous thrombosis and increased serum AFP levels are associated with a poor degree of differentiation of the tumor cells, in particular, these studies failed to comprehensively investigate the relationships with various parameters.[14,21,22,24,27]

Finally, another use of tumor markers is in the prediction of tumor recurrence. In theory, patients with HCC with elevated levels of AFP and/or DCP before treatment should also show elevated levels of the respective markers at the time of recurrence if the recurrence is metastatic. On the other hand, de novo secondary tumors also contribute to postoperative intrahepatic HCC recurrence.

In the present study, taking into account these unaddressed aspects of tumor markers of HCC, we comprehensively investigated the clinical significance of measurement of two tumor markers in cases of HCC, i.e., AFP and DCP, in a large cohort.

PATIENTS AND METHODS

Patients

The base population consisted of 714 consecutive patients who underwent curative liver resections for HCC at the Division of Hepato-Biliary-Pancreatic Surgery, Tokyo University Hospital, between January 1998 and November 2006. Curative resection was defined as removal of all recognizable tumors with a clear margin. The diagnosis of HCC was finally confirmed by pathological examination of the resected specimens in all cases.

Background characteristics of the patients are presented in Table 1. After discharge, monthly follow-up by tumor markers (AFP and DCP) and ultrasound as well as dynamic computed tomography (CT) scan every 4 months were conducted for 1 year. Then, we screened patients by tumor marker measurement and ultrasound every 2 months and dynamic CT scan every 6 months thereafter. We defined recurrence as the appearance of new lesions with radiological features typical of HCC, as confirmed by at least two imaging methods.[28]

AFP and DCP Assay

Samples for AFP and DCP were taken within 7 days prior to the liver resection. Serum AFP level was measured by commercially available immunometric assay (ST AIA-PACK AFP, Tosoh, Tokyo, Japan). Plasma DCP level was measured by two-step enzyme immunoassay (Picolumi PIVKA-II, Eizai, Tokyo, Japan).[10]

TABLE 1 Background characteristics of 714 patients with HCC

Variables	$n = 714$
Sex	
Male	556 (77.9%)
Female	158 (22.1%)
Age (years)[a]	67 (19–90)
Hepatitis B virus infection[b]	
No	560 (78.4%)
Yes	154 (21.6%)
Hepatitis C virus infection[b]	
No	250 (35.0%)
Yes	464 (65.0%)
Child–Turcotte–Pugh grade[c]	
A	601 (84.2%)
B	113 (15.8%)
Background liver status[d]	
Normal liver	14 (2.0%)
Chronic hepatitis	295 (41.3%)
Cirrhosis	405 (56.7%)

[a] Median with range

[b] Five patients were positive for both hepatitis B and C virus infections and 101 patients were negative for both hepatitis B and C virus infections

[c] No patient was Child–Turcotte–Pugh grade C

[d] Pathological findings assessed in the resected specimen

Assessment

Sensitivity/Specificity of AFP and DCP for Presence of HCC At 6 months post surgery, 25 out of the 714 patients were lost to follow-up in terms of serial tumor marker measurements, 190 had developed recurrence, 9 were disease-free at <6 months of follow-up, and the remaining 490 patients were confirmed to be disease free at this time point. The AFP and DCP values in 714 patients before the liver resection were defined as those of patients with HCC, while the values of these 490 patients at 6 months post surgery were defined as those of patients without HCC. Using these values, receiver operating characteristic (ROC) curves were constructed. The diagnostic performance of AFP and DCP was evaluated and compared through their areas under the receiver operating characteristic curves (AUROC). The cutoff values for AFP and DCP used in this study are those that have been conventionally used and/or have been proposed in previous reports: 20 ng/ml for AFP and 40 mAU/ml for DCP.[29]

AFP and DCP Levels as Tools for Evaluating Therapeutic Response to HCC In these 490 patients, complete tumor remission was thought to be achieved at 6 months after the liver resection. We examined whether this treatment response was correctly reflected in the alterations in the marker values. According to the cutoff values defined above, we classified the 490 patients into marker-positive or marker-negative status both before and at 6 months after the liver resection. We then investigated the changes of AFP- and DCP-positive/negative status following the liver resection.

AFP and DCP as Complementary Tumor Markers for HCC We first evaluated the relationship between AFP and DCP values in a total of 714 patients. Second, we classified these patients into four categories according to their positive/negative status for AFP and/or DCP according to the cutoff values.

AFP and DCP as Markers of Clinicopathological Variables Representative of Tumor Invasiveness and Prognosis We assessed the association of AFP and DCP values with clinicopathological variables that have been reported as prognostic factors for HCC in the 714 patients. The variables investigated are shown in Table 2. All variables were assessed pathologically on the resected specimens. Vascular invasion was defined as presence of portal vein invasion, venous invasion or biliary invasion. Multiple primary tumor nodules and intrahepatic metastases were differentiated using the guidelines proposed by the Liver Cancer Study Group of Japan.[30]

AFP and DCP Levels as Indices for Predicting the Pattern of Recurrence At the time of data collection, recurrence was observed in 444 patients. We classified these patients with recurrence into two groups, i.e., a group in which the recurrence occurred ≤6 months post surgery ($n = 190$),

TABLE 2 Tumor-related factors

Variables	$n = 714$	AFP (ng/ml)[a]	DCP (mAU/ml)[a]
Tumor size (mm)			
≤20	223 (31.2%)	18.0 (7.0–69.0)	24.0 (16.0–61.0)
20–50	335 (46.9%)	22.0 (7.0–144.0)	57.0 (21.0–328.0)
>50	156 (21.9%)	57.0 (8.5–3007)	1251.0 (118.5–7486.0)
		$rs = 0.19$	$rs = 0.51$
Tumor number			
1	483 (67.7%)	19.0 (1.0–216.0)	55.0 (20.0–456.0)
2	138 (19.3%)	26.0 (8.0–177.5)	53.0 (19.50–254.0)
≥3	93 (13.0%)	49.0 (13.5–162.5)	59.0 (19.5–329.5)
		$P = 0.07$	$P = 0.73$
Capsular formation			
No	169 (23.7%)	25.0 (8.0–148.0)	32.0 (18.0–163.0)
Yes	545 (76.3%)	21.0 (7.0–207.5)	72.0 (21.0–489.5)
		$P = 0.83$	$P < 0.05$
Capsular infiltration[b]			
No	137 (25.1%)	14.0 (6.0–78.5)	64.0 (10.0–364.0)
Yes	408 (74.9%)	27.0 (7.0–278.0)	83.5 (21.5–579.5)
		$P < 0.01$	$P = 0.21$
Vascular invasion[c]			
No	495 (69.3%)	17.0 (7.0–76.0)	38.0 (18.0–189.0)
Yes	219 (30.7%)	88.0 (12.0–1271.0)	233.0 (31.0–2110.0)
		$P < 0.0001$	$P < 0.0001$
Intrahepatic metastases			
No	601 (84.2%)	19.0 (7.0–137.0)	44.0 (10.0–310.5)
Yes	113 (15.8%)	81.0 (9.5–1261.0)	235.0 (40.0–2544.0)
		$P < 0.001$	$P < 0.0001$
Tumor differentiation			
Well	104 (14.5%)	12.5 (6.0–31.0)	29.0 (17.0–87.5)
Moderate	511 (71.6%)	20.0 (1.0–174.0)	63.0 (10.0–441.0)
Poorly	99 (13.9%)	165.0 (25.0–2326.0)	145.0 (26.0–2455.0)
		$P < 0.0001$	$P < 0.0001$

[a] Median with interquartile range

[b] We assessed 545/714 patients who had capsular formation

[c] Macroscopic invasion was observed in 45/219 (20.5%) patients, while microscopic invasion was found in 174/219 (79.5%) patients

and another in which the recurrence occurred >6 months post surgery ($n = 254$). We first compared the preoperative levels of AFP and DCP as well as the levels at time of recurrence between the two groups of patients. Then, we further classified the two groups of patients into two subgroups according to site of recurrence, i.e., intrahepatic or extrahepatic recurrence. We investigated the correlations between the preoperative marker values and the site of recurrence.

Etiological Association Between the Primary and Recurrent Tumors Investigated Through AFP and DCP Marker Values We investigated the correlations of the tumor marker values at the time of recurrence with those measured before the liver resection. We classified 444 patients who developed recurrences into four groups according to time to recurrence, as follows: recurrence at ≤6 months ($n = 190$), recurrence between 7 and 12 months ($n = 70$), recurrence between 13 and 24 months ($n = 70$), and recurrence after 2 years ($n = 114$). Then, we examined the chronological alterations in the correlation of values of the respective tumor markers measured before the liver resection with those measured at the time of recurrence.

Statistical Analysis

Marker values are expressed as median with interquartile range. The AUROC for markers was compared by Wilcoxon's rank-sum test.[31] Correlations between marker values were analyzed by Spearman's rank correlation. Categorical binary variables were compared by Fisher's exact test. Associations between marker values and clinicopathological variables were analyzed by Wilcoxon's rank-sum test or by the Kruskal–Wallis test, as appropriate. P values of < 0.05 were accepted as statistically significant. All statistical analyses were performed using the GraphPad Prism® computer software, version 5 (GraphPad Software Inc., San Diego, CA).

RESULTS

Sensitivity/Specificity of AFP and DCP for Presence of HCC

The median (interquartile range) AFP and DCP levels in 714 patients before liver resection were as follows: 22.0 (7.0–195.0) ng/ml and 55.0 (20.0–443.0) mAU/ml. The AFP and DCP levels in 490 patients who had no evidence of tumor recurrence at 6 months post surgery were 5.0 (3.0–9.0) ng/ml and 11.0 (10.0–15.0) mAU/ml, respectively. The sensitivity and specificity of AFP and DCP were assessed by ROC curves (Fig. 1). The AUROC (95% confidence interval, CI) for AFP and DCP were 0.79 (0.76–0.81) and 0.91 (0.89–0.92), respectively ($P < 0.001$). The sensitivities and specificities at various cutoff values including those adopted in the present study (AFP, 20 ng/ml; DCP, 40 mAU/ml) and proposed in previous reports are presented in Table 3.

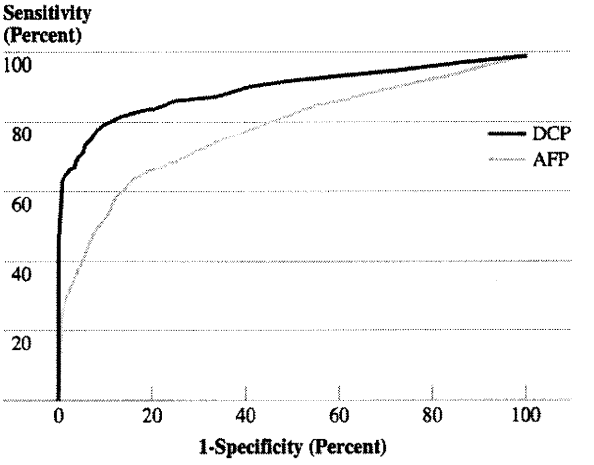

FIG. 1 ROC curves for AFP and DCP. The yellow line represents AFP and the blue line represents DCP. The AUROC (95% CI) for AFP and DCP were 0.79 (0.76–0.81) and 0.91 (0.89–0.92), respectively ($P < 0.001$)

TABLE 3 Sensitivities and specificities of AFP and DCP values according to various cutoff values

AFP (ng/ml)	11	13	20	100	200
Sensitivity (%)	64.9	60.8	51.3	30.4	24.7
Specificity (%)	82.9	86.1	90.8	98.6	99
DCP (mAU/ml)	20	30	40	100	125
Sensitivity (%)	73.4	62.8	55.9	41.9	39.1
Specificity (%)	94.7	99.4	99.8	100	100

In the present study, the cutoff values adopted were 20 ng/ml for AFP and 40 mAU/ml for DCP

AFP alpha-fetoprotein, *DCP* des-γ-carboxy prothrombin

AFP and DCP as Tools for Evaluating Response to Therapy of HCC

Among the 490 patients who were confirmed to be disease free at 6 months postoperatively, 229 (46.7%) and 246 (50.2%) were classified as AFP positive and DCP positive, respectively, before the liver resection under the present cutoff values. At 6 months post surgery, when complete tumor remission was thought to have been achieved, marker-negative status was achieved in 184/229 (80.3%) and 245/246 (99.6%) patients for AFP and DCP, respectively ($P < 0.0001$) (Table 4). Out of 45 patients

TABLE 4 Pre- and postoperative marker status in 490 disease-free patients at 6 months

Preoperative status		Postoperative status	
AFP			
(+)	229/490 (46.7%)	(−)	184/229 (80.3%)
		(+)	45/229 (19.7%)
(−)	261/490 (53.3%)	(−)	261/261 (100%)
		(+)	0/261 (0%)
DCP			
(+)	246/490 (50.2%)	(−)	245/246 (99.6%)
		(+)	1/246 (0.4%)
(−)	244/490 (49.8%)	(−)	244/244 (100%)
		(+)	0/244 (0%)

Cutoff values were set at 20 ng/ml for AFP and 40 mAU/mL for DCP, respectively

AFP alpha-fetoprotein, *DCP* des-γ-carboxy prothrombin

who showed AFP-positive status without recurrence at 6 months post surgery, 33 remained disease free at 12 months post surgery, whereas 12 had developed recurrence by this time point. In retrospect, the AFP values at 6 months post surgery were not thought to be indicative of recurrence at least in 6/12 patients. A single patient positive for DCP at 6 months post surgery was also disease free 5 years later. In all the 261 (53.3%) and 244 (49.8%) patients who were negative for AFP and DCP, respectively, before the surgery, the marker status for both of these markers remained negative at 6 months post surgery (Table 4).

AFP and DCP as Complementary Tumor Markers for HCC

The correlation between the levels of these markers in the 714 patients is shown in Fig. 2; no association was seen ($rs = 0.23$). These patients were classified into four categories by the cutoff values used in the present study, as follows: AFP(+)/DCP(+): 229 (32.1%), AFP(+)/DCP(−): 137 (19.2%), AFP(−)/DCP(+): 170 (23.8%), and AFP(−)/DCP(−): 178 (24.9%) (Fig. 2).

AFP and DCP as Markers of Clinicopathological Variables Representative of Tumor Invasiveness and Prognosis

The correlations of the AFP and DCP levels with clinicopathological findings are shown in Table 2. Although the DCP levels increased with increasing tumor size ($rs = 0.51$), this relationship was not found for AFP ($rs = 0.19$). While no statistical correlation was found between DCP levels and tumor number ($P = 0.73$), AFP levels tended to increase with increasing tumor number

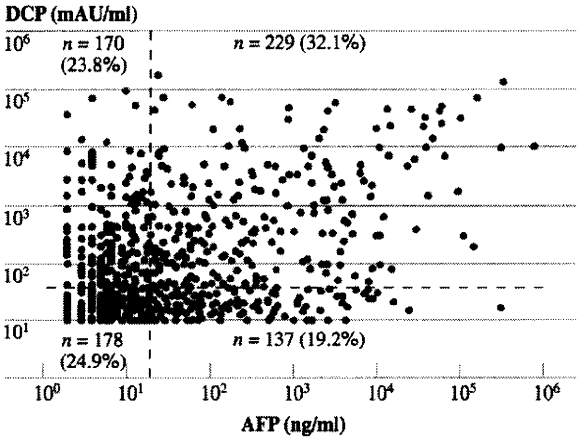

FIG. 2 Correlation between AFP and DCP values in 714 patients. No correlation was found between the two markers ($rs = 0.23$, $P < 0.0001$). Dotted line represents cutoff values, i.e., 20 ng/ml for AFP and 40 mAU/ml for DCP. Patients were placed into four categories: either positive or negative for AFP and/or DCP according to these cut-off values. Number of patients in the each category was shown

($P = 0.07$). AFP and DCP levels increased to similar extent in the presence of indices of tumor invasiveness, such as vascular invasion and intrahepatic metastases. Likewise, both marker levels increased with increasing tumor cell differentiation.

AFP and DCP Levels as Indices for Predicting the Pattern of Recurrence

The preoperative AFP and DCP values in HCC patients who developed recurrence ≤6 months ($n = 190$) versus patients who developed recurrence >6 months post surgery ($n = 254$) are shown in Table 5. Patients who developed recurrence ≤6 months post surgery showed higher preoperative AFP and DCP values than those who developed recurrence >6 months post surgery. Similarly, the AFP and DCP values measured at the time of recurrence in the two groups are shown separately in Table 5. Again, patients who developed HCC recurrence ≤6 months post surgery showed higher AFP and DCP values at the time of recurrence.

Out of 190 recurrences observed ≤6 months post surgery, 32 (16.8%) were extrahepatic: 18/32 (59 %) in the lung, 6/32 (19%) in the lymph node, 4/32 (13%) in the bone, 2/32 (6%) in the peritoneal membrane, and 1/32 (3%) in the adrenal gland.

On the other hand, the overall rate of extrahepatic recurrence in the patients who developed recurrence >6 months post surgery was 3/254 (1.2 %). Since extrahepatic recurrence was a rare event >6 months post surgery, we analyzed the correlations between the

TABLE 5 AFP and DCP values in patients who developed HCC recurrence ≤6 months ($n = 190$) and >6 months ($n = 254$) post surgery

	Preoperative values		Values at recurrence	
	Recurrence ≤6 months	Recurrence >6 months	Recurrence ≤6 months	Recurrence >6 months
AFP (ng/ml)	54.0 (9.0–624.5)[a]	18.5 (7.0–76.0)	17.5 (6.0–163.5)[a]	13.0 (6.0–43.0)
DCP (mAU/ml)	237.5 (22.8–2553.0)[b]	37.5 (19.0–142.0)	25.0 (14.0–131.0)[c]	18.0 (13.0–34.3)

Values are expressed as median (interquartile range)

[a] $P < 0.0001$ compared with recurrence >6 months

[b] $P < 0.005$ compared with recurrence >6 months

[c] $P < 0.0005$ compared with recurrence >6 months

TABLE 6 Preoperative AFP and DCP values in patients who developed intrahepatic ($n = 158$) and extrahepatic ($n = 32$) recurrence ≤6 months post surgery

	Intrahepatic recurrence	Extrahepatic recurrence
AFP (ng/ml)	50.0 (9.0–337.8)[a]	255.0 (10.8–9636.0)
DCP (mAU/ml)	188 (22.8–184.0)[b]	543.0 (34.3–10179.0)

Values are expressed as median (interquartile range)

One patient who developed intra- and extrahepatic recurrences simultaneously was classified into those with extrahepatic recurrence

[a] $P < 0.05$ compared with extrahepatic recurrence

[b] $P = 0.08$ compared with extrahepatic recurrence

preoperative marker values and the site of recurrences exclusively in the 190 patients who developed recurrence ≤6 months post surgery (Table 6). Patients who developed intrahepatic recurrence ($n = 158$) showed higher preoperative marker values than those who developed extrahepatic recurrence ($n = 32$).

AFP and DCP as Markers Reflecting the Association Between the Primary and Recurrent Tumors

The values of AFP and DCP measured before the liver resection are plotted against the values measured at the time of recurrence separately according to their time to recurrences in Fig. 3A–D and Fig. 4A–D, respectively. The AFP values in patients with recurrence at ≤6 months showed a close relationship with those measured before the liver resection ($rs = 0.78$, Fig. 3A). The strength of this relation became weaker in the groups with longer time to recurrence (Fig. 3B–D).

A similar trend was found in regard to the relationship of DCP values, although the correlations were weaker than those observed for AFP (Fig. 4A–D).

DISCUSSION

The diagnostic accuracy of tumor markers should be evaluated on the basis of a trade-off between sensitivity and specificity, ideally by drawing ROC curves.[31] To date, three cross-sectional studies have compared the accuracy of AFP and DCP levels for the diagnosis of HCC through ROC curves, each using the present sensitive assay method for measuring DCP.[17,19,20] Two studies reported superiority of DCP.[17,20] However, a third reported better overall diagnostic accuracy of AFP.[19] The distribution of the etiology of the underlying liver disease in the present study population was similar to that in the populations studied by Marrero et al. and Nakamura et al., except that the former included a quantifiable proportion of alcoholic patients.[19,20] In regard to the distribution of the Child–Turcotte–Pugh (CPT) grade, our cohort is thought to lie in between the study cohorts of Marrero et al. and Nakamura et al., since 84.2% of our patients were classified into CPT grade A.[19,20]

In this study, we defined patients without recurrence at 6 months post surgery as a cohort without HCC. Although this approach may be different from that of former studies, this is advantageous in that the background characteristics are uniform in the patients with and without HCC.[17,19,20] This situation, which is an essential requirement in prospective screening studies of tumor markers, is not necessarily guaranteed in a cross-sectional study.[32] This study showed similar ROC results to those reported by Marrero et al. and Wang et al., which demonstrated superiority of DCP by approximately 10% (0.73–0.83 versus 0.85–0.93 for AFP versus DCP) (Fig. 1).[17,20]

In the present study, we used the cutoff values for AFP (20 ng/ml) and DCP (40 mAU/ml) proposed by previous studies and used most commonly in clinical settings.[29] Considering that much higher AFP values, e.g., 100 ng/ml or 200 ng/ml, have often been proposed as cutoff points, it is noteworthy that the present cutoff value showed better performance than these cutoff values, and even lower cutoff values can be adopted in terms of ROC performance (Table 3, Fig. 1). The cutoff value for DCP in the present study (40 mAU/ml), showing similar sensitivity to that of AFP, was thought to be the lowest among the values proposed until now (40–125 mAU/ml). Again, analysis of the ROC curve revealed that this value can be reduced even further in terms of a trade-off between sensitivity and specificity.

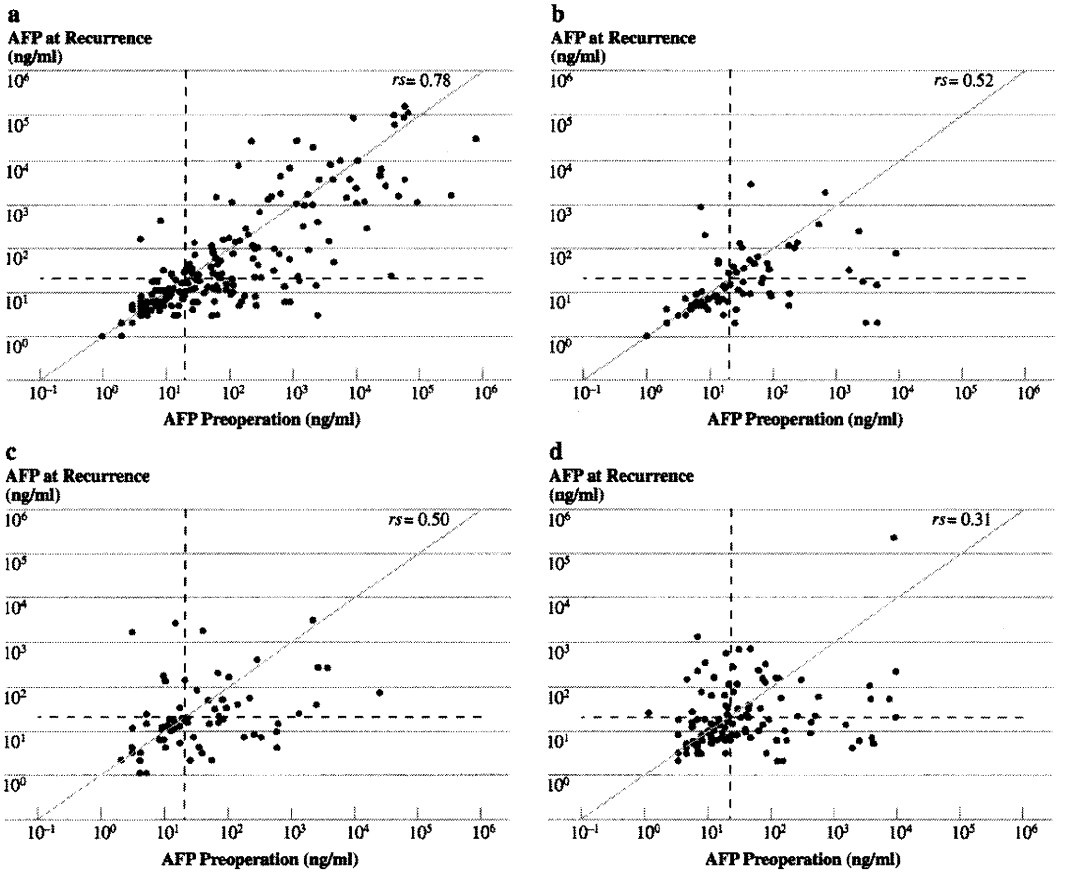

FIG. 3 Correlations between preoperative AFP values and AFP values at recurrence stratified according to period of recurrence: (**a**) recurrence ≤6 months (n = 190), (**b**) recurrence from 7 to 12 months (n = 70), (**c**) recurrence from 13 to 24 months (n = 70), and (**d**) recurrence >2 years (n = 114). The dotted lines represent 20 ng/ml

In addition, DCP is a superior marker for monitoring response to therapy, that is, it was confirmed that positive DCP status converted to negative status in 99.6% (245/246) of patients at 6 months post surgery in the absence of tumor recurrence; in contrast, conversion from AFP-positive to AFP-negative status was achieved in only 80.3% of the patients (184/229). This high false-positive rate of AFP is thought to reflect the observed elevation in the levels of this marker also in conditions such as acute and/or chronic hepatitis and cirrhosis, which is an inherent drawback of AFP as a HCC-specific tumor marker.[3] Whereas high DCP values have been reported in patients with vitamin K deficiency, such as in cases of obstructive jaundice or cases receiving vitamin K antagonists, e.g., warfarin, these uncommon clinical situations can be easily discriminated in HCC patients.[12,33] Rather, it must be noted that patients with chronic alcoholism, another high-risk cohort for HCC, often show nonspecific DCP elevation, reportedly in 5–8% of patients.[34,35] The higher DCP cutoff value adopted by Marrero et al. in their study (125 mAU/mL) may be partially ascribed to the fact that their cohort included a considerable proportion of alcoholic patients (5%).[20]

In the present study, no correlation was found between the levels of AFP and DCP. This observation is consistent with previous reports.[11–17,21] These results strongly suggest that these markers are complementary to each other and that, although DCP might be superior to AFP as a single marker, the two should be evaluated in combination in clinical practice.

Although the association of tumor markers with various clinicopathological variables has been evaluated in many studies, the majority of these works assessed the associations solely with variables of interest and/or exclusively for AFP or DCP. Bearing this in mind, we investigated these associations in a comprehensive manner. While serum DCP values increased with increasing tumor size, no similar association was found for AFP (Table 2). This result is consistent with the results of previous

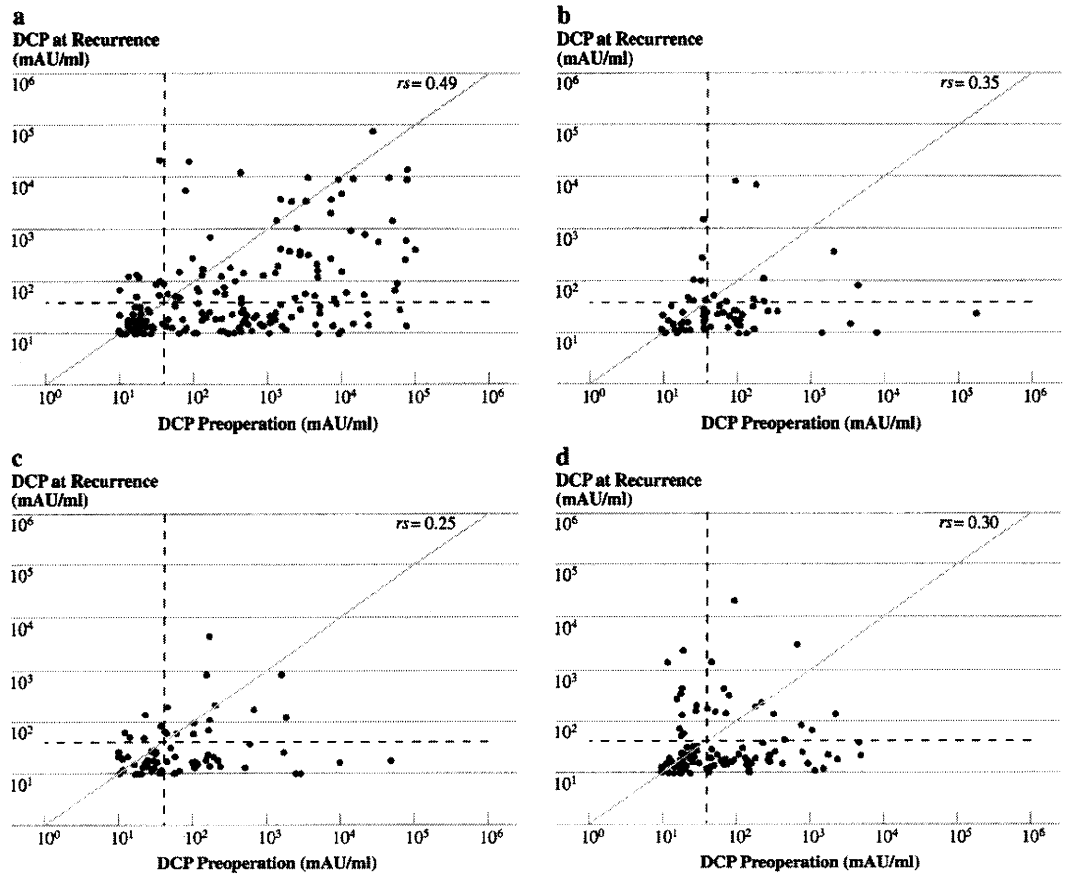

FIG. 4 Correlations between preoperative DCP values and DCP values at recurrence stratified according to period of recurrence: (**a**) recurrence ≤6 months ($n = 190$), (**b**) recurrence from 7 to 12 months ($n = 70$), (**c**) recurrence from 13 to 24 months ($n = 70$), and (**d**) recurrence >2 years ($n = 114$). The dotted lines represent 40 mAU/ml

studies.[8,13,14,16,17,26,36] These findings suggest that the interindividual variations in the capacity of the tumor cells to synthesize AFP far exceed the elevation in the marker values with increasing tumor cell number.

While serum AFP levels tended to increase with increasing tumor number, this association was not observed for plasma DCP (Table 2). This finding is consistent with those of Kasahara et al. and Carr et al., who found a significant relationship between AFP and tumor number.[13,22] Considering that tumor number is thought to be a variable representing the degree of carcinogenicity in the background liver, the finding of the association for AFP but not for DCP is most probably explained by the elevation of AFP with advancing severity of background liver disease.[3,36,37]

In the present cohort ($n = 714$), both increased AFP and DCP values were related to presence of indices of tumor invasiveness, such as vascular invasion, and intrahepatic metastases. To date, several studies with 72–161 patients have investigated the association of AFP and/or DCP with these indices, three of which assessed these pathological variables on surgically resected specimens.[14,21,24] A closer and/or specific relationship between these indices and DCP has been reported. Thus, the results of the present and former studies were partially contradictory. In our study, the AFP and DCP values were associated to a similar extent with the tumor cell differentiation grade (Table 2). Again, this observation is partially contradictory to the results of previous studies with 56–354 patients that claimed a specific close association with AFP or DCP.[24,26,27] The results of the present large cohort strongly suggests that both increased levels of AFP and DCP indicate the overall presence of pathological indices representing tumor invasiveness and/or increased malignant potential; however, they do not necessarily signify the presence of any specific entity.

Elevated preoperative AFP and/or DCP levels were correlated with early postoperative recurrence (≤6 months), and recurrence in the early phase was characterized by high serum levels of tumor markers. These results can most reasonably be interpreted as follows: high tumor marker levels signify an increased malignant

potential of the tumor, and the majority of recurrences in the early phase represent recurrence by metastasis, while the later phase of recurrences most often represent secondary de novo tumors whose malignant potential has not yet increased during the process of multistep carcinogenesis. This contention is further supported by the observed association of elevated tumor marker levels with a higher frequency of extrahepatic recurrence.

Two different underlying mechanisms are thought to contribute to postoperative HCC recurrence. In theory, recurrence by metastasis takes place in the early period after surgery, whereas recurrence in the late phase largely represents a new primary lesion.[37,38] Likewise, it can be hypothesized that (1) metastatic recurrence exhibits similar tumor characteristics to the primary lesion, while de novo lesions are independent of the primary tumors in terms of the marker expression profile, and (2) tumor marker levels in recurrent tumors in the early phase show a close relationship with those before hepatectomy, while this relationship becomes obscure in recurrent tumors in the late phase. Chronological alterations in the correlation coefficients (Figs. 3 and 4) support this hypothesis. Moreover, this correlation was stronger for AFP than for DCP across all the study groups. This observation suggests that the increased AFP values both before hepatectomy and at the time of recurrence are at least partially accounted for by the background liver diseases.

A limitation of this investigation is that all of the study patients underwent curative liver resections. They would therefore be supposed to exhibit relatively well-preserved liver function, despite the presence of cirrhosis, from the viewpoint of screening. Likewise, they would be expected to have relatively early stage of HCC as compared with patients undergoing transcatheter arterial embolization, from the standpoint of prediction of response to therapies.

In conclusion, although DCP might be more accurate than AFP for the differentiation of HCC from nonmalignant chronic liver disease, the two markers are complementary to each other. The levels of both markers increased with tumor growth, but no specific association of either with any specific pathological entities was noted. The observed relationship between the preoperative marker values and the values measured at the time of recurrence may serve as a basis for predicting the pattern of recurrence of HCC, i.e., recurrence by metastasis or de novo secondary lesions.

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Genome-wide DNA methylation profiles in liver tissue at the precancerous stage and in hepatocellular carcinoma

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To clarify genome-wide DNA methylation profiles during hepatocarcinogenesis, bacterial artificial chromosome (BAC) array-based methylated CpG island amplification was performed on 126 tissue samples. The average numbers of BAC clones showing DNA hypo- or hypermethylation increased from noncancerous liver tissue obtained from patients with hepatocellular carcinomas (HCCs) (N) to HCCs. N appeared to be at the precancerous stage, showing DNA methylation alterations that were correlated with the future development of HCC. Using Wilcoxon test, 25 BAC clones, whose DNA methylation status was inherited by HCCs from N and were able to discriminate 15 N samples from 10 samples of normal liver tissue obtained from patients without HCCs (C) with 100% sensitivity and specificity, were identified. The criteria using the 25 BAC clones were able to discriminate 24 additional N samples from 26 C samples in the validation set with 95.8% sensitivity and 96.2% specificity. Using Wilcoxon test, 41 BAC clones, whose DNA methylation status was able to discriminate patients who survived more than 4 years after hepatectomy from patients who suffered recurrence within 6 months and died within a year after hepatectomy, were identified. The DNA methylation status of the 41 BAC clones was correlated with the cancer-free and overall survival rates of patients with HCC. Multivariate analysis revealed that satisfying the criteria using the 41 BAC clones was an independent predictor of overall outcome. Genome-wide alterations of DNA methylation may participate in hepatocarcinogenesis from the precancerous stage, and DNA methylation profiling may provide optimal indicators for carcinogenetic risk estimation and prognostication.

Key words: bacterial artificial chromosome array-based methylated CpG island amplification; hepatocellular carcinoma; multistage carcinogenesis; precancerous condition; prognostication

Alteration of DNA methylation is one of the most consistent epigenetic changes in human cancers.[1,2] It is known that DNA hypomethylation results in chromosomal instability as a result of changes in the chromatin structure, and that DNA hypermethylation of CpG islands silences tumor-related genes in cooperation with histone modification in human cancers.[3,4]

With respect to hepatocarcinogenesis, we have shown that alterations of DNA methylation at multiple chromosomal loci can be detected even in noncancerous liver tissue showing chronic hepatitis or cirrhosis, which are widely considered to be precancerous conditions, but not in normal liver tissue, using classical Southern blotting analysis.[5] This was one of the earliest reports of alterations of DNA methylation at the precancerous stage. Multiple tumor-related genes, such as the *E-cadherin*[6,7] and *hypermethylated-in-cancer (HIC)-1*[8] genes, are silenced by DNA hypermethylation in hepatocellular carcinomas (HCCs). DNA methyltransferase (DNMT) 1 expression is significantly higher even in noncancerous liver tissue showing chronic hepatitis or cirrhosis than in the normal liver tissue and is even higher in HCCs.[9,10] DNMT1 overexpression is also correlated with poorer tumor differentiation, portal vein involvement and intrahepatic metastasis of HCCs and poorer patient outcome.[11] On the other hand, overexpression of DNMT3b4, an inactive splice variant of DNMT3b, may lead to chromosomal instability through induction of DNA hypomethylation in pericentromeric satellite regions during hepatocarcinogenesis.[12]

Because aberrant DNA methylation is one of the earliest molecular events during hepatocarcinogenesis and also participates in malignant progression,[13,14] it may be possible to estimate the future risk of developing more malignant HCCs on the basis of DNA methylation status. However, only a few previous studies focusing on HCCs have used recently developed array-based technology for assessing genome-wide DNA methylation status,[15] and such studies have focused mainly on identification of tumor-related genes that are silenced by DNA methylation. DNA methylation profiles, which could become the optimum indicator for carcinogenetic risk estimation and prediction of patient outcome, should therefore be further explored during hepatocarcinogenesis using array-based approaches.

In this study, to clarify genome-wide DNA methylation profiles during multistage hepatocarcinogenesis, we performed bacterial artificial chromosome (BAC) array-based methylated CpG island amplification (BAMCA)[16–18] using a microarray of 4,361 BAC clones[19] in the normal liver tissue obtained from patients without HCCs, noncancerous liver tissue obtained from patients with HCCs, and in HCCs themselves.

Material and methods

Patients and tissue samples

As a learning cohort, 15 samples of the noncancerous liver tissue (N1 to N15) and 19 primary HCCs (T1 to T19) were obtained from surgically resected specimens from 16 patients who underwent partial hepatectomy at the National Cancer Center Hospital, Tokyo, Japan. The patients comprised 13 men and 3 women with a mean (±SD) age of 64.9 ± 7.4 years. Of these, 7 were positive for hepatitis B virus (HBV) surface antigen (HBs-Ag), 8 were positive for anti-hepatitis C virus (HCV) antibody (anti-HCV) and 1 was negative for both. Histological examination of the noncancerous liver tissue samples revealed findings compatible with chronic hepatitis in 5 and cirrhosis in 9 and no remarkable histological findings in 1.

Additional Supporting Information may be found in the online version of this article.

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For the comparison, 10 normal liver tissue samples (C1 to C10) showing no remarkable histological findings were also obtained from 10 patients without HCCs who were both HBs-Ag- and anti-HCV-negative. The patients comprised 7 men and 3 women with a mean age of 58.4 ± 9.7 years. Nine patients underwent partial hepatectomy for liver metastases of primary colon cancers, and 1 patient did so for liver metastases of gastrointestinal stromal tumor of the stomach.

In addition, for the comparison, 7 liver tissue samples (V1 to V7) were obtained from 7 patients who were positive for HBs-Ag or anti-HCV, but who had never developed HCCs. The patients comprised 4 men and 3 women with a mean age of 62.4 ± 5.2 years. Three patients underwent partial hepatectomy for liver metastases of primary colon or rectal cancers, and 1 patient did so for liver metastases of gastric cancer. Three patients underwent partial hepatectomy for cholangiocellular carcinomas.

As a validation cohort, 26 normal liver tissue samples (C11 to C36) showing no remarkable histological features were obtained from 26 patients without HCCs who were both HBs-Ag- and anti-HCV-negative. Twenty-four noncancerous liver tissue samples (N16 to N 39) and 25 primary HCCs (T20 to T44) were obtained from surgically resected specimens from 24 patients who underwent partial hepatectomy were added. The patients from whom C11 to C36 were obtained comprised 21 men and 5 women with a mean age of 59.9 ± 10.9 years. The patients with HCCs from whom N16 to N 39 and T20 to T44 were obtained comprised 22 men and 2 women with a mean age of 61.6 ± 11.4 years. Of the 24 patients with HCCs from whom N16 to N 39 and T20 to T44 were obtained, 5 were positive for HBs-Ag, 16 were positive for anti-HCV and 3 were negative for both. Histological examination of N16 to N 39 revealed findings compatible with chronic hepatitis and cirrhosis in 16 and 8 samples, respectively.

This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan.

BAMCA

High molecular weight DNA from fresh-frozen tissue samples was extracted using phenol-chloroform followed by dialysis. Because DNA methylation status is known to be organ specific, the reference DNA for analysis of the developmental stages of HCCs should be obtained from the liver and not from other organs or peripheral blood. Therefore, a mixture of normal liver tissue DNA obtained from 5 male patients (C37 to C41) and 5 female patients (C42 to C46) was used as a reference for analyses of male and female test DNA samples, respectively.

DNA methylation status was analyzed by BAMCA using a custom-made array (MCG Whole Genome Array-4500) harboring 4,361 BAC clones located throughout chromosomes 1 to 22 and X and Y,[19] as described previously.[16–18] Briefly, 5-μg aliquots of test or reference DNA were first digested with 100 units of methylation-sensitive restriction enzyme *Sma* I and subsequently with 20 units of methylation-insensitive *Xma* I. Adapters were ligated to *Xma* I-digested sticky ends, and polymerase chain reaction (PCR) was performed with an adapter primer set. Test and reference PCR products were labeled by random priming with Cy3- and Cy5-dCTP (GE Healthcare, Buckinghamshire, UK), respectively, and precipitated together with ethanol in the presence of Cot-I DNA. The mixture was applied to array slides and incubated at 43°C for 72 hr. Arrays were scanned with a GenePix Personal 4100A (Axon Instruments, Foster City, CA) and analyzed using GenePix Pro 5.0 imaging software (Axon Instruments) and Acue 2 software (Mitsui Knowledge Industry, Tokyo, Japan). The signal ratios were normalized in each sample to make the mean signal ratios of all BAC clones 1.0.

Statistics

Differences in the average number of BAC clones that showed DNA methylation alterations between groups of samples were analyzed using the Mann-Whitney *U* test or the Kruskal-Wallis test. Correlations between DNA methylation alterations in noncancerous liver tissue samples and the incidence of metachronous development and recurrence of HCCs were analyzed using the chi-squared test. Differences at $p < 0.05$ were considered significant. BAC clones whose signal ratios yielded by BAMCA were significantly different between groups of samples were identified by Wilcoxon test ($p < 0.01$). A support vector machine algorithm and a leave-one-out cross-validation were used to identify BAC clones by which the cumulative error rate for discrimination of sample groups became minimal. Two-dimensional hierarchical clustering analysis of noncancerous liver tissue samples and the BAC clones, and such analysis of HCCs and the BAC clones, were performed using the Expressionist software program (Gene Data, Basel, Switzerland). Survival curves of patient groups with HCCs were calculated by the Kaplan-Meier method, and the differences were compared by the log-rank test. The Cox proportional hazards multivariate model was used to examine the prognostic impact of DNA methylation status, histological differentiation, portal vein tumor thrombi, intrahepatic metastasis and multicentricity. Differences at $p < 0.05$ were considered significant.

Results

Genome-wide DNA methylation alterations during multistage hepatocarcinogenesis

Figures. 1*a* and 1*b* show examples of scanned array images and scattergrams of the signal ratios (test signal/reference signal), respectively, for normal liver tissue from a patient without HCC (Panel C), and both noncancerous liver tissue (Panel N) and cancerous tissue (Panel T) from a patient with HCC. In all normal liver tissue samples, the signal ratios of 97% of the BAC clones were between 0.67 and 1.5 (red bars in Fig. 1*b*). Therefore, in noncancerous liver tissue obtained from patients with HCCs and HCCs, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypomethylation and DNA hypermethylation of each BAC clone compared with normal liver tissue, respectively.

In samples of noncancerous liver tissue obtained from patients with HCCs, many BAC clones showed DNA hypo- or hypermethylation (Panel N of Fig. 1*b*). In the learning cohort, all 9 patients (100%) showing DNA hypo- or hypermethylation on 70 or more than 70 BAC clones in their noncancerous liver tissue samples developed metachronous or recurrent HCCs after hepatectomy, whereas only 2 (30%) of the 6 patients showing DNA hypo- or hypermethylation on less than 70 BAC clones in their noncancerous liver tissue samples did so ($p = 0.0235$).

In HCCs themselves, more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation, *i.e.*, deviation of the signal ratio from 0.67 or 1.5, was increased (Panel T of Fig. 1*b*) in comparison with noncancerous liver tissue obtained from patients with HCCs. The average numbers of BAC clones showing a signal ratio of less than 0.67 ($p = 0.0000063$) and more than 1.5 ($p = 0.00000052$) were increased significantly relative to normal liver tissue, to noncancerous liver tissue obtained from patients with HCCs, and to HCCs (Table I).

There were no significant differences in the number of BAC clones showing DNA hypo- or hypermethylation in samples of normal liver tissue obtained from male and female patients without HCCs (66.0 ± 30.1 and 98.7 ± 55.9, $p = 0.362$) and noncancerous liver tissue (111.2 ± 68.4 and 60.7 ± 46.9, $p = 0.279$) and cancerous tissue (521.5 ± 255.8 and 626.7 ± 329.0, $p = 0.539$) obtained from male and female patients with HCCs, respectively. Although there were no significant differences in the number of BAC clones showing DNA hypo- or hypermethylation between HBV- and HCV-positive patients with HCCs in both noncancerous liver tissue (108.3 ± 80.5 and 98.4 ± 60.0, $p = 1.000$) and cancerous tissue (475.6 ± 323.8 and 497.0 ± 247.8, $p = 0.689$), Wilcoxon test ($p < 0.01$) identified BAC clones in which DNA methylation status differed significantly between HBV- and

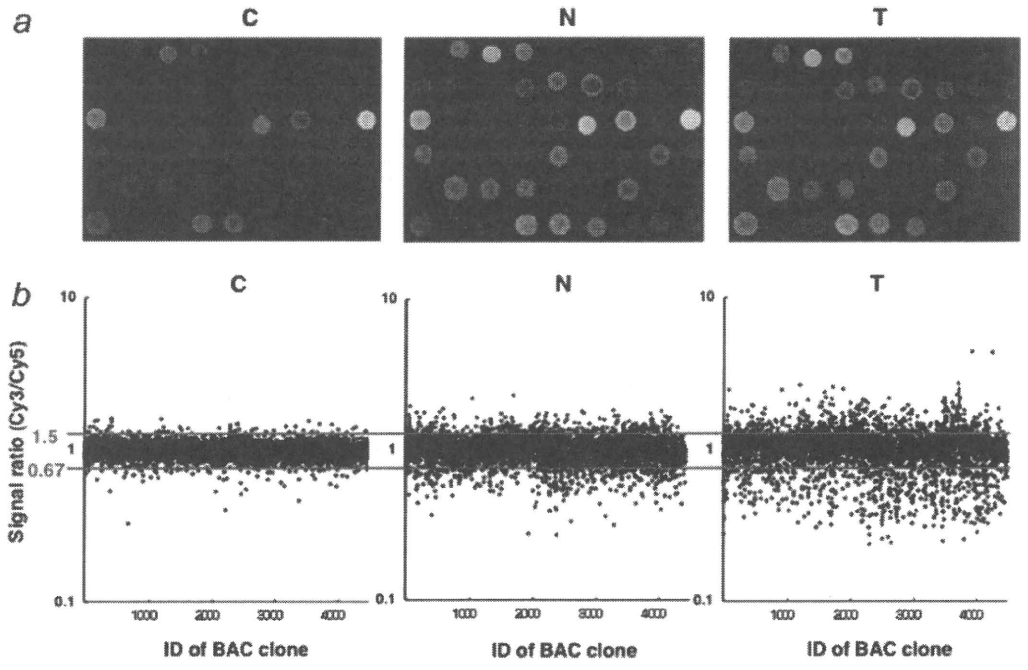

FIGURE 1 – Genome-wide DNA methylation alterations during multistage hepatocarcinogenesis. (*a*) Scanned array images yielded by BAMCA in normal liver tissue obtained from a patient without HCC (C) and noncancerous liver tissue (N) and cancerous tissue (T) obtained from a patient with HCC. (*b*) Scattergrams of the signal ratios yielded by BAMCA. In all C samples, the signal ratios of 97% of BAC clones were between 0.67 and 1.5 (red bars). In N and T, DNA methylation status corresponding to a signal ratio of less than 0.67 and more than 1.5 was defined as DNA hypomethylation and DNA hypermethylation on each BAC clone compared with C, respectively. Even in N, many BAC clones showed DNA hypo- or hypermethylation. In T, more BAC clones showed DNA hypo- or hypermethylation, and the degree of DNA hypo- or hypermethylation, *i.e.*, deviation of the signal ratio from 0.67 or 1.5 was increased in comparison with N.

TABLE I – GENOME-WIDE DNA METHYLATION ALTERATIONS DURING MULTISTAGE HEPATOCARCINOGENESIS

Tissue samples	Average number of BAC clones (mean ± SD)					
	Signal ratio <0.67 (DNA hypomethylation)	*p*	Signal ratio >1.5 (DNA hypermethylation)	*p*	Signal ratio <0.67 or >1.5(DNA hypo- or hypermethylation)	*p*
Normal liver tissue samples obtained from patient without HCCs (C, $n = 10$)	39.9 ± 20.8	0.0000063[1]	38.9 ± 24.9	0.00000052[1]	75.8 ± 39.3	0.00000061[1]
Noncancerous liver tissue samples obtained from patient with HCCs (N, $n = 15$)	61.2 ± 46.8	0.000102[2]	39.9 ± 27.3	0.0000026[2]	101.1 ± 66.5	0.0000065[2]
HCCs (T, $n = 19$)	278.9 ± 167.7	–	228.9 ± 125.7	–	507.8 ± 281.9	–

p values <0.05, which indicate significant differences.
[1]Kruskal-Wallis test among C, N and T.–[2]Mann-Whitney *U* test between N and T.

HCV-positive patients with HCCs in noncancerous liver tissue (18 BAC clones) and cancerous tissue (15 BAC clones), respectively.

DNA methylation profiles discriminating noncancerous liver tissue obtained from patients with HCCs from normal liver tissue

The above findings indicating accumulation of clinicopathologically significant genome-wide DNA methylation alterations in noncancerous liver tissue prompted us to estimate the degree of carcinogenetic risk based on DNA methylation profiles. Wilcoxon test ($p < 0.01$) revealed that the signal ratios of 512 BAC clones differed significantly between normal liver tissue samples and noncancerous liver tissue samples obtained from patients with HCCs. To omit potentially insignificant BAC clones associated only with inflammation and/or fibrosis and focus on BAC clones for which DNA methylation status was inherited by HCCs from the precancerous stage, we defined Groups I, II, III and IV. Group I: BAC clones in which the average signal ratio of noncancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue and the average signal ratio of HCCs was even higher than that of noncancerous liver tissue obtained from patients with HCCs (41 BAC clones), Group II: BAC clones in which the average signal ratio of noncancerous liver tissue obtained from patients with HCCs was higher than that of normal liver tissue and the average signal ratio of HCCs did not differ from that of noncancerous liver tissue obtained from patients with HCCs (146 BAC clones), Group III: BAC clones in which the average signal ratio of noncancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue and the average signal ratio of HCCs was even lower than that of noncancerous liver tissue obtained from patients with HCCs (40 BAC clones), and Group IV: BAC clones in which the average signal ratio of noncancerous liver tissue obtained from patients with HCCs was lower than that of normal liver tissue and the average