Detection of glypican-3-specific CTLs in chronic hepatitis and liver cirrhosis

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Abstract. Glypican-3 (GPC3) is one of carcinoembryonic antigens known to be overexpressed in hepatocellular carcinoma (HCC). It has been suggested that GPC3 may be related to the development of HCC in a background of chronic hepatitis (CH) and liver cirrhosis (LC). Therefore, in an attempt to establish an early diagnostic marker of HCC, we quantified the number of GPC3-specific CTLs in the peripheral blood of CH and LC patients. We selected CH and LC patients who were HCV-RNA (+) or HBs antigen (+) within 6 months prior to the study and had no HCC nodules as detected by imaging. A total of 56 patients with CH and LC, and 45 patients with HLA-A24$^+$ or HLA-A2$^+$ were enrolled for this investigation. After isolation of mononuclear cells from each patient's peripheral blood specimens, we performed ELISPOT assay using HLA-A24- and HLA-A2-restricted GPC3 peptides. In the ELISPOT assay, GPC3-specific CTLs were detected in 10 of the 45 CH and LC cases (22%). In addition, the plasma titers of anti-GPC3 IgG were increased in the CH and LC patients as compared with those in healthy donors. GPC3-specific CTLs were found to be present not only in patients with HCC, but also in patients with CH and LC. This suggests the possibility of GPC3-specific CTLs serving as a marker for the early diagnosis of imaging-invisible HCC.

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Abbreviations: GPC3, glypican-3; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma

Key words: glypican-3, CTL, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma

Introduction

The prevalence of hepatocellular carcinoma (HCC) is increasing rapidly in both Asian and Western countries. It is clear that patients with hepatitis B- or C-associated liver cirrhosis are at a higher risk of developing HCC (1), and patients with hepatitis treated surgically or by other therapies are also at a higher risk of recurrence (2). Furthermore, the liver function of these patients is often very poor, which restricts further treatment options for recurrence. As a result, the prognosis of HCC remains poor, and the development of new therapies for the prevention of cancer development and recurrence, that is, adjuvant therapy, is urgently needed.

Glypican-3 (GPC3) has been reported to be overexpressed in most types of HCC (3-10) and melanoma in humans (6,8,9). GPC3 belongs to the six-member family of glypicans in mammals (11). GPC3 is a heparan sulfate proteoglycan that is bound to the outer surface of the plasma membrane by a glycosylphosphatidylinositol anchor. GPC3 has been shown to regulate the signaling mediated by Wnts (12,13), Hedgehogs (14), fibroblast growth factors (15,16) and bone morphogenetic proteins (15,17). These signaling pathways are only partially dependent on the heparan sulfate chains (11,16,18). However, whether GPC3 plays an oncogenic role in HCC is still controversial.

We recently identified both HLA-A24 (A*2402) and H-2K^d-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2 (A*0201)-restricted $GPC3_{144-152}$ (FVGEFFTDV), both of which can induce GPC3-reactive cytotoxic T cells (CTLs) (19). We previously reported a preclinical study conducted in a mouse model with a view to designing an optimal schedule for clinical trials of a GPC3-derived peptide vaccine (20). We predicted that overexpression of GPC3 in HCC is related to the development of HCC in a background of chronic hepatitis (CH) and/or liver cirrhosis (LC). Towards establishing the possibility of early diagnosis of imaging-invisible HCC and vaccine therapy, we determined the number of GPC3-specific CTLs in the peripheral blood of CH and LC patients.

Materials and methods

Patients, blood samples and cell lines. Blood samples from patients with CH and LC were collected during routine diagnostic procedures after obtaining their written consent at the Tokyo Rosai Hospital between October 2006 and October 2007. CH and LC patients who were confirmed to be HCV-RNA(+) or HBs antigen(+) within six months prior to registration were eligible for the study. The diagnosis of CH or LC was made clinically by imaging and laboratory data. The patients had no medical history of HCC, and no evidence of HCC on ultrasonography, CT (computed tomography) or MRI (magnetic resonance imaging) conducted prior to the registration.

Human liver cancer cell lines SK-Hep-1/GPC3, HepG2 and K562 were maintained *in vitro* in RPMI-1640 or DMEM supplemented with 10% FCS. SK-Hep-1/GPC3 has been described previously (19). HepG2 endogenously expressing GPC3 was kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). HLA-class I deficient K562 was obtained from Kumamoto University. The origins and HLA genotypes of these cell lines have been described in previous reports (21,22).

Ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay. We isolated peripheral blood mononuclear cells (PBMCs) from the heparinized blood of HLA-A2$^+$ and/or HLA-A24$^+$ Japanese CH, LC or HCC patients and healthy donors by means of Ficoll-Conray density gradient centrifugation. IFN-γ production by the CTLs present in the PBMCs in the presence or absence of the GPC3 peptide was assessed by the ELISPOT assay (BD™ Bioscience, San Diego, CA), as described previously. Briefly, defrosted PBMCs (1x10^6/well) were cultured in 96-well flat-bottomed plates for the ELISPOT assay (BD Bioscience) with HLA-A2-restricted $GPC3_{44-52}$ (A2-1) (RLQPGLKWV), $GPC3_{144-152}$ (A2-3) (FVGEFFTDV), $GPC3_{155-163}$ (A2-4) (YILGSDINV) and HLA-A24-restricted $GPC3_{298-306}$ (A24-8) (EYILSLEEL) (10 μM) with 100 units/ml recombinant human IL-2 overnight *in vitro*. The negative control consisted of medium alone and the positive control included HLA-A24- or -A2-restricted cytomegalovirus. The number and area of the spots were automatically determined and subsequently analyzed with the ELISPOT system (Minerva Tech, Tokyo, Japan).

Induction of GPC3-reactive human CTLs and cytotoxic assay. We evaluated the cytotoxic activity of the CTLs that were induced with the GPC3 A2-3 peptide in the PBMCs isolated from the CH4 patient. PBMCs were isolated from HLA-A2$^+$ CH4 patient, distributed into 4 wells (3x10^5 cells/24-well), and cultured with the GPC3 A2-3 peptide. After culture for 7 and 14 days, the PBMCs cocultured with irradiated autologous monocyte-derived DCs obtained by positive selection with human CD14 Micro Beads (Miltenyi, Bergisch Gladbach, Germany) were pulsed with the GPC3 A2-3 peptide. The CD14$^+$ cells were cultured in the presence of 100 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Inc.) and 100 ng/ml of IL-4 (R&D Systems, Inc.) in RPMI-1640 (Sigma-Aldrich Corp., St. Louis, MO) containing 2% heat-inactivated autologous serum and 1% penicillin-streptomycin-glutamine (Gibco, Invitrogen, Ltd.; Paisley, Scotland, UK). After 5 days, TNFα (PEPRPTECH EC., London, UK) was added at the concentration of 20 ng/ml to induce maturation of the DCs. After 7 days, mature DCs were harvested and pulsed with 10 μM of the candidate peptides for 4 h at room temperature in RPMI. The peptide-pulsed DCs were then irradiated (3500 rads) and mixed at a ratio of 1:20 with autologous PBMCs.

These DCs were set up in 48-well culture plates; each well contained 1.5x10^4 peptide-pulsed DCs, 3x10^5 PBMCs and 5 ng/ml IL-7 (PEPRPTECH EC.) in 0.5 ml of RPMI containing 10% autologous serum. Three days after the start of the incubation, IL-2 (R&D Systems, Inc.) was added to these cultures at a final concentration of 10 U/ml. On days 7 and 14, the T cells were restimulated with the autologous DCs pulsed with the peptide.

After 21 days, the cells were recovered and analyzed for their cytotoxic activity against the target cells with the TERASCAN VPC system (Minerva Tech), as previously described (23). Briefly, SK-Hep-1/GPC3 (GPC3$^+$, A2$^+$, A24$^+$), HepG2 (GPC3$^+$, A2$^+$, A24$^+$) and K562 (HLA-class I$^-$) cells were used as the target cells and labeled with calcein-AM solution for 30 min at 37°C. The labeled cells were washed three times and distributed into a 96-well culture plate (1x10^4 per well) and then incubated with the effector cells for 5 h. The fluorescence intensity was measured before and after 5-h culture, and the Ag-specific cytotoxic activity was calculated using the following formula: cytotoxicity (%) = [(sample release) - (spontaneous release)]/[(maximum release) - (spontaneous release)] x 100.

ELISA for the detection of anti-GPC3 IgG antibodies. Recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) was diluted in 10 x Block Ace (Dainippon Pharmaceutical, Osaka) to a final concentration of 1 μg/ml, dispensed into 96-well plates (100 μl/well) and incubated overnight at 4°C. Then, the plates were blocked with Block Ace for 1 h at room temperature. Plasma samples from CH and LC patients and healthy controls (100 μl, 1:100 dilution) were added to each well, followed by incubation for 2 h at room temperature. After washing three times with PBS containing 0.05% Tween-20 (PBST), Peroxidase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratories, Inc., W. Baltimore, USA) was reacted for 30 min. The plates were washed with PBST and developed with Stable Peroxide Substrate Buffer (Pierce, Rockford, IL) for 20 min. After stopping the reaction with 1 M H_2SO_4, the absorbance was measured at 490 nm. All plasma samples were measured in duplicate and were randomly dispensed into the plates.

Statistical analysis. The two-tailed Student's t-test was used to evaluate the statistical significance of differences in the data obtained by the ELISPOT assay. Unpaired Mann-Whitney U tests were used for the evaluation of the significance of differences in the data obtained by ELISA. $P<0.05$ was considered to denote significant difference.

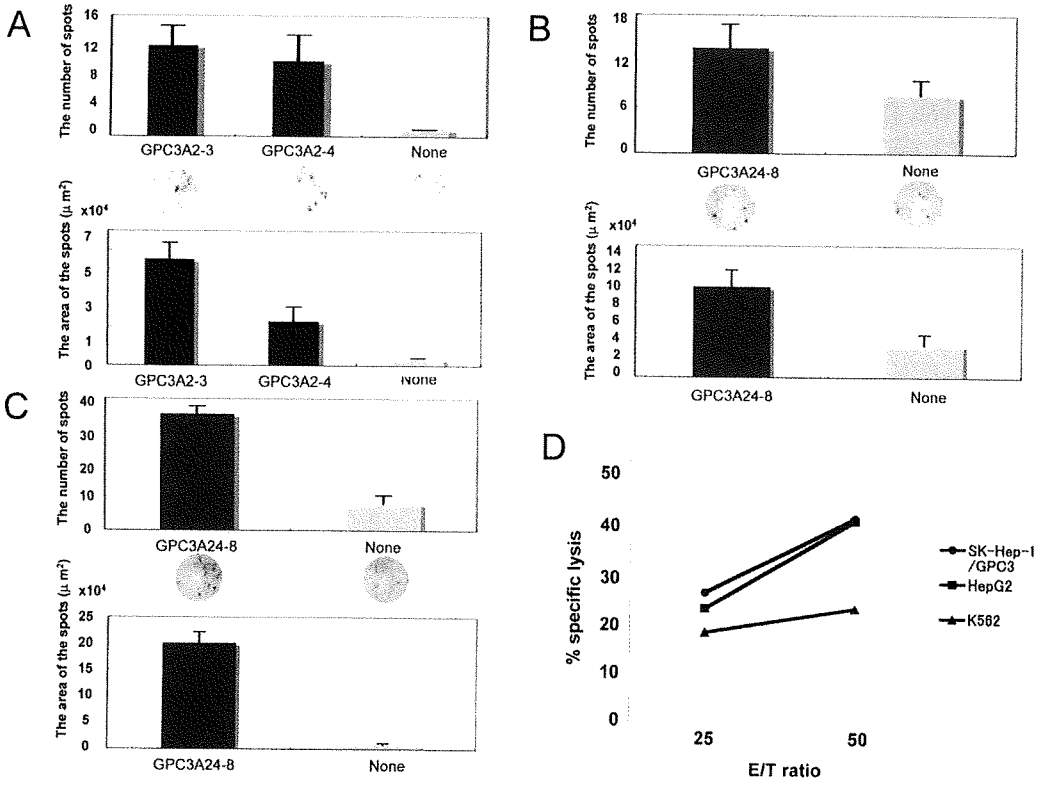

Figure 1. Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2+ or HLA-A24+ CH and LC patients and the cytotoxicity of the CTLs induced by stimulation with the GPC3 (A2-3) peptide. GPC3-specific CD8+ T cells were detected in the chronic hepatitis [(A), HLA-A2+ CH4 patient; (B), HLA-A24+ CH5 patient] and liver cirrhosis [(C), HLA-A24+ LC5 patient]. IFN-γ produced by the peptide-specific T cells was measured by the IFN-γ-ELISPOT assay (middle column). The number and area of spots are shown in the upper and lower panels, respectively. Lysis of human hepatoma cell lines SK-Hep-1/GPC3 (circles) and HepG2 (squares) expressing GPC3 and HLA-A2 by GPC3-specific CTLs was observed following stimulation with the GPC3 A2-3 peptide (FVGEFFTDV) [(D), HLA-A2+ CH4 patient]. An HLA-classI- K562 human erythromyeloblastoid leukemia cell line was used as the negative control (triangles).

Results

Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2+ or HLA-A24+ CH, LC and HCC patients. We evaluated the frequency of CTLs that recognized the GPC3 A2-1, A2-3, A2-4 or A24-8 peptide in the PBMCs of CH, LC and HCC patients. The CH and LC patients enrolled in this study were 34 male and 22 female patients. The average age of the patients was 64 years. HCV and HBV infection was found in 54 and 2 patients, respectively. The 56 patients were 33 CH and 23 LC cases. Mean serum α-fetoprotein (AFP) was 13.3±21.1 ng/ml (normal <20 ng/ml). In regard to the HLA genotype, 10, 22 and 13 patients, respectively, were HLA-A2+, HLA-A24+ and HLA-A2+/24+. On the other hand, there were 11 patients who were HLA-A2-/A24-. In this investigation, we enrolled the 45 patients who were HLA-A2+ or HLA-A24+.

We determined the presence of CTLs in the PBMCs of the CH and LC patients by ELISPOT assay using HLA-A24- and HLA-A2-restricted GPC3 peptides (Fig. 1, Table I). The representative data of the ELISPOT assay are highlighted. Interestingly, in the CH4 patient, the spots and areas were highly developed in the GPC3 A2-3 and A2-4 peptide-stimulated PBMCs (Fig. 1A). However, few spots and areas were detected in the negative control (no peptide). In addition, GPC3 A24-8 peptide-restricted CTLs were also detected in the CH5 and LC5 patients (Fig. 1B and C). These results suggest that GPC3-specific CTLs are present in the PBMCs of some of CH and LC patients.

Cytotoxicity of CTLs induced by stimulation with the GPC3 (A2-3) peptide. To clarify the cytotoxic activity of GPC3-specific CTLs induced by stimulation with the GPC3 peptide, the HCC cell line, SK-Hep-1/GPC3, transfected with GPC3 and expressing HLA-A2 and HLA-A24 were used as the target cells (Fig. 1D). The CTLs induced from the PBMCs of CH4 (Table I) patient by stimulation with the GPC3 A2-3 peptide showed specific cytotoxicity against the SK-Hep-1/GPC3 and HepG2 cells. On the other hand, no GPC3-specific cytotoxicity was observed against the HLA-classI- K562 cells. These results indicate that GPC3-peptide-specific CTLs induced from CH4 (Table I) patient are cytotoxic against the GPC3-expressing target HCC cells.

Frequency of HLA-A2+ or HLA-A24+ CH, LC and HCC patients positive for GPC3-peptide-specific CTLs in PBMC The frequency of patients with GPC3-specific CTLs in their PBMCs is shown in Fig. 2, while the clinical backgrounds of the CH, LC and HCC patients are summarized in Table II. CTL positivity was observed in 5 of 26 CH patients (19%), 5 of 19 LC patients (26%), and 21 of 54 HCC patients (39%). In addition, the percentage of CTL-positive patients tended to

Table I. Detection of GPC3-specific CTLs in the PBMCs of chronic hepatitis/liver cirrhosis patients by ELISPOT assay.

	Peptide/Peptide sequence									
	GPC3 A2-1/RLQPGLKWV		GPC3 A2-3/FVGEFFTDV		GPC3 A2-4/YILGSDINV		GPC3 A24-8/RYILSLEEL		No peptide	
	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)	No. of spots mean (±SD)	Area (μm^2) mean (±SD)
CH[a]1(A*0201)	**1.0±0.0**[c]	**25905.0±8487.8**	**2.0±1.0**	**2826.0±3079.5**	**1.6±1.1**	**13895.0±4486.8**	NT	NT	0.0±0.0	0.0±0.0
CH2 (A*0201)	**1.0±1.7**	**707.0±1223.6**	**1.6±1.1**	**6830.0±6934.2**	**2.6±1.1**	**3297.0±3263.1**	NT	NT	0.0±0.0	0.0±0.0
CH3 (A*0201)	NT[d]	NT	**18.3±5.5**	**85100.0±17050.1**	**15.6±2.5**	**20173.0±4728.4**	NT	NT	8.0±1.7	8045.0±1849.1
CH4 (A*0201)	NT	NT	**12.0±2.6**	**55187.0±8618.4**	**10.0±3.4**	**22832.0±7632.2**	NT	NT	1.0±0.0	3853.0±375.2
CH5 (A*2402)	NT	NT	NT	NT	NT	NT	**13.3±3.7**	**101736.0±54505.9**	7.0±1.0	36502.5±14892.4
LC[b]1 (A*0201)	**1.0±0.0**	**1060.0±815.7**	**2.1±0.2**	**2944.0±815.7**	**6.3±0.5**	**50162.0±4283.0**	NT	NT	0.5±0.0	354.0±0.0
LC2 (A*0201)	**24.0±3.0**	**55891.2±23304.1**	**8.0±2.0**	**45971.9±25440.5**	**8.0±1.0**	**103961.4±13618.6**	NT	NT	4.3±0.5	2098.3±2166.5
LC3 (A*0201)	**1.3±0.5**	**2355.0±2855.2**	**3.6±1.5**	**8007.0±6564.4**	**11.3±5.7**	**100323.0±70946.1**	NT	NT	2.0±3.4	2826.0±4894.7
LC4 (A*2402)	NT	NT	NT	NT	NT	NT	**14.0±8.0**	**41331.0±31472.6**	3.0±0.0	7065.0±3996.5
LC5 (A*2402)	NT	NT	NT	NT	NT	NT	**35.3±2.3**	**200882.0±21210.9**	8.3±2.3	8714.0±2855.5

[a]CH, chronic hepatitis. [b]LC, liver cirrhosis. [c]We show values higher than the value for 'No peptide' by a bold font. [d]NT, not tested.

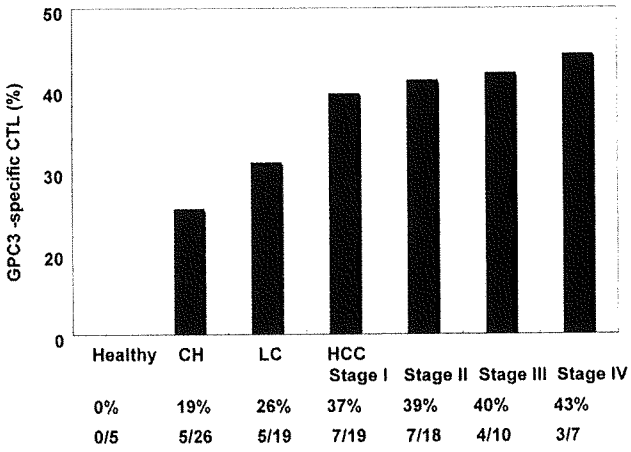

Figure 2. Frequency of HLA-A2[+] or HLA-A24[+] CH, LC and HCC patients positive for GPC3-peptide-specific CTLs in the PBMCs. GPC3-peptide-specific CTLs were detected in 19 and 26% of the patients with CH and LC, respectively. In the HCC patients, the percentage of these CTLs tended to increase with increasing stage of progression of the disease: 37% (stage I), 39% (stage II), 40% (stage III) and 43% (stage IV).

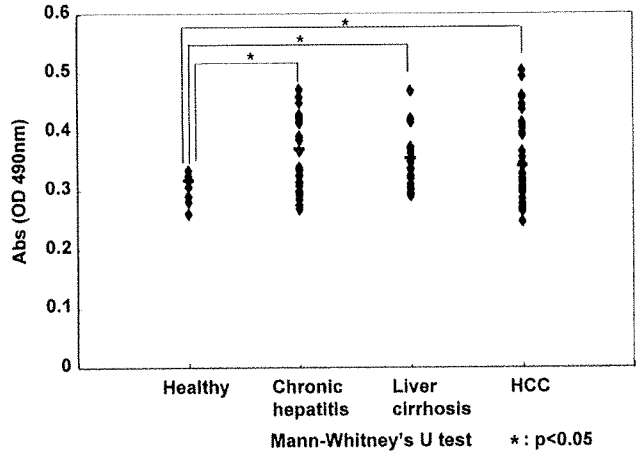

Figure 3. Plasma titers of anti-GPC3 IgG in the CH, LC and HCC patients. Anti-GPC3 IgG was detected by ELISA using recombinant GPC3 protein. A significantly higher titer of IgG to GPC3 was observed in the CH ($p<0.05$), LC ($p<0.05$) and HCC patients ($p<0.05$) as compared with that in healthy donors. *$p<0.05$ (Mann-Whitney U test).

increase with increasing clinical stage of HCC; stage I (7/19, 37%), stage II (7/18, 39%), stage III (4/10, 40%), and stage IV (3/7, 43%) (Table II). There were no CTL-positive cases (0/5, 0%) in healthy donors.

Anti-GPC3 IgG in the plasma in patients with CH, LC and HCC. To examine the quantitative titers of anti-GPC3 IgG in the plasma of patients with CH, LC and HCC, we carried out ELISA using the recombinant GPC3 protein (Fig. 3). The titers in the CH, LC and HCC patients were significantly higher as compared with the peak titer in healthy controls. These results indicate that the GPC3 antigen is expressed not only in HCC patients, but also in CH and LC patients.

Table II. Number of CTL-negative and -positive cases in chronic hepatitis, liver cirrhosis and HCC patients.

Group	Healthy (n=5)		Chronic hepatitis (n=33)		Liver cirrhosis (n=23)		HCC (n=54)	
	Negative (n=5) mean (±SD)	Positive (n=0) mean (±SD)	Negative (n=28) mean (±SD)	Positive (n=5) mean (±SD)	Negative (n=19) mean (±SD)	Positive (n=5) mean (±SD)	Negative (n=33) mean (±SD)	Positive (n=21) mean (±SD)
Age	31.2±7.1	-	61.6±11.2	60.6±12.9	67.3±10.1	71.0±2.7	65.8±7.9	64.0±10.5
Male	4	0	16	3	12	3	28	15
Female	1	0	12	2	6	2	5	6
HCV/HBV								
+/-	ND	ND	5	26	18	5	18	14
-/+	ND	ND	2	0	0	0	4	2
+/+	ND	ND	0	0	0	0	2	2
-/-	ND	ND	0	0	0	0	9	3
AFP (ng/ml)	ND	ND	9.5±18.9	9.6±7.3	21.2±25.4	8.8±7.7	26335.1±143782.5	1431.5±3574.9
HLA-								
$A02^+$	3	0	3	3	2	2	13	8
$A24^+$	2	0	12	1	7	2	18	11
$A02^+/24^+$	0	0	6	1	5	1	2	2
$A02^-/24^-$	0	0	7	0	4	0	0	0

Discussion

The oncofetal antigen GPC3 is known to be overexpressed in HCCs (3-10) and melanomas (6,8,9). We recently identified GPC3-specific peptides restricted to HLA-A24 (A^*2402) and H-$2K^d$, or HLA-A2 (A^*0201), both of which can induce GPC3-reactive cytotoxic T cells (CTLs) (19). We are currently conducting a phase I clinical trial of peptide vaccine prepared using these peptides against advanced HCC. In addition, in the near future, we propose to carry out a phase II clinical trial of the vaccine in HCC patients as well as CH and LC patients to evaluate its efficacy in preventing the onset of HCC. We report the finding of GPC3-specific CTLs in CH and LC patients for the first time in this study. Furthermore, the plasma titers of anti-GPC3 IgG in the CH and LC patients were also found to be significantly increased as compared with those in healthy donors.

It has been suggested that GPC3-specific CTLs may be derived from clinically invisible pre-neoplastic or neoplastic nodular lesions. In previous studies, expression of GPC3 was reported in 2/23 (8%) cirrhotic low-grade dysplastic nodules, and 2/9 (22%) (24), 2/22 (9%) (25) or 6/31 (19%) high-grade dysplastic nodules (26). In one study, among 5 adenomas with malignant characteristics, 3 (60%) showed immunoreactivity for GPC3 in the malignant regions (24). Other studies reported positive staining for GPC3 in 12/20 (60%) (24) and 22/32 (69%) cases (25) of early HCC. Meanwhile, the serum titers of the elevated GPC3 antigen in HCC cases were reported to be correlated with the clinical stage of HCC (19). In our study, we noted an increase of the plasma titers of anti-GPC3 IgG antibody in CH, LC and HCC patients. In addition, the frequency of patients with GPC3-specific CTLs appeared to increase with the stage of progression of the liver disease. These results suggest that GPC3 expression and the appearance of GPC3-specific CTLs may be prediagnostic markers of HCC.

On the other hand, the increase in the frequency of GPC3-specific CTLs and titers of anti-GPC3 IgG in the peripheral blood might be related to the expression of GPC3 in CH with high grade inflammation and LC. In this study, we did not perform immunohistochemical examination for GPC3, because needle biopsy of the liver in our patients was not conducted in our collaborative clinic. Previous studies have demonstrated GPC3 expression by immunohistochemistry in 25/30 (83%) cases of CH with high grade inflammation (27) and 11/95 (12%) cases of LC (26), indicating that GPC3 might be expressed in CH with high-grade inflammation and some LC patients, resulting in the appearance of GPC3-specific CTLs in the PBMCs of these patients.

During the 1-year follow-up of this study, onset of HCC was not observed in any of the 10 CH and LC patients who were positive for GPC3-specific CTLs in the peripheral blood; on the other hand, 2 (1CH and 1LC) patients who were negative for GPC3-specific CTLs showed development of HCC. It would, therefore, seem that the GPC3-specific CTLs might prevent the development of HCC or be predictive of a favorable prognosis of non-neoplastic liver lesions. However, our examination was limited to only HLA-A24- and A2-positive patients, and moreover, we followed up the patients for only one year. Therefore, careful long-term observation of a larger number of CH and LC cases is necessary to determine the role of GPC3-specific CTLs in patients with CH and LC.

In this study, we demonstrated an increase of GPC3-specific CTLs and high titers of anti-GPC3 IgG in CH and LC patients. Thus, GPC3-specific CTLs and anti-GPC3 IgG

may possibly be markers of early imaging-invisible HCC. In addition, active immunotherapy using GPC3 peptides may prevent the development of both non-neoplastic and neoplastic lesions of the liver.

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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

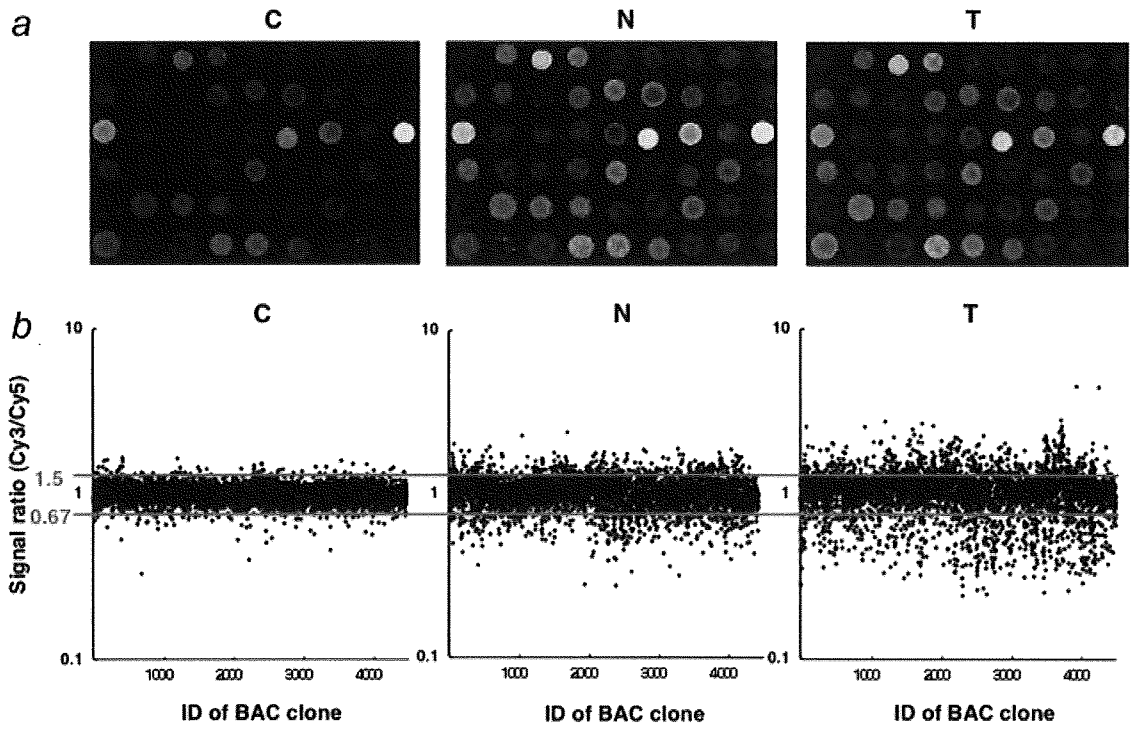

FIGURE 1 – Genome-wide DNA methylation alterations during multistage hepatocarcinogenesis. (*a*) Scanned array images yielded by BAMCA in normal liver tissue obtained from a patient without HCC (C) and noncancerous liver tissue (N) and cancerous tissue (T) obtained from a patient with HCC. (*b*) Scattergrams 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

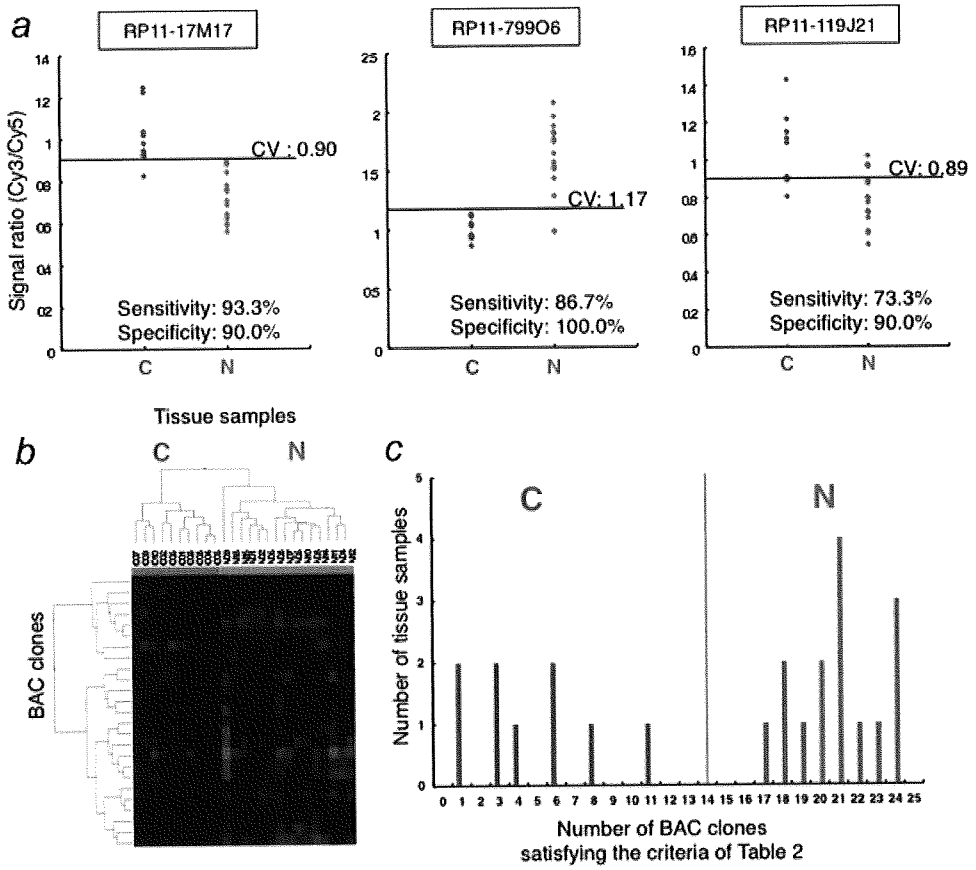

FIGURE 2 – DNA methylation profiles discriminating noncancerous liver tissue obtained from patients with HCCs from normal liver tissue. (*a*) Scattergrams of the signal ratios in normal liver tissue samples (C1 to C10) and noncancerous liver tissue samples obtained from patients with HCCs (N1 to N15) in the learning cohort on representative BAC clones, RP11-17M17, RP11-799O6 and RP11-119J21. Using the cutoff values (CV) described in each panel, noncancerous liver tissue samples obtained from patients with HCCs (N) in the learning cohort were discriminated from normal liver tissue samples (C) with sufficient sensitivity and specificity. (*b*) By 2-dimensional hierarchical clustering analysis using the 25 BAC clones selected by the process described in the Results section, normal liver tissue samples (C1 to C10) and noncancerous liver tissue samples obtained from patients with HCCs (N1 to N15) in the learning cohort were subclassified into the different subclasses without any error. The cluster trees for tissue samples and BAC clones are shown at the top and left of the panel, respectively. (*c*) Histogram showing the number of BAC clones satisfying the Table II criteria in samples C1 to C10 and N1 to N15. On the basis of this histogram, we established the following criteria: when the noncancerous liver tissue satisfied the criteria in Table II for 14 (green bar) or more than 14 BAC clones, it was judged to be at high risk of carcinogenesis.

signal ratio of HCCs did not differ from that of noncancerous liver tissue obtained from patients with HCCs (131 BAC clones). From the 512 BAC clones, 358 (Groups I, II, III and IV), in which the DNA methylation status was inherited by HCCs from noncancerous liver tissue, were selected. From the 358 BAC clones, the first 40 were identified by spot ranking analysis using the support vector machine algorithm for discrimination of noncancerous liver tissue obtained from patients with HCCs from normal liver tissue. Figure 2*a* shows scattergrams of the signal ratios in normal liver tissue samples and noncancerous liver tissue samples obtained from patients with HCCs on representative examples of the 40 BAC clones. Using the cutoff values described in each panel, noncancerous liver tissue obtained from patients with HCCs in the learning cohort was discriminated from normal liver tissue with sufficient sensitivity and specificity (Fig. 2*a*). From the 40 BAC clones, 25, for which such discrimination was performed with a sensitivity or specificity of 70% or more than 70%, were selected (Supporting Information Table S1). The cutoff values of the signal ratios for the 25 BAC clones, and their sensitivity and specificity, are shown in Table II. Two-dimensional hierarchical clustering analysis using the 25 BAC clones is shown in Figure 2*b*: 10 normal liver tissue samples (C1 to C10) and 15 noncancerous liver tissue samples obtained from patients with HCCs (N1 to N15) in the learning cohort were subclassified into different subclasses without any error. The number of BAC clones satisfying the criteria listed in Table II in noncancerous liver tissue samples showing chronic hepatitis (20.6 ± 1.8) was not significantly different from that showing cirrhosis (21.3 ± 2.4, $p = 0.542$) in the learning cohort.

A histogram showing the number of BAC clones satisfying the criteria listed in Table II for samples C1 to C10 and N1 to N15 is shown in Figure 2*c*. On the basis of this figure, we finally established the following criteria: when noncancerous liver tissue satisfied the criteria of Table II for 14 or more BAC clones (green bar in Fig. 2*c*), it was judged to be at high risk of carcinogenesis, and when noncancerous liver tissue satisfied the criteria of Table II for less than 14 BAC clones, it was judged not to be at high risk of carcinogenesis. Based on these criteria, both the sensitivity and specificity for diagnosis of noncancerous liver tissue samples obtained from patients with HCCs in the learning cohort as being at high risk of carcinogenesis were 100%.

To confirm these criteria, an additional 50 liver tissue samples were analyzed by BAMCA as a validation study (Supporting Information Figure S1). Twenty-three of 24 validation samples satisfying the criteria of Table II for 14 or more BAC clones were noncancerous liver tissue samples obtained from patients with HCCs (N16 to N36 and N38), and 24 of 26 validation samples satisfying the criteria of Table II for less than 14 BAC clones were normal

TABLE II – 25 BAC CLONES WHICH COULD DISCRIMINATE NONCANCEROUS LIVER TISSUES (N) FROM NORMAL LIVER TISSUES (C)

BAC clone ID	Location	Cutoff value	DNA methylation status[1]	Sensitivity (%)	Specificity (%)
RP11-104J13	1p35-1p36	1.01	C>N	93.3	70.0
RP11-52I2	1p34-1p35	1.00	C<N	80.0	60.0
RP11-29M22	1p11-1p12	1.11	C<N	86.7	90.0
RP11-21K1	2q37.2	1.00	C>N	86.7	70.0
RP11-109B15	5q33	1.04	C<N	66.7	90.0
RP11-88B24	6q26	0.95	C>N	80.0	70.0
RP11-112B7	7p13-7p14	1.00	C>N	80.0	70.0
RP11-48D21	8p11.2	1.00	C>N	80.0	90.0
RP11-120E20	11p15.4-11p15.5	0.90	C>N	73.3	100.0
RP11-334E6	11q23	1.00	C>N	86.7	80.0
RP11-17M17	11q25	0.90	C>N	93.3	90.0
RP11-319E16	12p13.32a	1.00	C>N	80.0	90.0
RP11-1100L3	12q13.13c-12q13.13d	1.04	C<N	86.7	80.0
RP11-799O6	12q13.3a-12q13.3b	1.17	C<N	86.7	100.0
RP11-119J21	12q24.33	0.89	C>N	73.3	90.0
RP11-332N6	14q11.2b	0.95	C>N	86.7	100.0
RP11-529E4	14q12c	1.00	C>N	93.3	50.0
RP11-89M4	16p13.2-16p13.3	1.20	C<N	86.7	100.0
RP11-215M5	15q15-15q21.1	1.00	C<N	86.7	70.0
RP11-348B12	19p13	1.00	C<N	80.0	80.0
RP11-134G22	20p11.2-20p12	1.01	C>N	80.0	90.0
RP11-328M17	22q13.2-22q13.33	0.93	C>N	86.7	100.0
RP11-354I12	22q13.31-22q13.33	1.00	C>N	93.3	80.0
RP11-55J11	22q13.2-22q13.33	1.00	C>N	80.0	70.0
RP11-480M11	Xq27.1-Xq28	0.90	C>N	80.0	90.0

[1]C>N, when the signal ratio was lower than the cutoff value, the tissue sample was considered to be at high risk for carcinogenesis; C<N, when the signal ratio was higher than the cutoff value, the tissue sample was considered to be at high risk for carcinogenesis.

liver tissue samples (C11 to C31, 33, 34 and 36). That is, our criteria enabled diagnosis of noncancerous liver tissue samples obtained from patients with HCCs in the validation set as being at high risk of carcinogenesis with a sensitivity of 95.8% and a specificity of 96.2%. The number of BAC clones satisfying the criteria listed in Table II in noncancerous liver tissue samples showing chronic hepatitis (17.6 ± 2.5) was not significantly different from that showing cirrhosis (19.4 ± 1.8, $p = 0.128$) in the validation cohort.

In addition, the average number of BAC clones satisfying the criteria in Table II was significantly lower in 7 samples of liver tissue obtained from patients who were infected with HBV or HCV, but who had never developed HCCs (V1 to V7, 13.14 ± 4.78), than that in N1 to N39 (19.21 ± 2.67, $p = 0.00419$).

Association of HCC DNA methylation profiles with patient outcome

To establish criteria for prognostication of patients with HCCs, in the learning cohort, 5 of 19 HCC samples obtained from patients who had survived more than 4 years after hepatectomy and 6 of 19 HCC samples from patients who had suffered recurrence within 6 months and died within a year after hepatectomy were defined as a favorable-outcome group and a poor-outcome group, respectively. Wilcoxon test ($p < 0.01$) revealed that the signal ratios of 41 BAC clones (Supporting Information Table S1) differed significantly between the favorable-outcome group ($n = 5$) and the poor-outcome group ($n = 6$). Figure 3*a* shows scattergrams of the signal ratios in samples from the favorable- and poor-outcome groups for representative examples of the 41 BAC clones. Using the cutoff values described in Figure 3*a* and Table III for the 41 BAC clones, samples from the poor-outcome group were discriminated from favorable-outcome group samples with sufficient sensitivity and specificity (Fig. 3*a* and Table III). Two-dimensional hierarchical clustering analysis using the 41 BAC clones is shown in Figure 3*b*: 5 HCCs in the favorable-outcome group and 6 HCCs in the poor-outcome group were subclassified into different subclasses without any error (Fig. 3*b*). A histogram showing the number of BAC clones satisfying the criteria in Table III is shown in Fig. 3*c*. In all 19 HCCs in the learning cohort, multivariate analysis revealed that satisfying the criteria in Table III for 32 or more BAC clones was a predictor of overall patient outcome and was independent of parameters that are already known to have prognostic impact,[20] such as histological differentiation, portal vein tumor thrombi, intrahepatic metastasis and multicentricity (Table IV).

To confirm these criteria, an additional 25 HCC samples were analyzed by BAMCA as a validation study, and then evaluated based on the criteria in Table III. All 44 HCCs were divided into 2 groups according to the number of BAC clones satisfying the criteria (32 or more BAC clones *vs.* less than 32 BAC clones). The period covered ranged from 11 to 3,413 days (mean, 1,349 days). The cancer-free and overall survival rates of patients with HCCs satisfying the criteria in Table III for 32 or more BAC clones was significantly lower than that of patients with HCCs satisfying the criteria in Table III for less than 32 BAC clones (Fig. 3*d*, $p = 0.000000002$ and $p = 0.0013$, respectively).

Discussion

Although many researchers in the field of cancer epigenetics use promoter arrays to identify the genes that are methylated in cancer cells,[21–23] we used a BAC array[19] in this study. The efficiency of identification of specific genes that are silenced by DNA methylations around the promoter regions and may become a target of therapy may be generally lower using the BAMCA approach than with conventional promoter array-based analysis. However, the promoter regions of specific genes are not the only target of DNA methylation alterations in human cancers. DNA methylation status in genomic regions not directly participating in gene silencing, such as the edges of CpG islands, may be altered at the precancerous stage before the alterations of the promoter regions themselves occur.[24] Moreover, aberrant DNA methylation of large regions of chromosomes, which are regulated in a coordinated manner in human cancers due to a process of long-range epigenetic silencing, has recently attracted attention.[25] BAMCA methods may be suitable for overviewing the DNA methylation status of individual large regions among all chromosomes and for

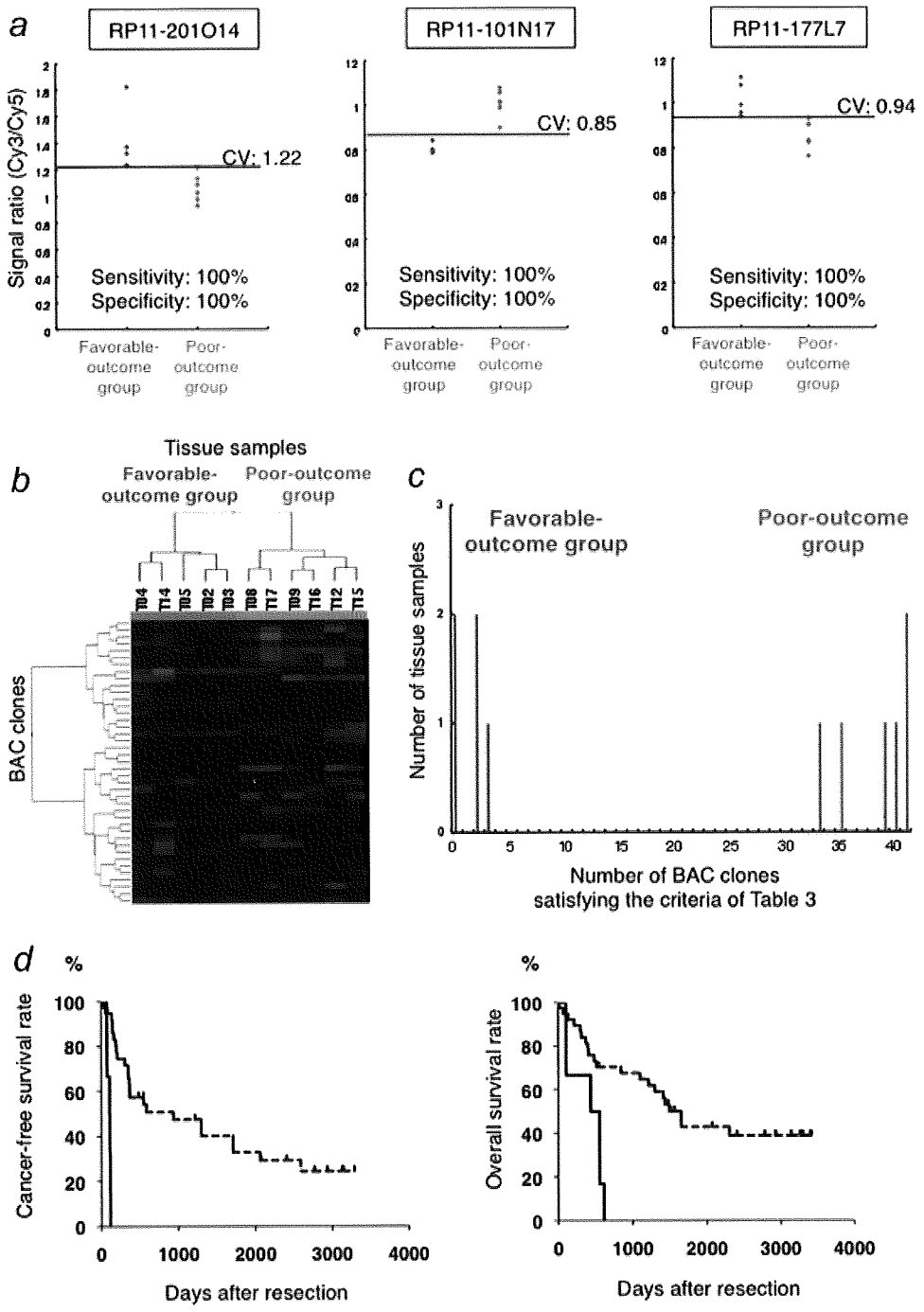

FIGURE 3 – DNA methylation profiles in HCCs associated with patient outcome. (*a*) Scattergrams of the signal ratios in HCCs from patients who survived more than 4 years after hepatectomy (favorable-outcome group, $n = 5$) and HCCs from patients who suffered recurrence within 6 months and died within a year after hepatectomy (poor-outcome group, $n = 6$) in the learning cohort for representative BAC clones, RP11-201O14, RP11-101N17 and RP11-177L7. Using the described cutoff values (CV), the poor-outcome group was discriminated from the favorable-outcome group with 100% sensitivity and specificity. (*b*) By 2-dimensional hierarchical clustering analysis using the 41 BAC clones selected by Wilcoxon test, HCCs in the favorable-outcome group and those in the poor-outcome group in the learning cohort were subclassified in the different subclasses without any error. The cluster trees for tissue samples and BAC clones are shown at the top and left of the panel, respectively. (*c*) Histogram showing the number of BAC clones satisfying the Table III criteria in HCCs of the favorable- and poor-outcome groups in the learning cohort. (*d*) Kaplan-Meier survival curves of all patients with HCCs (T1 to T44). The cancer-free (left panel, $p = 0.000000002$) and overall (right panel, $p = 0.0013$) survival rates of patients with HCCs satisfying the Table III criteria for 32 or more than 32 BAC clones (solid lines) were significantly lower than that of patients with HCCs satisfying the Table III criteria for less than 32 BAC clones (broken lines).

TABLE III – 41 BAC CLONES WHICH COULD DISCRIMINATE HCCS IN POOR-OUTCOME GROUP (P) FROM THOSE IN FAVORABLE-OUTCOME GROUP (F)

BAC clone ID	Location	Cutoff value	DNA methylation status[1]	Sensitivity (%)	Specificity (%)
RP11-89K16	1p35	1.50	F<P	83.3	100.0
RP11-201O14	1p34.3-1p36.13	1.22	F>P	100.0	100.0
RP11-156K6	1p31.1-1p31.3	1.15	F>P	100.0	80.0
RP11-553K8	1q31.2-1q31.3	1.16	F>P	100.0	100.0
RP11-89E10	1q31.3	0.91	F<P	100.0	100.0
RP11-180L21	2p16-2p21	1.29	F>P	100.0	80.0
RP11-90B13	2p14-2p15	1.13	F>P	83.3	100.0
RP11-449B19	2q11.2	0.75	F<P	100.0	80.0
RP11-30M1	2q32.3	1.10	F<P	100.0	100.0
RP11-89B13	2q32.3-2q33.1	1.11	F>P	83.3	80.0
RP11-255O19	3p24.3-3p25	1.08	F>P	100.0	100.0
RP11-421F9	3p24.2a	0.97	F>P	83.3	100.0
RP11-122D19	3p21.2	0.99	F<P	100.0	80.0
RP11-36K8	4q22	0.91	F>P	83.3	100.0
RP11-101N17	4q26	0.85	F<P	100.0	100.0
RP11-177L7	4q32	0.94	F>P	100.0	100.0
RP11-13O14	4q34-4q35	0.88	F<P	83.3	100.0
RP11-88H16	5p14	0.85	F<P	100.0	100.0
RP11-91G9	5q22-5q23	1.45	F<P	83.3	100.0
RP11-79K22	6q16	0.98	F<P	83.3	100.0
RP11-126B8	7q21.3	1.06	F>P	100.0	100.0
RP11-89P11	7q35	0.83	F>P	83.3	100.0
RP11-88N8	8q21.11d	1.02	F>P	100.0	100.0
RP11-85C21	9q33.3-9q34.2	0.95	F<P	83.3	100.0
RP11-714M16	10q26.11-10q26.3	1.00	F<P	100.0	100.0
RP11-48A2	10q26.2	0.69	F<P	100.0	80.0
RP11-206I1	11p11.2	1.20	F<P	100.0	100.0
RP11-35F11	11q12	1.30	F<P	100.0	80.0
RP11-158I9	11q23	1.04	F>P	83.3	100.0
RP11-74I8	12q13	1.13	F<P	100.0	100.0
RP11-167B4	16p13.3	0.97	F>P	83.3	100.0
RP11-368N21	16p11.2-16p12	1.10	F>P	83.3	100.0
RP11-303G21	16q12.1b	0.80	F>P	83.3	100.0
RP11-151M19	16q22	1.05	F>P	100.0	100.0
RP11-135N5	17p13.2	1.00	F>P	100.0	100.0
RP11-398A1	17q11.2d	1.00	F>P	100.0	100.0
RP11-15A1	19q13	1.08	F>P	83.3	100.0
RP11-697B10	19q13.3	0.90	F>P	83.3	100.0
RP11-79A3	19q13.3	1.05	F<P	100.0	100.0
RP11-29H19	20q12	1.00	F>P	100.0	100.0
RP11-36N5	22q11.2	1.15	F>P	83.3	100.0

[1]F>P, when the signal ratio was lower than the cutoff value, the tissue sample was considered to have been obtained from a patient with poor prognosis; F<P, when the signal ratio was higher than the cutoff value, the tissue sample was considered to have been obtained from a patient with poor prognosis.

identifying reproducible indicators for carcinogenetic risk estimation and prognostication. In fact, we have successfully obtained optimal indicators for carcinogenetic risk estimation and prognostication of renal cell carcinomas[26] and urothelial carcinomas (data will be published elsewhere) by BAMCA using the same array as that used in this study.

Our previous studies indicated that alterations of DNA methylation are one of the earliest events of multistage hepatocarcinogenesis and participate in malignant progression of HCCs.[5,7-14,27-29] However, since in previous studies we examined DNA methylation status on only a restricted number of CpG islands or chromosomal loci, it has not yet been clarified whether DNA methylation status on only restricted regions is simply altered at the precancerous stage, or whether genome-wide alterations of DNA methylation status have certain clinicopathological significance. As shown in Panel N of Figure 1*b*, genome-wide DNA methylation alterations (both hypo- and hypermethylation) were confirmed even in noncancerous liver tissue samples obtained from patients with HCCs. The number of BAC clones showing DNA methylation alterations and the degree of DNA methylation alterations were found to increase stepwise from the precancerous stage to the HCC stage (Fig. 1*b* and Table I). This study revealed that alterations of DNA methylation during multistage hepatocarcinogenesis occur in a genome-wide manner. Genome-wide DNA methylation alterations may participate in multistage hepatocarcinogenesis potentially through the induction of chromosomal instability and silencing of tumor-suppressor genes. DNA methylation alterations in noncancerous liver tissue were correlated with the future development of HCCs, suggesting that DNA methylation alterations at the precancerous stage may not occur randomly but are prone to further accumulation of genetic and epigenetic alterations.

Although mass vaccination against HBV has been initiated, this will not have a major impact for many years, as the age at presentation of HBV is older than 50 years mainly in Asia and Africa.[30] The spread of HCV in Japan that occurred in the 1950s and 1960s has resulted in a rapid increase in the incidence of HCC since 1980. In other countries including the United States, where HCV infection spread more recently, an increase in the incidence of HCC is imminent.[31] 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, Wilcoxon test identified BAC clones in which DNA methylation status differed significantly between HBV- and HCV-positive patients with HCCs in both noncancerous liver tissue and cancerous tissue, suggesting that the HBV-related carcinogenetic

TABLE IV – MULTIVARIATE ANALYSIS OF CLINICOPATHOLOGICAL PARAMETERS AND DNA METHYLATION PROFILES ASSOCIATED WITH OVERALL OUTCOME IN PATIENTS WITH HCCS

Parameters	Hazard ratio (95% CI)	χ^2	p
Histological differentiation			
Well differentiated	1 (Reference)	0.031	0.8594
Moderately or poorly differentiated	0.817 (0.088-7.616)		
Portal vein tumor thrombi			
Negative	1 (Reference)	2.095	0.1478
Positive	4.474 (0.588-34.033)		
Intrahepatic metastasis[1]			
Negative	1 (Reference)	0.090	0.7647
Positive	1.248 (0.292-5.336)		
Multicentricity[1]			
Negative	1 (Reference)	1.499	0.2209
Positive	0.328 (0.055-1.955)		
The criteria of Table 3			
Satisfying for less than 32 BAC clones	1 (Reference)	4.997	0.0254
Satisfying for 32 or more BAC clones	4.466 (1.202-16.585)		

CI, confidence interval.
[1]In patients with multiple lesions, whether the lesions other than the main tumor from which tissue samples were obtained for this study were intrahepatic metastases of the main tumor or second primary lesions was judged by microscopic observation of hepatectomy specimens based on the previously described criteria.[35]

pathway may result in distinct DNA methylation profiles. These findings are in accordance with a previous report showing that HBV-related proteins can induce DNA methylation alterations.[32]

The effectiveness of surgical resection for HCC is limited, unless the disease is diagnosed early at the asymptomatic stage. Therefore, surveillance at the precancerous stage will become a priority. To reveal the baseline liver histology, microscopic examination of liver biopsy specimens is performed in patients with HBV or HCV infection prior to interferon therapy.[33,34] Therefore, carcinogenetic risk estimation using such liver biopsy specimens will be advantageous for close follow-up of patients who are at high risk of HCC development. Because even subtle alterations of DNA methylation profiles at the precancerous stage are stably preserved on DNA double strands by covalent bonds, they may be better indicators for risk estimation than mRNA and protein expression profiles that can be easily affected by the microenvironment of precursor cells.

The present genome-wide analysis revealed DNA methylation profiles that were able to discriminate noncancerous liver tissue obtained from patients with HCCs from normal liver tissue and diagnose it at high risk of HCC development in the learning set. The sensitivity and specificity in the validation set were 95.8 and 96.2%, respectively, and the criteria listed in Table II were validated. For carcinogenetic risk estimation using liver biopsy specimens obtained prior to interferon therapy, DNA methylation profiles actually associated with carcinogenesis should be discriminated from those associated with inflammation and/or fibrosis. Therefore, we first omitted potentially insignificant BAC clones associated only with inflammation and/or fibrosis and focused on BAC clones for which DNA methylation status was inherited by HCCs from the precancerous stage (Groups I, II, III and IV). In fact, it was confirmed that there were no significant differences in the number of BAC clones satisfying the criteria in Table II between noncancerous liver tissue samples showing chronic hepatitis and noncancerous liver tissue samples showing cirrhosis, not only in the learning set ($p = 0.542$) but also in the validation set ($p = 0.128$), indicating that our criteria were not associated with the degree of inflammation or fibrosis. In addition, the average numbers of BAC clones satisfying the criteria in Table II were significantly lower in liver tissue of patients without HCCs (V1 to V7) than in noncancerous liver tissue of patients with HCCs (N1 to N39), even though the patients from whom V1 to V7 were obtained were infected with HBV or HCV. Therefore, our criteria not only discriminate noncancerous liver tissue obtained from patients with HCCs from normal liver tissue but may also be applicable for classifying liver tissue obtained from patients who are followed up because of HBV or HCV infection, chronic hepatitis or cirrhosis into that which may generate HCCs and that which will not. Our criteria are applicable to both patients with chronic hepatitis and liver cirrhosis, although liver cirrhosis is known to show a more pronounced tendency to lead to HCC development than chronic hepatitis.[20] We intend to validate the reliability of such risk estimation prospectively using liver biopsy specimens obtained prior to interferon therapy from a large cohort of patients. On the basis of the present data, we now consider it justifiable to propose that clinicians can apply a portion of biopsy cores for this type of prospective study.

Because a sufficient quantity of good-quality DNA can be obtained from liver biopsy specimens, PCR-based analyses focusing on individual CpG sites are not always required. Although cut-off values should be modified for widely available standardized reference DNA, array-based analysis that overviews aberrant DNA methylation in each BAC region is immediately applicable to routine laboratory examinations. Moreover, because DNA methylation status of CpG sites is often regulated in a coordinated manner in each individual large region on chromosomes,[13,14,25] an overview of the DNA methylation tendency (hypo- or hypermethylation) in the whole BAC region can be a more reproducible diagnostic indicator than one focusing on individual CpG sites.

The present genome-wide analysis revealed DNA methylation profiles that were able to discriminate a poor-outcome group from a favorable-outcome group. Correlation between the DNA methylation profiles and both cancer-free and overall survival rates of patients with HCCs (Fig. 3*d*) validated the criteria in Table III. Prognostication based on our criteria may be promising for supportive use during follow-up after surgical resection, because multivariate analysis revealed that our criteria can predict overall patient outcome independently of parameters observed in hepatectomy specimens that are already known to have prognostic impact.[20] Such prognostication using liver biopsy specimens obtained before transarterial embolization, transarterial chemoembolization and radiofrequency ablation may be advantageous even to patients who undergo such therapies. The reliability of such prognostication needs to be validated again prospectively in surgically resected specimens or biopsy specimens.

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Transarterial chemotherapy alone versus transarterial chemoembolization for hepatocellular carcinoma: A randomized phase III trial☆

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***Background/Aims*: Transcatheter arterial chemoembolization (TACE) is a combination of transarterial infusion chemotherapy (TAI) and embolization, and has been widely used to treat patients with hepatocellular carcinoma (HCC). However, since the impact of adding embolization on the survival of patients treated with TAI had never been evaluated in a phase III study, we conducted a multi-center, open-label trial comparing TACE and TAI to assess the effect of adding embolization on survival.**

***Methods*: Patients with newly diagnosed unresectable HCC were randomly assigned to either a TACE group or a TAI group. Zinostatin stimalamer was injected into the hepatic artery, together with gelatin sponge in the TACE group and without gelatin sponge in the TAI group. Treatment was repeated when follow-up computed tomography showed the appearance of new lesions in the liver or re-growth of previously treated tumors.**

***Results*: Seventy-nine patients were assigned to the TACE group, and 82 were assigned to the TAI group. The two groups were comparable with respect to their baseline characteristics. At the time of the analysis, 51 patients in the TACE group and 58 in the TAI group had died. The median overall survival time was 646 days in the TACE group and 679 days in the TAI group ($p = 0.383$).**

***Conclusions*: The results of this study suggest that treatment intensification by adding embolization did not increase survival over TAI with zinostatin stimalamer alone in patients with HCC.**

Keywords: **Zinostatin stimalamer; Survival benefit; Overall survival; Lipiodol emulsion; Gelatin sponge**

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Abbreviations: HCC, hepatocellular carcinoma; AFP, α-fetoprotein; TACE, transarterial chemoembolization; TAI, transarterial infusion chemotherapy; SMANCS, zinostatin stimalamer; CT, computed tomography; TE, therapeutic effect; SMA, styrene maleic acid; NCS, neocarzinostatin.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and a major cause of cancer mortality [1]. Although the screening of populations with a high risk of HCC using ultrasonography and serum α-fetoprotein (AFP) measurements have recently increased the number of candidates for effective local treatments such as hepatic resection and local ablation therapy, many patients exhibit HCCs that are unsuitable for local treatments at the time of the initial diagnosis or at the time of recurrence after local treatment. In these patients, transcatheter arterial chemoembolization (TACE) has been widely used, because TACE induces a marked antitumor effect in HCC.

Several randomized controlled studies have been conducted to assess the survival benefit of TACE compared with conservative therapy [2–9], and an improvement in survival with TACE has been shown in two recent phase III studies [7,8], in both of which TACE was compared with no treatment, and in two meta-analyses [10,11]. However, the impact of adding embolization on the overall survival of patients treated with transarterial infusion chemotherapy (TAI) has never been evaluated in a randomized controlled phase III study. We conducted a multi-centre, open-label trial to compare the effects of TACE and TAI alone to clarify the possible benefits of treatment intensification using embolization in addition to infusion chemotherapy. In this study, zinostatin stimalamer (SMANCS) was selected as the chemotherapeutic agent for use with both TACE and TAI. SMANCS is a lipophilic anti-cancer agent that dissolves in lipiodol to form a stable solution, retaining selectively in HCC. TAI with SMANCS has been widely used in clinical practice to treat patients with advanced HCC in Japan, because it has been reported to have fewer deleterious effects than TACE, especially on liver function, and to have an antitumor effect superior to TAI with other water-soluble agents in non-randomized studies [12,13].

2. Methods

Consecutive new patients with HCC were eligible if they had no indications for resection and/or local ablation therapy. The diagnosis was confirmed histologically and/or clinically using angiography and computed tomography (CT). Each patient was required to meet the following criteria: intrahepatic lesions that showed tumor staining by angiography and those in which the total size was less than 50% of the entire liver; adequate hematological function (white blood cells $\geqslant 3000/mm^3$, platelets $\geqslant 50{,}000/mm^3$, and hemoglobin $\geqslant 9.0$ g/dL), adequate hepatic function (serum total bilirubin $\leqslant 2.0$ mg/dL, serum albumin $\geqslant 3.0$ g/dL, serum AST [aspartate aminotransferase] $\leqslant 5$ times the upper limit of normal, serum ALT [alanine aminotransferase] $\leqslant 5$ times the upper limit of normal); adequate renal function (serum creatinine < the upper limit of normal, and serum blood urea nitrogen < the upper limit of normal); an Eastern Cooperative Oncology Group performance status of 0–1; an age of between 20 and 74 years of age; technically eligible for intra-arterial therapy; and written informed consent. Patients were excluded if they met any of the following criteria: a history of allergy to iodine-containing agents and/or contrast material; concomitant malignancy; a history of anti-cancer treatment for HCC; extrahepatic metastasis or tumor thrombus in the portal vein and/or the hepatic vein; intrahepatic arteriovenous shunting; ascites and/or pleural effusion not controlled by diuretics; pregnant or lactating woman and fertile patients not using effective contraception; myocardial infarction within the previous 6 months; or any serious physical and/or mental conditions. The study was performed in accordance with the Declaration of Helsinki, and approved by the ethics committee of each participating center. The study was investigator-designed and investigator-driven, and it received no support from any pharmaceutical companies.

Patients who met the eligibility criteria were provisionally registered before undergoing angiography. After confirmation of technical eligibility and reconfirmation of indications for the protocol intra-arterial treatments in regard to tumor status, including the number of tumors, their vascularity, and vascular invasion based on the angiographic findings, confirmatory registration was completed by each participating investigator. Central randomization to either a TACE group or TAI group was performed by using a telephone randomization system with stratification according to AFP level and treatment center. First, participants were stratified according to AFP level into a group with levels less than 400 ng/mL and a group with levels of 400 ng/mL or more. The group with AFP levels less than 400 ng/mL was further stratified according to treatment center. Randomization was achieved using a computer-generated allocation by permutation of blocks in equal proportions.

The treatments were performed by the participating investigators at 10 Japanese centers. Zinostatin stimalamer (SMANCS; Astellas Pharm Inc., Tokyo, Japan)/lipiodol emulsion (1 mg/mL) was injected slowly under fluoroscopic monitoring into the artery feeding the HCC using a catheter in a superselective manner in both the TACE and the TAI groups. The emulsion was prepared by suspending the SMANCS in lipiodol and shaking just before injection. The volume of the emulsion, up to a maximum of 6 mL (containing 6 mg of SMANCS), was adjusted according to the tumor size and tumor distribution. In the patients in the TACE group, gelatin sponge particles were utilized after the injection of the SMANCS-lipiodol emulsion. Treatment was repeated when a follow-up CT examination showed new lesions in the liver or re-growth of previously treated tumors. Treatment was discontinued if the size of the tumor treated had increased by more than 25% one month after the previous treatment; if there were any vascular contraindications, any exclusion criteria, or any severe adverse effects (defined as grade 4 leucopenia, grade 4 neutropenia, or grade 3 febrile leucopenia/neutropenia, a serum total bilirubin elevation of more than or equal to 5.0 mg/dL, a serum creatinine elevation of more than or equal to 1.5 times the upper normal limit, or grade 3 or greater non-hematological toxicity excluding nausea, vomiting, anorexia, pain, fever, hyperglycemia, fatigue, and serum transaminase elevation), or if the patient so requested.

The primary outcome measure was survival calculated from the date of randomization. Secondary outcome measures were tumor response and toxicity. Antitumor effect was evaluated by CT performed 1 month after the completion of treatment and every 3–4 months thereafter according to the response evaluation criteria proposed by the panel of experts of the Liver Cancer Study Group of Japan [14], which resemble the criteria proposed by the European Association for the Study of the Liver (EASL) Panel of Experts on HCC [15]. Tumor size was measured using the sum of the products of the perpendicular longest diameters of all measurable lesions. In the response evaluation criteria, lipiodol accumulation in the tumors is regarded as an indication of necrosis because significant positive correlations have been reported between lipiodol accumulation observed on CT images and the necrotic regions in resected tumors examined pathologically after TACE and after TAI with SMANCS [13,16,17]. Therapeutic effect V (TE V) is defined as the disappearance or 100% necrosis of all tumors, TE IV as a more than a 50% reduction in tumor size and/or more than 50% necrosis, and TE III as a more than 25% reduction and/or more than 25% necrosis. TE I is defined as a more than 25% increase in tumor size. TE II is defined as disease not qualifying for classification as TE V, IV, III, or I. The serum AFP level of each patient was also measured 1 month after treatment and every 3–4 months thereafter. Toxicity was assessed according to the criteria of the Japan Society for Cancer

Therapy [18], whose criteria are essentially the same as the WHO criteria [19]. The follow-up period was defined in the protocol as 2 years after the enrollment of the last patient.

2.1. Statistical analysis

Based on our previous phase II studies, in which we reported a 2-year survival rate of 80% in patients treated with TACE and of 60% in patients treated with TAI, 70 patients were required in each group to achieve a 90% power to detect superior survival in the TACE group by using a two-sided alpha level of 5% [13,20]. After sensitivity analyses of combinations of survival parameters, we targeted the recruitment of 80 patients in each group. All analyses were conducted based on the intention-to-treatment principle. Survival curves were calculated from the day of randomization using the Kaplan–Meier method and compared using the log-rank test. Comparisons between groups were made using the Wilcoxon test for continuous variables and Fisher's exact test for categorical variables. Analyses were conducted using SAS ver. 8.

3. Results

Between October 1999 and June 2003, 222 patients were provisionally enrolled in the study at 10 Japanese centers (Fig. 1). Sixty-one of the 222 patients were excluded because of ineligibility for intra-arterial treatment based on the angiographic findings or withdrawal of consent; too few or too many definitive tumors that required reconsideration of the treatment strategy (46), tumor thrombus in the portal vein (3), tumors without sufficient tumor staining (3), intrahepatic arteriovenous shunting (2), allergy to contrast material (1), and withdrawal of consent (6). The most common reason for exclusion was having too few definitive tumors (37/61). The patients who were excluded because of having too few definitive tumors had been considered eligible based on the detection of several small hypervascular nodules on pre-treatment CT imaging that were diagnosed as HCC, but treatment had been switched to local ablation therapy or monitoring based on angiographic findings suggesting that the nodules represented dysplastic nodules. All of the patients who withdrew consent requested TACE for their treatments. The remaining 161 patients were allocated randomly to the TACE group (79 patients) or the TAI group (82 patients). Follow-up was continued through to June 17, 2005, two years after the enrollment of the last patient. Although the baseline data of some eligible patients did not meet the eligibility criteria after they were enrolled, the study protocol permitted initiation of treatment when according to the judgment of the investigator, treatment could be performed safely. Two patients had a pre-treatment serum albumin level that was below the eligibility criterion, but there were no statistically significant differences in baseline characteristics between the two groups (Table 1).

3.1. Treatment

The total number of treatment courses was 170 with a mean of 2.2 courses per patient (range, 1–9 courses) in the TACE group and 193 with a mean of 2.4 courses (range, 1–6 courses) in the TAI group. Eight patients in the TACE group and two patients in the TAI group were scheduled for the continuation of protocol treatment as of the date of the last follow-up. The remaining 71 patients in the TACE group and 80 patients in the TAI group had discontinued treatment. The reasons for treatment discontinuation were similar in both groups (Table 2).

3.2. Survival

At the time of the final analysis, 51 patients in the TACE group and 58 patients in the TAI group had died. Seven patients in the TACE group and eight in the TAI group were lost to follow-up after the cessation of protocol treatment. The median overall survival time was 646 days in the TACE group and 679 days in the TAI group. The estimated 2-year survival rate was 48.2% for the TACE group and 49.6% for the TAI group. No significant difference in survival was seen between the two groups ($p = 0.383$, Fig. 2).

3.3. Antitumor effect

The tumor response on CT was determined in 156 patients (77 in the TACE group and 79 in the TAI group). In the TACE group, there were 8 TE V, 29 TE IV, 31 TE III, 7 TE II, and 2 TE I responses. In the TAI group, there were 5 TE V, 22 TE IV, 30 TE III, 21 TE II, and 1 TE I response. The proportion of patients with TE V or IV among the measurable patients was not significantly different between the TACE group and the TAI group (48.1% vs. 34.2%; $p = 0.11$). There was no significant difference between the two groups in the proportion of patients with a pre-treatment AFP level > 200 ng/mL whose AFP level decreased by more than half (16.5% vs. 13.4%; $p = 0.66$).

3.4. Toxicity

Hematological toxicity was relatively mild and transient in both groups, although 2 patients (2.6%) in the TACE group and 3 (3.7%) in the TAI group developed grade 4 thrombocytopenia (Table 3). Major non-hematological toxicities were hyperbilirubinemia, elevations in serum liver enzymes, fever and abdominal pain in both groups. The grade of elevated ALT levels was significantly higher in the TACE group than in the TAI group, although there were no significant differences in any other toxicities between the two groups. No treatment-related death was observed in either group. Two patients in the TACE group and six in the TAI group manifested a grade 1–2 allergic reaction immediately after injection of the SMANCS-lipiodol emulsion. Shivering in the form of trembling of the whole body lasting

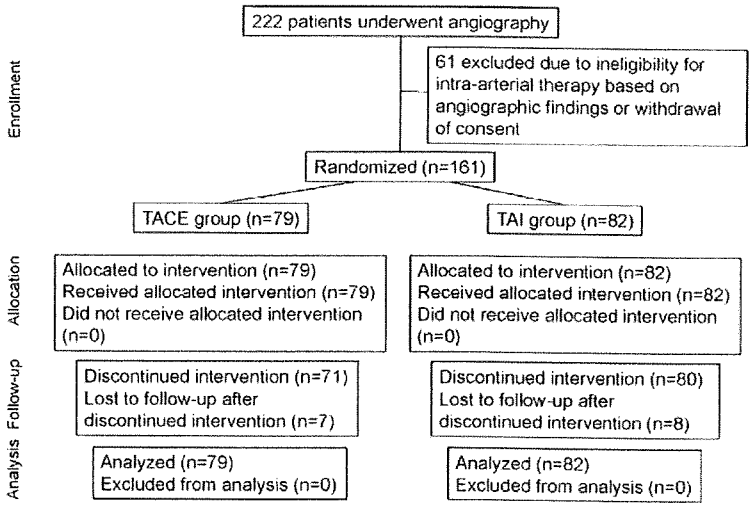

Fig. 1. Study flow diagram.

several minutes after the injection was noted in 12 patients in the TACE group and 14 patients in the TAI group, and it was thought to have been caused by SMANCS.

4. Discussion

We initiated this randomized study in 1999 because the impact of adding embolization on overall survival

Table 1
Baseline characteristics.

No. of patients		79	82
Age, year	Median (range)	65.0 (42–74)	67.0 (44–74)
Gender	Male	61 (77.2%)	70 (85.4%)
Performance status	0	76 (96.2%)	77 (93.9%)
	1	3 (3.8%)	5 (6.1%)
HBsAg	+	11 (13.9%)	7 (8.5%)
HCVAb	+	57 (72.2%)	60 (73.2%)
Alcohol abuse	+	33 (41.8%)	28 (34.1%)
Albumin, g/dL	Median (range)	3.6 (2.8–4.6)	3.6 (3.0–4.6)
Total bilirubin, mg/dL	Median (range)	1.0 (0.4–2.0)	0.9 (0.3–2.0)
AST, IU/L	Median (range)	63 (16–243)	69 (18–232)
ALT, IU/L	Median (range)	60 (12–184)	60 (10–213)
Prothrombin time, %	Median (range)	80 (41–129)	78.5 (43–111)
Platelet count, $\times 10^9$/L	Median (range)	110 (48–280)	120 (44–290)
	$<100 \times 10^9$/L	29 (36.7%)	28 (34.1%)
Ascites	+	3 (3.8%)	3 (3.7%)
Stage*	I	2 (2.5%)	4 (4.9%)
	II	18 (22.8%)	17 (20.7%)
	III	28 (35.4%)	25 (30.5%)
	IV-A	31 (39.2%)	36 (43.9%)
Tumor distribution	Unilateral	40 (50.6%)	36 (43.9%)
	Bilateral	39 (49.4%)	46 (56.1%)
Maximum tumor diameter, mm	Median (range)	35 (10–330)	35 (12–350)
Number or tumors	1	13 (16.5%)	11 (13.4%)
	2–5	43 (54.4%)	52 (63.4%)
	6	23 (29.1%)	19 (23.2%)
AFP, ng/ml	Median (range)	68.3 (2.8–79170)	93.8 (3.1–40,000)
	≥400 ng/ml	26 (32.9%)	27 (32.9%)
Serum creatinine, mg/dL	Median (range)	0.7 (0.4–1.3)	0.8 (0.5–1.1)

Abbreviations: AFP, α-fetoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibody.
Alcohol abuse was defined as ethanol intake ≥80 g/day for ≥5 years.
* According to the staging system of the Liver Cancer Study Group of Japan.

Table 2
Reasons for treatment discontinuation.

	TACE group		TAI group	
Ineffectiveness of protocol treatment	10	13%	10	12%
Adverse event caused by protocol treatment				
Elevation of serum creatinine level	1	1%	1	1%
Elevation of alkaline phosphatase level	2	3%	2	2%
Dyspnea	0	0%	1	1%
Hypotension	1	1%	1	1%
Shivers	0	0%	1	1%
Abdominal pain	0	0%	2	2%
Ascites	1	1%	0	0%
Deterioration before subsequent protocol treatment				
Extrahepatic metastasis	4	5%	7	9%
Portal vein thrombosis	6	8%	3	4%
Tumor rupture	2	3%	0	0%
Ascites	9	11%	11	13%
Liver dysfunction	9	11%	11	13%
Poor general condition	2	3%	2	2%
Other disease	1	1%	6	7%
Technical problem preventing subsequent protocol treatment	13	16%	9	11%
Patient's request	10	13%	11	13%
Indication for tumor ablation	1	1%	2	2%
Protocol treatment ongoing	7	9%	2	2%
Total	79		82	

for patients with advanced HCC treated with TAI had not been fully evaluated and because the efficacy of TACE was still being debated at that time in various countries. Moreover, several differences in TACE methods had been noted between clinical practice in East Asian countries, including Japan, and randomized studies conducted in Europe, including differences in the selection of embolization materials, anti-cancer agents and their doses, in treatment intervals, and in patient characteristics such as tumor stage and liver function. In this study, in which our TACE method was introduced, we selected SMANCS as a chemotherapeutic agent for both TACE and TAI. SMANCS is an anti-cancer drug that has been approved by the Japanese government for administration with lipiodol into the artery feeding HCC, and TAI with SMANCS has been widely used instead of TACE in many hospitals because of its favorable antitumor effect and mild toxicity profile.

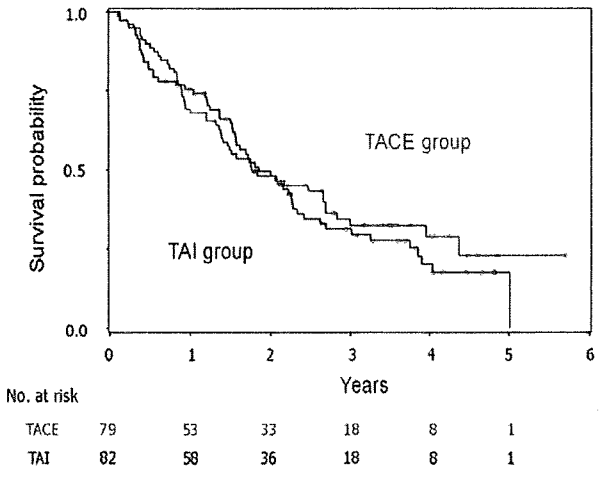

Fig. 2. Survival curves in the TACE group and in the TAI group.

This study did not confirm any significant survival advantage of TACE over TAI. A German group also reported that adding transient occlusion using degradable starch microspheres improved neither tumor response nor survival for patients treated with TAI using cisplatin and doxorubicin in a randomized phase II trial [21]. Llovet and Bruix showed that survival benefits were identified with TACE (doxorubicin or cisplatin) but not with embolization alone in their meta-analysis [11]. The survival benefit of TACE can be ascribed to the combination of embolization and chemotherapy.

It could be argued that the absence of a significant difference in survival rates between the TACE group and TAI group in this study is attributable to our methodological strategy for selecting SMANCS as the anti-cancer agent, because the agent may have produced favorable results in the TAI group. SMANCS is a high molecular weight chemical conjugate of a synthetic copolymer of styrene maleic acid (SMA) and the anti-cancer antibiotic protein, neocarzinostatin (NCS) [22,23]. SMANCS is lipophilic and dissolves in lipiodol to form a stable emulsion (SMANCS-lipiodol), which prevents rapid washout of SMANCS into plasma from trapped lipiodol. Furthermore, because of the enhanced permeability of the tumor vasculature and/or poor lym-