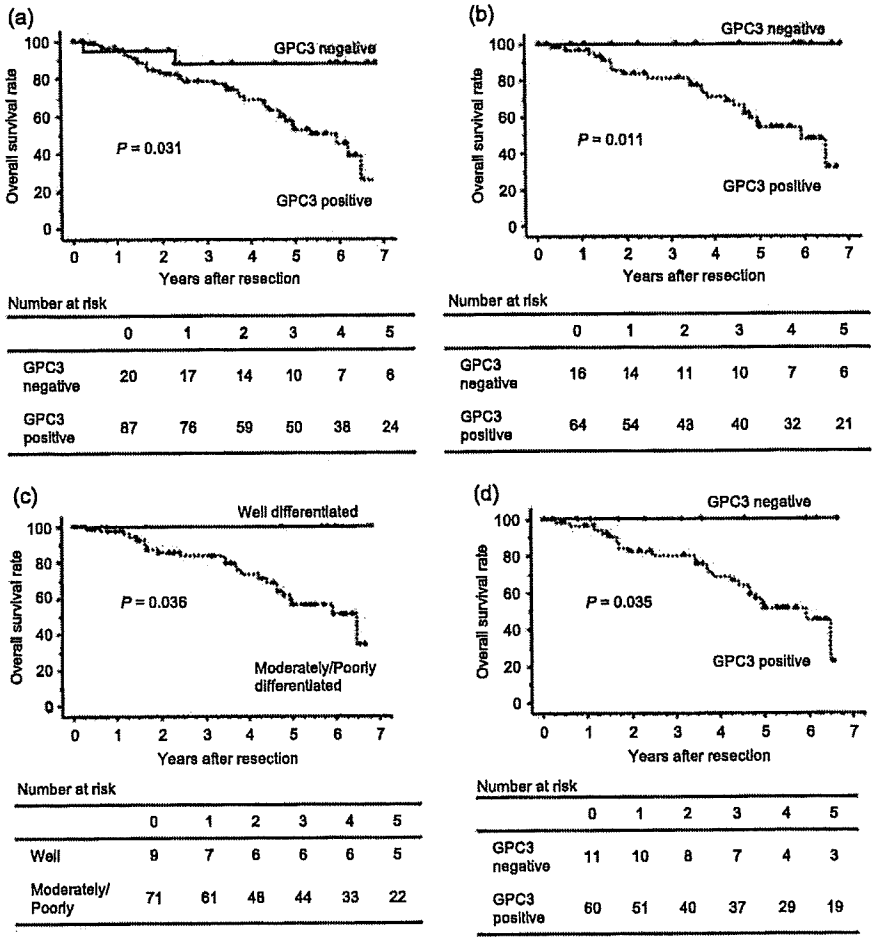

Fig. 2. Overall survival curves for the 107 hepatocellular carcinoma (HCC) patients stratified into those with glypican (GPC)-3-positive and GPC3-negative HCC. (a) Overall survival of patients with GPC3-positive HCC was shorter than those with GPC3-negative HCC ($P = 0.031$). (b) Overall survival curves in 80 of 107 HCC patients with initial treatment who underwent hepatectomy with positive and negative GPC3 expression. Patients with GPC3-positive HCC had a lower 5-year survival than those with GPC3-negative HCC ($P = 0.011$). (c) Overall survival curves in the 71 HCC patients with initial hepatectomy who exhibited well- and moderately and poorly differentiated HCC on histopathological examination. The 5-year survival rate was lower in the moderately and poorly differentiated GPC3-positive HCC than in the corresponding GPC3-negative HCC ($P = 0.036$). (d) Overall survival curves in the 71 initial treatment patients who underwent hepatectomy and exhibited moderately and poorly differentiated HCC on pathological examination with positive and negative GPC3 expression. The 5-year survival rate was lower in the GPC3-positive HCC patients than in the GPC3-negative HCC patients ($P = 0.035$).

Table 2. Prognostic factors for overall survival by univariate and multivariate analyses

Variable	No. patients	Univariate analysis		Multivariate analysis		
		5-year survival rate (%)	*P*-value	RR	95% CI	*P*-value
Age (years) (≥65/<65)	51/56	65.8/53.4	0.531			
Sex (male vs female)	85/22	56.1/72.7	0.403			
HBsAg (positive vs negative)	29/78	51.0/62.3	0.011	1.14	0.31–4.16	0.844
HCV (positive vs negative)	62/45	66.7/46.4	0.004	2.41	0.75–7.69	0.138
ICG R15 (%) (≥15 vs <15)	50/57	70.3/46.8	0.047	0.69	0.31–1.54	0.362
AFP (ng/mL) (≥50 vs <50)	45/62	49.1/65.1	0.132			
PIVKA-II (mAU/mL) (≥700 vs <700)	30/77	35.0/65.6	0.016	1.91	0.730–5.02	0.188
Tumor occurring (first vs recurrence)	80/27	62.8/50.2	0.019	1.83	0.78–4.31	0.167
No. tumors (solitary vs multiple)	75/32	65.7/42.7	0.009	3.53	1.41–8.00	0.006
Resection (trisegmentectomy, lobectomy, or segmentectomy/subsegmentectomy or partial resection)	29/78	36.5/67.1	0.005	1.71	0.52–5.60	0.374
Operation time (min) (>300 vs ≤300)	49/58	43.9/72.3	0.053			
Intraoperative blood loss (mL) (≥1300 vs <1300)	42/65	42.3/68.8	0.097			
Perioperative transfusion (present vs absent)	54/53	49.6/66.5	0.599			
Tumor size (mm) (>50 vs ≤50)	38/69	51.5/62.5	0.154			
Histological differentiation (well vs moderately and poorly)	12/95	77.8/56.4	0.102			
pStage (I vs II/III)	41/66	64.2/56.5	0.071			
Portal vein involvement (present vs absent)	47/60	64.9/58.5	0.369			
Hepatic vein involvement (present vs absent)	10/97	44.4/60.5	0.060			
Bile duct involvement (present vs absent)	12/95	20.0/62.7	0.004	0.94	0.31–2.85	0.912
Intrahepatic metastasis (present vs absent)	24/83	29.0/66.6	0.001	3.57	1.13–10.50	0.027
Non-cancerous lesion (cirrhosis vs non-cirrhosis)	40/67	53.6/61.9	0.232			
GPC3 staining (positive vs negative)	87/20	54.5/87.7	0.025	5.26	1.13–24.39	0.034

AFP, alpha-fetoprotein; CI, confidence interval; HBsAg, hepatitis B s antigen; HCV, hepatitis C virus; ICG-R15, indocyanine green-retention at 15 min; PIVKA-II, protein induced by vitamin K absence II; RR, relative risk; UICC, International Union against Cancer.

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In this study, the patients who were HCV positive, had higher ICG-R15 values, or portal vein involvement showed longer survival times, especially the patients who were HCV-positive or had higher ICG-R15 values, showed statistical significance in the univariate analysis. However, there was no statistical significance in these variables in the multivariate analysis. The reasons for these contradictive results in the univariate analysis are unclear.

In contrast, subgroup analysis did not reveal any significant difference in the disease-free survival rate between the GPC3-positive and GPC3-negative HCC patients (data not shown). The rate of recurrence in patients after surgery was 63.8% within the first 2 years after surgery among the previously treated patients in this study. Tumor recurrence in the GPC3-positive HCC patients occurred earlier than that in the GPC3-negative HCC patients until 9.7 months after the surgery among the patients who had received previous treatment. Two mechanisms of postoperative recurrence of HCC have been suggested: one is intrahepatic metastasis in the residual liver in a metachronous manner, and the other is multicentric hepatocarcinogenesis based on chronic hepatitis.[20–23] Some authors have suggested that early recurrence arises most often from intrahepatic metastases, whereas late recurrence is more likely to be multicentric in origin. Poon *et al.* and Portolani *et al.* reported that tumor factors like neoplastic vascular infiltration, but not host factors, were linked to early recurrence, whereas the risk of late recurrence was dependent on the underlying liver status.[21,22] These results indicate that GPC3 expression may indicate a high risk of intrahepatic recurrence.

Most of the GPC3 expression patterns in HCC cells showed the cytoplasmic pattern. There was no case that showed only the membrane pattern. Almost half of the HCC cases showed the mixed pattern (cytoplasm and membrane) and the other half showed only the cytoplasmic pattern.

There was no statistical significance between the mixed pattern (cytoplasm and membrane) and cytoplasmic pattern ($P = 0.297$) in Kaplan–Meier survival analysis. The functional difference between cytoplasmic GPC3 and membrane GPC3 is unknown, so further investigations are needed to clarify whether the different localization of staining has a different significance.

In addition to the investigation of its role as a prognostic indicator, a phase I clinical trial of a GPC3-derived peptide vaccine for advanced HCC is now underway; GPC3 is an ideal target for this therapy because it is more effective in patients with increased expression of GPC3, which is frequently observed in the later stages of HCC, as shown in the present study. The poor prognosis of patients with GPC3-positive HCC also prompted us to develop a strategy of anticancer immunotherapy,[24,25] that is, we may expect the effect of hepatocarcinogenesis prevention after surgery in patients with GPC3-positive HCC.

In summary, our study evaluated the prognostic significance of GPC3 expression at the protein level in clinical tissue specimens of HCC. The overall survival rate was significantly poorer in patients with elevated GPC3 expression in the tumor than in those with lower levels of GPC3 expression. Further functional characterization of GPC3 may be expected to lead to a better understanding of the molecular mechanisms underlying the development and progression of HCC.

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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.

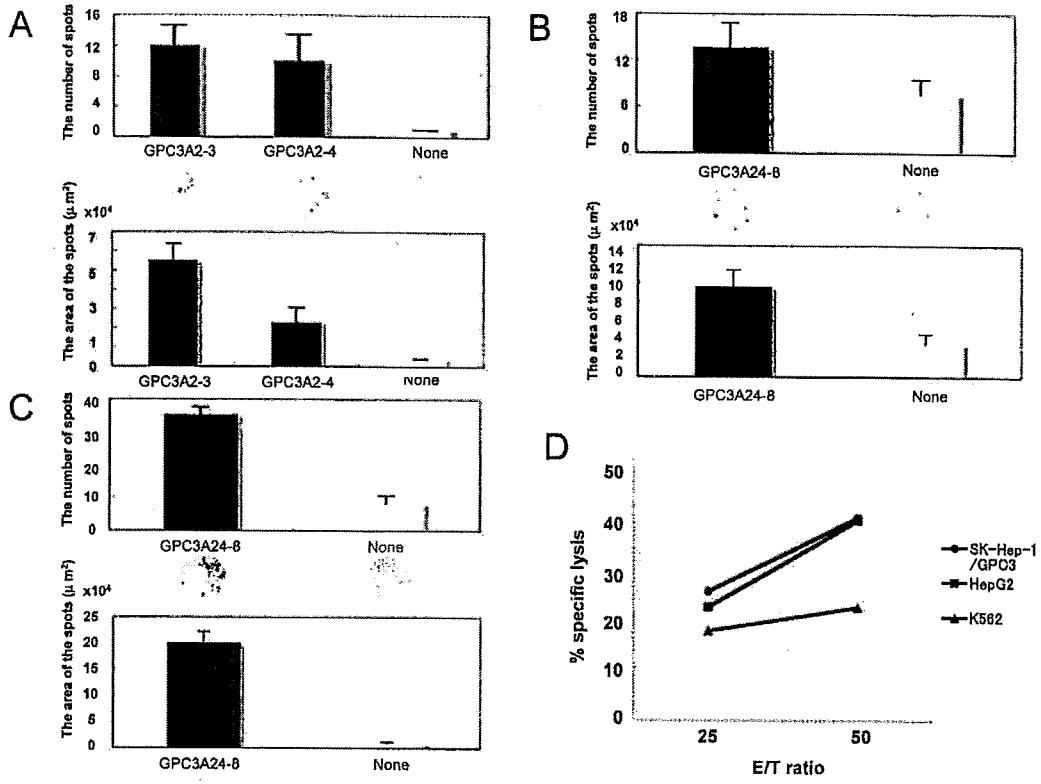

Figure 1. Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2+ or HLA-A24+ CH and LC patients and the cytotoxicity of the CTLs induced by stimulation with the GPC3 (A2-3) peptide. GPC3-specific CD8+ T cells were detected in the chronic hepatitis [(A), HLA-A2+ CH4 patient; (B), HLA-A24+ CH5 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 $H\text{-}2K^d$, 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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Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer

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Abstract. Primary liver cancers are classified into three types based on their morphology and cytogenetic characteristics hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined hepatocellular and cholangiocarcinoma (CHC). It is often difficult to distinguish these liver tumors. Glypican-3 (GPC3) is serological and histochemical marker of hepatocellular carcinoma. In order to separate these three types of liver cancers, we analyzed the GPC3 expression in 85 liver resection specimens, including 46 HCCs, 28 ICCs and 11 CHCs. GPC3 immunohistochemical staining was used to distinguish HCC from ICC by comparing with the conventional biomarker, α-fetoprotein (AFP). The immunostaining of GPC3 was identified in 78.3% (36/46) of HCCs, 60% (9/15) of well differentiated, 88.9% (16/18) of moderately differentiated and 84.6% (11/13) of poorly differentiated HCCs. It was negative in the ICCs. We confirmed that GPC3 expression is specific to HCC component (8/11, 72.7%) but few samples also showed weakly in ICC component (2/11, 18.2%) of CHC sections among 11 cases compared with HCC biomarkers including AFP and hepatocyto paraffin 1 (HepPar1), and ICC biomarkers cytokeratin (CK) 7 and CK19. Three cases in which the macroscopic features resembled ICC did not express GPC3 even in the pathological HCC component. Most (10/11, 91%) of the pathological cholangiocarcinoma components in CHC showed positive staining for CK7 and CK19. The results of this study suggest that GPC3 is a biomarker that is sensitive and specific to HCC component of CHC, and CK7 and CK19 are markers for pathological cholangiocarcinoma component of CHC.

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Abbreviations: HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; CHC, combined hepatocellular and chorangiocarcinoma; GPC3, glypican-3; AFP, α-fetoprotein; HepPar1, hepatocyto paraffin 1; CK, cytokeratin; CC, cholangiocarcinoma; cp, component

Key words: hepatocellular carcinoma, intrahepatic cholangiocarcinoma, combined hepatocellular and chorangiocarcinoma, glypican-3, CK7, CK19, immunohistochemical analysis

Introduction

Liver cancer is one of the common malignancies that are rapidly increasing throughout the world. Primary liver cancers are classified into three types based on their morphology and cytogenetic characteristics, hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined hepatocellular and cholangiocarcinoma (CHC). HCC is hepatocyto-origin, and ICC is from the epithelium of the intrahepatic bile duct. CHC is a rare type of liver cancer with features of both hepatocellular and biliary differentiation (1-3). The pathological structure of CHC is composed of hepatocellular element showing bile production, an intercellular bile canaliculi or trabecular growth pattern and cholangiocellular component showing mucin production or gland formation.

Because of their rapid growth rate and the lack of accurate ways of diagnosis in the early stages, the prognosis and the survival rate for liver cancer patients remain poor. Currently, ultrasound sonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and histopathological examination for tumor biopsy are used for diagnosis. However, distinguishing the three different primary liver tumors is often a challenging task in diagnosis, for which immunohistochemical analysis for specific antigens is a helpful tool: α-fetoprotein (AFP) and hepatocyto paraffin 1 (HepPar1) for HCC (4-8) and cytokeratin (CK) 7 and CK19 for ICC (9-11).

Glypican-3 (GPC3) was discovered as a potential serological and histochemical marker whose expression is specific for HCC (12-16). GPC3 belongs to glypican family that is a group of heparan sulfate proteoglycans linked to the outer surface of cell membrane through a glycosylphosphatidylinositol anchor (17). In mammals, six members of GPCs have been reported, GPC1 to GPC6. GPCs are released from the cell surface by a lipase called Notum to regulate the signaling of Wnts, Hedgehogs, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) (18-25). Depending on the cellular context, their function can be stimulatory or inhibitory activity, or signaling. The expression of GPC3 is detected in placenta and fetal liver, but not in other normal organs. During hepatic carcinogenesis, GPC3 have been reported to reappear in HCC and to be released into serum (12,13,15,26). Its expression is also detected in melanoma (27-29). The functions of GPC3 in cancer cells are still unclear.

In this study, we examined whether immunohistochemical analysis for GPC3 can be used to distinguish HCC from ICC, if so, how effectively GPC3 can be detected, compared to other biomarkers that are conventionally used. We demonstrate that distinguishing HCC from ICC by detecting the expression of GPC3 enables more accurate diagnosis.

Materials and methods

Case selection. We selected 85 cases of liver tumors from the surgical pathology files from 1992 to 2006 of National Cancer Center Hospital East, Kashiwa, Chiba, Japan. The cases included 46 primary HCCs, 28 ICCs, and 11 CHCs that underwent hepatectomy. All identifiers were eliminated to protect patients' identities. Size of the tumor and any clinicopathologic factors (age, sex and grade of tumor) were matched between HCC and ICC. The 46 cases of HCCs occurred in 33 men and 13 women with a mean of age at 65.3 years (range, 44-80 years). HCC was subclassified into well (n=15), moderately (n=18), and poorly (n=13) differentiated types according to the World Health Organization classification criteria. The 28 cases of ICC consisted of 18 men and 10 women. Their mean age was 65.7 years (range, 51-82 years). All 28 resected cases of ICC were confirmed by hematoxylin-eosin (H.E.) staining.

The 11 cases of CHC included 7 men and 4 women with a mean age of 62.5 years (range, 47-76 years). All CHCs were pathologically confirmed after surgery.

Tissue samples. Liver tissue sections were retrieved from the files of the Department of Pathology in our institution. All liver specimens were prepared from surgically resected tumors and adjacent parenchyma. They were fixed in 10% formalin and paraffinized for routine histological examination.

Immunohistochemical staining procedure. Six-micrometer-thick sections were made from the paraffin-embedded blocks. Subsequently the sections were deparaffinized in xylene and rehydrated through ethanol to water. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 20 min. For antigen retrieval, Sections were heated in 10 mM citrate buffer (pH 6.0) with microwave for 15 min in a water bath at 95°C. Only for CK7 immunostaining, sections were digested by Proteinase K (DakoCytomation, Carpenteria, CA) for 5 min at room temprature. Slides were then allowed to cool down. The prediluted primary antibodies, monoclonal anti-GPC3 (dilution 1:300, 1G12; Biomosaics, Inc., Burlington, VT), anti-AFP (dilution 1:400, DakoCytomation), anti-HepPar1 (dilution 1:100, DakoCytomation), anti-CK7 (dilution 1:100, DakoCytomation), and CK19 (dilution 1:200, DakoCytomation) were added to cover each slide, and the slides were incubated for 2 h at room temperature. Slides were washed 3 times in phosphate-buffered saline (PBS)/Tween for 5 min each. Mouse Envision Polymer (DakoCytomation) was used as a secondary antibody for 30 min at room temperature followed by washes in PBS/Tween 3 times for 5 min each. Diaminobenzidine chromagen (DakoCytomation) was added to each slide and incubated for 2 min. Slides were washed in distilled water, counterstained with hematoxylin and dehydrated in xylene. To analyze GPC3 expression, the immunohistochemical results were classified according to the number of positive cells as follows: -, negative (<10%); ±, weakly positive (10-30%); + positive (>30%). To validate the data in GPC3 as a marker for HCC, parallel staining for AFP of 46 cases were further analyzed. For 11 CHC cases, AFP, HepPar1, CK7 and CK19 were stained and compared with GPC3 staining pattern.

The slides were examined independently by 3 observers (Shirakawa H, Kuronuma T and Nakatsura T) and then collectively by 2 more pathologists (Hasebe T and Nakano M).

Statistical analysis. Differences in proportion were tested by the χ^2 test. Differences in the means of each subgroup were tested using the Student's t-test. P-value of <0.05 was considered statistically significant.

Results

GPC3 was present in 80% of HCC and negative in ICC. In order to examine the levels and pattern of GPC3 expression, 46 cases of HCC and 28 cases of ICC were immunohistochemically analyzed. GPC3 was detected in 36 cases (78%) of HCC (Fig. 1a), and no expression of GPC3 was found in any of the ICC patients (Fig. 1b). The GPC3 staining was diffused throughout (Fig. 1c) or localized in a granular pattern in the cytoplasm (Fig. 1d). In other cases, GPC3 was observed at the plasma membrane (Fig. 1e). Previously GPC3 is shown to bind to the cell membrane (16), however, those cases with membranous GPC3 had staining in the cytoplasm as well, but there was no case of GPC3 located only at the plasma membrane. When sensitivity of GPC3 was evaluated, 36 cases (78%) were positive for GPC3 when only 16 cases (35%; P<0.0001) were stained for AFP in HCC suggesting that GPC3 is more sensitive than AFP. Thus, GPC3 was confirmed to be specific and sensitive to HCC compared to AFP.

GPC3 expression increased in moderately and poorly differentiated HCC. In terms of GPC3 expression and tumor

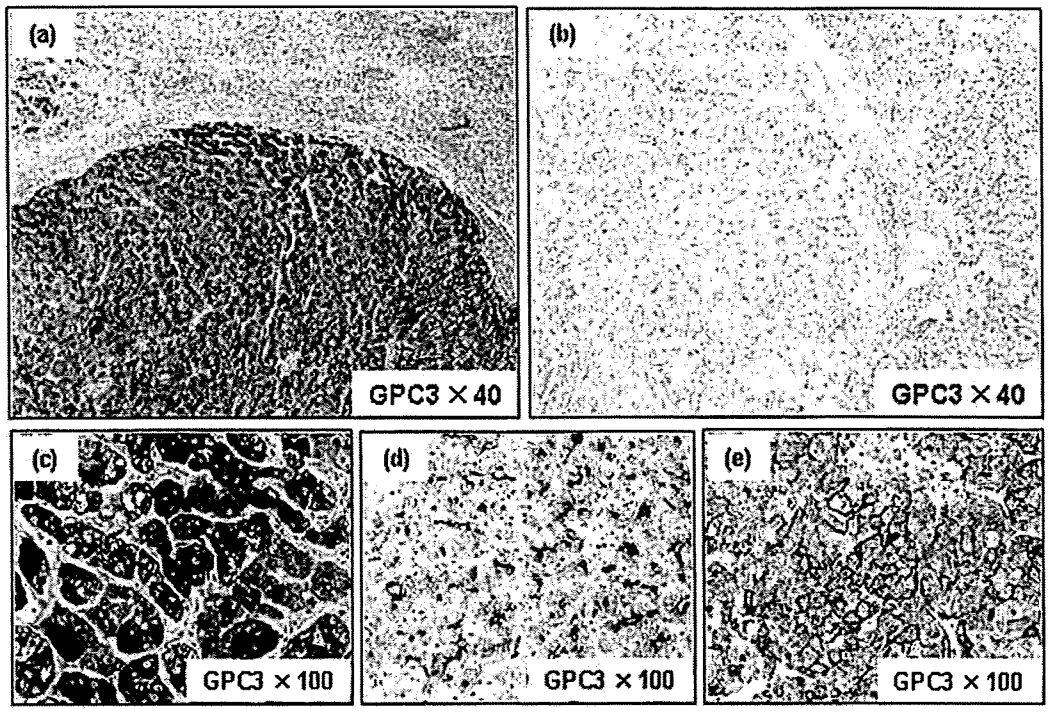

Figure 1. GPC3 expression was specific to HCC and absent in ICC. Immunohistochemical detection of GPC3 expression in HCC (a) and ICC (b) (magnification, x40). Immunostaining patterns of HCC: (c) diffuse in cytoplasm, gulanular in cytoplasm (d), and membranous (e).

Table I. Correlation of positive for GPC3 staining and tumor grade.

Grade of tumor	HCC					ICC		
		GPC3						
	No. of case	-	±	+	positivity	No. of case	GPC3 positivity	P-value
Well-differentiated	15	6	5	4	9 (60%)	8	0 (0%)	
Moderately differentiated	18	2	4	12	16 (89%)	10	0 (0%)	
Poorly differentiated	13	2	5	6	11 (85%)	10	0 (0%)	
Total	46				36 (78%)	28	0 (0%)	<0.0001

-, negative (<10%); ±, weakly positive (10-30%); +, positive (>30%).

differentiation level, GPC3 was expressed in 9 (60%) of 15 well differentiated, 16 (89%) of 18 moderately differentiated and in 11 (85%) of 13 poorly differentiated HCC (Table I). AFP was expressed in 3 (20%) of 15 well differentiated, 6 (33%) of 18 moderately differentiated and in 7 (54%) of 13 poorly differentiated HCC (data not shown). The expression level of GPC3 was lower in well differentiated HCC than in the other HCC grades, though the difference was not statistically significant (well- vs. moderately differentiated: P=0.054, well- vs. poorly differentiated: P=0.150). Thus, GPC3 expression is also a good indicator for malignancy levels.

GPC3 expression was observed specifically in pathological HCC component in CHC. There are discrepancies between preoperative diagnosis and pathological findings for CHC patients. Diagnostic results and the expression of tumor markers of 11 CHC patients are summarized in Table II. Initial diagnosis was carried out by H.E. staining. Among these 11 patients, 7 patients (63.6%) were diagnosed as HCC and 3 (27.3%) were ICC. Only 1 patient (9%) of the 11 CHC was correctly diagnosed as CHC. To seek the possibility to use GPC3 immunostaining to detect HCC component (cp) in CHC, combination of antibodies against GPC3, AFP, HepPar1, CK7 and CK17 were used. In addition to AFP, HepPar1 is frequently used as marker for HCC (4-8) and CK 7 and CK19 for ICC (9-11).

Among 11 CHC cases, 4 cases preoperatively diagnosed as HCC were chosen to represent the collision and transitional type of CHCs based on the macroscopic features

Table II. Correlation of immunostaining varieties and pathological components of CHC.

Pt. no.	Preoperative diagnosis	Macroscopic diagnosis	Pathological hepatocellular carcinoma component					Pathological cholangiocarcinoma component				
			GPC3	AFP	HepPar1	CK7	CK19	GPC3	AFP	HepPar1	CK7	CK19
1	HCC	CHC	+	+	-	+	+	-	-	-	-	-
2	HCC	HCC	+	-	-	-	-	-	-	+	+	+
3	HCC	HCC	+	-	+	-	-	±	-	-	+	+
4	CHC	HCC	+	+	+	-	-	±	-	-	+	+
5	HCC	CHC	+	-	+	-	-	-	-	-	+	+
6	HCC	CHC	+	-	-	-	-	-	-	+	+	+
7	ICC	CHC	±	-	-	±	+	-	-	-	+	+
8	HCC	HCC	+	+	-	-	-	-	+	-	+	+
	Total ±		8/8	3/8	3/8	3/8	2/8	2/8	1/8	2/8	7/8	7/8
	positive rate (%)		100	38	38	38	25	25	13	25	88	88
9	ICC	ICC	-	-	-	-	-	-	-	-	+	+
10	HCC	ICC	-	-	-	+	±	-	-	-	+	+
11	ICC	ICC	-	-	-	+	+	-	-	-	+	+
	Total ±		0/3	0/3	0/3	2/3	2/3	0/3	0/3	0/3	3/3	3/3
	positive rate (%)		0	0	0	67	67	0	0	0	100	100

-, negative (<10%); ±, weakly positive (10-30%); +, positive (>30%); HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; CHC, combined hepatocellular and cholangiocarcinoma; GPC3, glypican-3; AFP, α-fetoprotein; HepPar1, hepatocyto-paraffin 1; CK, cytokeratin; CC, cholangiocarcinoma.

in cut surface. In Fig. 2, macroscopic observation and the immunostained histological sections are shown. These sections include 2 elements with pathological HCC cp forming bile production and trabecular growth pattern by eosinophilic staining and cholangiocarcinoma (CC) cp forming mucin production or gland formation by basophilic staining. Cases 1-8 were GPC3 positive, and cases 9-11 were negative for GPC3 in the HCC cp. Macroscopic, histological and immunohistochemical features of cases 2, 6, 8 and 10 are shown in Fig. 2a, b, c and d. Case 2 had greenish white and yellow nodules within the same tumor mass in the cut surface. HCC subtypes such as simple nodular and confluent multinodular type exist. Case 2 exhibited the features of HCC with multinodular type (Fig. 2a-i). Pathological diagnosis by H.E. staining revealed CHC pathologically (Fig. 2a-ii and -iii), which was so-called 'collision'-type tumor as reported by Goodman *et al* (30). A 'collision'-type tumor is coincidental occurrence of HCC and CC within the same tumor mass (31). GPC3 was positive (Fig. 2a-iv), but AFP and HepPar1 were not detected in HCC cp (Fig. 2a-v and -vi). Although HepPar1 is generally used as HCC marker, it was unexpectedly stained in CC region as well as CK7 and CK19 (Fig. 2a-vii and -viii).

Case 6 showed pale and lobulated phenotype in the cut surface macroscopically (Fig. 2b-i), and pathological diagnosis was also confirmed by H.E. staining (Fig. 2b-ii and -iii). This was so-called 'transitional' type tumor (30). A 'transitional' type tumor has an area of HCC that appears to transform into CC (31). GPC3 was stained in pathological HCC cp (Fig. 2b-iv) where AFP was negative (Fig. 2b-v). The HCC region was surrounded by pathological CC cp with the staining for CK7 (Fig. 2b-vii). HepPar1 and CK19 were detected in the same region with CC cp (Fig. 2b-vi and -viii). HepPar1 stained the CC cp as in case 2. The immunoreactivity of CK19 was not consistent with that of CK7.

Case 8 was diagnosed as HCC similarly to cases 2 and 6, but mixed tumor masses with white and gray in the cut surface were observed (Fig. 2c-i and c-ii). Both GPC3 and AFP were positive in HCC cp (Fig. 2c-iv and -v). HepPar1 was stained in CC cp (Fig. 2c-vi). CK7 and CK19 were positive in CC cp (Fig. 2c-vii and -viii), especially CK19 was more specific for CC cp than CK7. These three cases (cases 2, 6 and 8) indicated that detecting GPC3 can compensate for AFP and enhance the ability to identify the presence of HCC cp in CHC.

Cases 9, 10 and 11 were negative for GPC3 expression in several tumors. Macroscopically, they had the features of ICC with irregular shaped, white solid tumor masses. As an example, case 10 is shown in Fig. 2d. Althogh case 10 was diagnosed as HCC preoperativerly, it showed macroscopic freatures of ICC with the presence of abundant fibrous stroma and indistinct tumor margin (Fig. 2d-i). This case was later diagnosed as CHC based on the pathological examination (Fig. 2d-ii and d-iii). GPC3, AFP and HepPar1 were not detected in either HCC cp or CC cp (Fig. 2d-iv, -v, and -vi). CK7 was stained diffusely in the tumor (Fig. 2d-vii), and CK19 expression was more specific in CC cp than CK7 (Fig. 2d-viii). These 3 cases showed positive staining

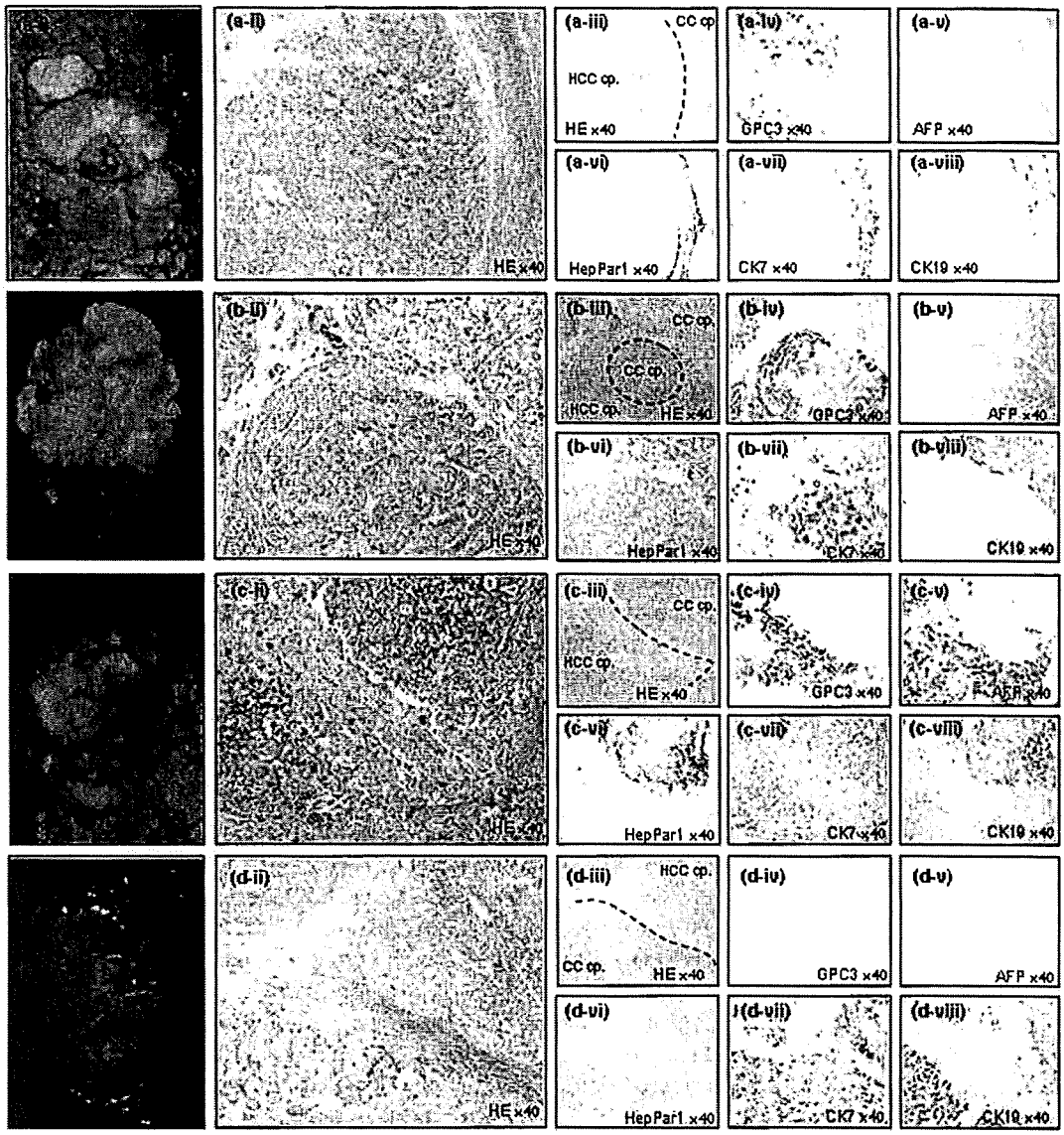

Figure 2. Macroscopic, histological and immunohistochemical features of four cases of CHC, a, case 2; b, case 6; c, case 8; d, case 10 in Table II. (a-i) Macroscopic feature in cut surface of case 2 tumor. (a-ii) The histological structure can be also devided into 2 types. HCC component showed expansive growth oppressing the cholangiocarcinoma component. (a-iii) Collision border between hepatocellular carcinoma and cholangiocarcinoma component are indicated as dots. The tumor cells within mainly hepatocellular carcinoma component showed only expression of GPC3 (a-iv) without expression of AFP (a-v). In the opposite side, the glandular area with cholangiocarcinoma component shows HepPar1 (a-vi), CK7 (a-vii) and CK19 expression (a-viii). (b-i) Case 6 shows macroscopic CHC feature in tumor cut surface that was suspected out HCC preoperatively. (b-ii) The histological cholangiocarcinoma component forming trabeculae with columnar appearance was surrounded by HCC component forming hepatoid structure. (b-iii) A dotted line is a boundary of HCC in the H.E. staining. The tumor cells within transitional region were positive for GPC3 (b-iv), CK 7 (b-vii) and CK 19 (b-viii). The difference was recognized between hepatocellular carcinoma component and cholangiocarcinoma component because GPC3 positive area encircled the CK7 area. The expressions of AFP (b-v) and HepPar1 (b-vi) were not observed. (c-i) Though case 8 was also suspected to be HCC preoperatively, the macroscopic features showed atypical HCC with mixed white and gray and indistinct tumor border. (c-ii) The cholangiocarcinoma component was obviously composed of structural gland formation. (c-iii) Collision area was distinguished histopathologically by a dotted line. The tumor cells of HCC component showed not only GPC3 (c-iv) but also AFP expression (c-v). In the glandular area of cholangiocarcinoma component, HepPar1 was expressed (c-vi), but CK7 not at all (c-vii) and CK19 shows weak positive expression (c-viii). (d-i) Case 10 shows macroscopic ICC features in tumor cut surface that was suspected as HCC preoperatively. (d-ii) The histological structure can be devided into 2 types with cholangiocarcinoma component forming trabeculae with columnar appearance and HCC component forming hepatocellular structures. (d-iii) A dotted line is a boundary of HCC in the H.E. GPC3 (d-iv), AFP (d-v) and HepPar1 (d-vi) were not stained, but CK7 (d-vii) and CK19 (d-viii) stained the cholagiocarcinoma component.

for CK7 and CK19 in CC cp, but not AFP or HepPar1 in HCC cp. Therefore, accuracy of CHC diagnosis can be achieved by combination of multiple tumor markers in addition to morphological characteristics: GPC3 that is specific for pathological HCC cp of CHC, and CK7 and CK19 that are specific for pathological CC cp of CHC.

Discussion

The diagnosis for HCC, ICC and CHC has been routinely performed by histopathological examination. Additionally, diagnosis of HCC is done by supplementary immunohistochemical analysis for AFP and HepPar1. Until now, though

the sensitivity is limited, AFP has been regarded as the most useful marker for HCC (4,32-34). HepPar1 is also widely used for HCC to distinguish between primary HCC and ICC. However, both markers are limited for the ability to discriminate different levels of malignancy in HCC because its sensitivity drops substantially in poorly differentiated HCC, and it does not discriminate between benign and malignant liver cancers (35). As these biomarkers frequently results in misdiagnosis, in this study, we showed that GPC3 is more sensitive to detect HCC compared to AFP. Due to the fact that GPC3 was downregulated in ICC (36), GPC3 may help to separate HCC from ICC.

CHC is the least common primary cancer of the liver but followed by an aggressive growth, it tends to metastasize to many organs leading to significantly poorer prognosis than HCC and ICC (31,37,38). Correct diagnosis leads to both appropriate treatment and better outcome for the patients. Nishie, *et al* reported that one third (nine of 27 cases) of patients with CHC were correctly diagnosed by enhanced computed tomography (39). In our study, only one of the 11 (9.1%) patients with CHC was correctly diagnosed before operation without fine needle aspiration biopsy. The difficulty to pathologically distinguish CHC from HCC and ICC comes from glandular or pseudoglandular structures in HCC and solid or trabecular patterns in CC (37,38). We believe that combination with histopathological examination with GPC3 immunostaining and radiological examination can bring an accurate diagnosis and improved clinical therapies for the patients leading to a better prognosis.

We showed that the immunostaining for GPC3 is specific for HCC patients and not detected in ICC patients. This confirmed that detecting GPC3 may improve the method to diagnose CHC. Of the 11 cases of CHC, 8 displayed GPC3 expression in restricted area of HCC cp. We demonstarated that immunohistochemical staining of GPC3 in liver tumor helps to recognize the pathological HCC cp more precisely. GPC3 expression was observed with high frequency in the HCC cp compared with AFP and HepPar1. HepPar1 was unexpectedly stained in CC cp, but this has been observed previously as well (7,40). This could be due to a transition from HCC to ICC where HepPar1 is one of the molecules that is downregulated at later stages in the process. CK7 and CK19 have been already reported as good markers of biliary epithelial differentiation (41). These were highly expressed in pathological CC cp (10/11, 91%) in CHC. The positive immunoreactivity of CK19 was more distinct than that of CK7 whose staining was weaker. Our immunohistochemical data disclosed that GPC3 can be a better marker specific for HCC leading to a better confirmation for HCC component of CHC as well as for HCC. Moreover, it provided evidence of the biologic behavior of such combined tumors, which are phenotypically and genetically leaning toward either ICC with predominant biliary differentiation or HCC with hepatocellular differentiation (42,43).

Employing multiple tumor markers may also allow the accurate diagnosis of CHC containing both hepatocellular and biliary differentiation. Concerning sensitivity and specificity, the combination of GPC3 for HCC cp and CK19 for ICC cp seems to be useful in the diagnosis of liver cancer. For CHC, GPC3 positive/CK19 negative profile suggests HCC, GPC3 positive/CK19 positive indicates CHC, and GPC3 negative/CK19 positive essentially rules out HCC and suggests the possibility of CC or CHC.

We developed a new anti-cancer immunotherapy with GPC3 as a target (44-47), and the phase I clinical trial of GPC3-derived peptide vaccination for advanced HCC is now on going. Because this new immunotherapy is not indicated for ICC, immunohistochemical staining of GPC3 is a useful method to select eligible patients. Furthermore, if CHC would be justified as a target of our immunotherapy in future, immunohistochemical analysis for GPC3 expression is indispensable for the process of patient selection.

GPC3 is expressed in the group of cells that are AFP-positive and/or CK7/19-positive in injured livers with activation of oval cell compartment; an indication for liver repair and regeneration (48). In addition, CK7, CK19 and AFP are frequently expressed in biliary epithelial cells (49,50) and in immature fetal hepatoblasts (51,52). Liver progenitor cells originate from the canal of Hering, lined by both hepatocytes and biliary ductular epithelial cells (53). It is not clear whether GPC3 is expressed in hepatic embryonic progenitor cells or cancer stem cells, but GPC3 may be a marker for hepatic progenitor/stem cells. In CHC cases of 2, 3 and 4, GPC3, CK7 and CK19 coincided in the regions of HCC and CC. Although HCC and ICC are two different kinds of primary liver malignancies arising from different cell types as hepatocytes and cholangiocytes, co-localization of GPC3 and CK7/19 suggest that the CHC is originated from progenitor or oval cell. In addition, case 6 showed an HCC lesion with GPC3 positive immunostaining surrounded by CC (Fig. 2b). This finding suggests that GPC3-positive HCC tumor cells are derived from GPC3-negative CC mass. Moreover, we predict from the fact that GPC3 is expressed in embryonic liver and downregulated after birth in normal liver but reappears in cancer is due to its regulatory role in proliferative and dedifferentiated cells, like cancer cells that acquired a progenitor- or cancer stem cell-like characteristics.

In summary, we confirmed that GPC3 is a marker sensitive and specific for HCC, but not ICC. Moreover, we revealed that GPC3 was expressed specifically in the HCC cp in the CHC. Therefore, GPC3 is a molecule that is significant not only in clinical but also biological field. It is clinically an important biomarker that can be used for accurate diagnosis leading to a better treatment and prognosis. Also, biologically, it may be an indicator for the identity and the origin of the cancer cells.

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Identification of the H2-K^d-restricted cytotoxic T lymphocyte epitopes of a tumor-associated antigen, SPARC, which can stimulate antitumor immunity without causing autoimmune disease in mice

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We previously reported that the secreted protein acidic and rich in cystein (SPARC) was overexpressed in melanoma in humans, and the serum SPARC level was useful as a novel tumor marker for melanoma. SPARC was also reported to be overexpressed in various human cancers. In this study, we asked whether SPARC-specific cytotoxic T lymphocytes (CTL) could induce antitumor immunity to SPARC-expressing tumor in mice or not as a preclinical study of SPARC-directed anticancer immunotherapy. Because of similarities in the structural motifs of major histocompatibility complex-binding peptides between H2-K^d and HLA-A24 (*A*2402*), the most common human leukocyte antigen class I allele in the Japanese population, we attempted to identify the H2-K^d-restricted SPARC epitope for CTL in BALB/c mice and we found that the mouse SPARC$_{143-151}$ (DYIGPCKYI) and SPARC$_{225-234}$ (MYIFPVHWQF) peptides could induce peptide-reactive CTL in BALB/c mice without causing autoimmune diseases. The immunization of mice with SPARC$_{225-234}$ peptide-pulsed bone marrow-derived dendritic cells (BMDC) inhibited the growth of s.c. inoculated mouse mammary cancer cell line, N2C, expressing SPARC and these mice lived longer than the mice immunized with peptide-unpulsed BMDC. In conclusion, our study indicated that SPARC peptide-based cancer immunotherapy was effective and safe at least in a mouse tumor prevention model. (*Cancer Sci* 2009; 100: 132–137)

Secreted protein acidic and rich in cystein (SPARC), also called osteonectin or BM-40, was identified in 1981 as a major non-collagenous constituent of bovine bone.[1] SPARC is a matricellular glycoprotein secreted by many cells types,[2] that modulates cellular interaction with extracellular matrix during tissue remodeling.[3] SPARC plays an important role in wound repair, cell proliferation, cell migration, morphogenesis, cellular differentiation and angiogenesis.[2–5] Targeted disruption of the *SPARC* gene in mice results in early cataractogenesis,[6,7] osteopenia[8] and curly tails.[2]

SPARC was reported to be overexpressed in various human cancers,[9–12] including primary and metastatic melanomas. The overexpression of SPARC by melanoma cells was associated with an invasive phenotype *in vivo*.[13,14] We previously reported that serum SPARC levels observed in melanoma patients were higher than those observed in healthy donors.[15] Increased level of serum SPARC was observed in 33% of all melanoma patients, irrespective of the clinical stages and even in the sera of patients with stage 0 *in situ* melanoma. Moreover, the combined use of SPARC and glypican-3, which was reported by us as a novel tumor marker for melanoma,[16] enabled a 66.2% detection rate of melanoma patients at an early stage (0–II). Thus, SPARC is considered to be a useful tumor marker for melanoma. However, the usefulness of SPARC as a target for cancer immunotherapy has not been previously investigated.

One of the actual methods of the immunotherapy for cancer was vaccination of epitope peptides derived from tumor-associated antigen. Recently, several investigators have reported the effect of peptide vaccination on cancer.[17,18] However, the effect was partial, and more useful antigens were required. We previously identified several tumor-associated antigens, including glypican-3, heart shock protein 105, proliferation potential-related protein, KM-HN-1, cell division cycle associated 1 and cadherin-3/P-cadherin.[16,19–27] In addition, we identified several HLA-A2- or HLA-A24-restricted cytotoxic T lymphocytes (CTL) epitopes derived from these antigens. The immunization with these epitopes was effective in a mouse tumor model and some of these were applied to phase I clinical trials of cancer immunotherapy.

In this study, we identified the H2-K^d-restricted and SPARC-derived CTL epitopes useful for SPARC-directed immunotherapy, and the vaccination with these peptides elicited effective antitumor immunity with no evidence of autoimmune diseases in mice.

Materials and Methods

Cell lines. Mouse cancer cell lines, B16, B16F1, B16F10, EL4, MCA, NIH3T3, 3LL, BALB/3T3, Colon26, A20, RL male1 and MethA were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan). A mouse mammary cancer cell line, N2C, was provided by Dr Sangaletti Sabina of the National Institute of Tumors (Milan, Italy). T2K^d, a TAP-deficient T2 cell transfected with *K^d*-gene expression vector, was provided by Dr Paul M. Allen of Washington University School of Medicine (St Louis, MO, USA). These cells were maintained *in vitro* in RPMI-1640 or Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Expression of H2-K^d was examined with flow cytometry analysis by using a fluorescein isothiocyanate (FITC)-conjugated antimouse H2-K^d-specific antibody (clone SF1-1.1, mIgG2ak; BD Biosciences Pharmingen, San Diego, CA, USA).

Mice. Seven-week-old female BALB/c mice (H-2^d), purchased from Charles River Laboratories Japan (Yokohama, Japan),

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were kept in the Center for Animal Resources and Development of Kumamoto University and handled in accordance with the animal care policy of Kumamoto University.

Identification of the CTL epitopes of SPARC in BALB/c mice. Mouse SPARC-derived peptides (purity, >90%), sharing the amino acid sequences with human SPARC and carrying binding motifs for both H2-K^d and HLA-A24 (*A*2402*), were searched for using BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD, USA), and we purchased four kinds of peptides (Table 1) from AnyGen (Gwangju, Korea). Identification of the CTL epitopes of SPARC was done using BALB/c mice as described.[19] In brief, the BALB/c mice were immunized i.p. with bone marrow-derived dendritic cells (BMDC) pulsed with the mixture of SPARC candidate peptides once a week for 2 weeks. Seven days after the last immunization, CD4$^-$ spleen cells collected from immunized BALB/c were stimulated with syngeneic BMDC pulsed with each peptide *in vitro*. Then, 6 days later, CD4$^-$ T cells were collected from the culture and the CTL-producing γ-interferon (IFN-γ) was detected by an enzyme-linked immunospot (ELISPOT) assay. Moreover, after 5 days culture *in vitro* under the same conditions, cytotoxic activities of these cells directed against target cells were tested by standard 6-h ^{51}Cr release assays as described previously.[19]

Table 1. SPARC-derived peptides conserved between human and mouse SPARC and predicted to bind to H2-K^d and HLA-A24

Designation	Position	Subsequence residue listing	Binding score	
			H2-K^d	HLA-A24
SPARC-1	143–151	DYIGPCKYI	4000	75
SPARC-2	123–131	HFFATKCTL	1382	20
SPARC-3	161–170	EFPLRMRDWL	960	30
SPARC-4	225–234	MYIFPVHWQF	120	210

These peptides were searched for using BIMAS (Bioinformatics and Molecular Sections, Center for Information Technology, NIH, Bethesda, MD, USA) software (http://www-bimas.cit.nih.gov/molbio/hla_bind/).

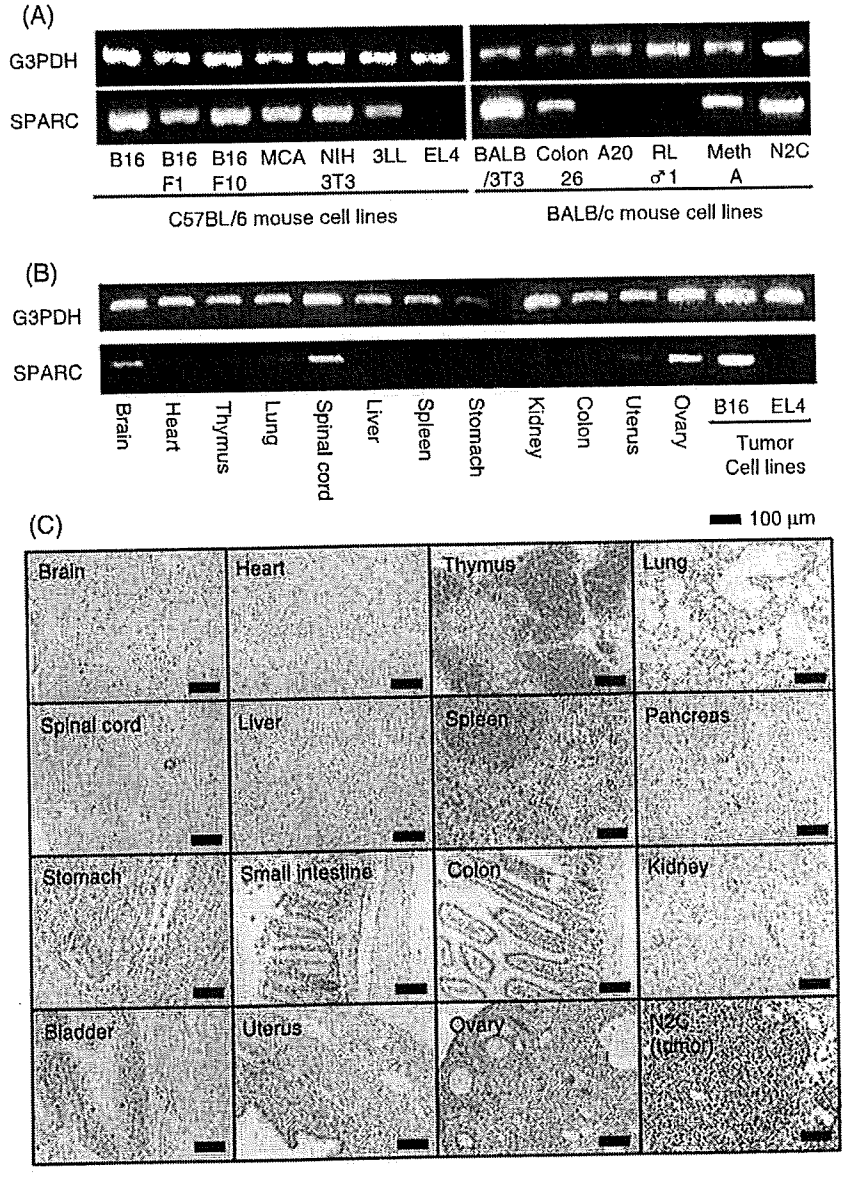

Fig. 1. The reverse transcription polymerase chain reaction (RT-PCR) analysis of *SPARC* mRNA expression in mouse cancer cell lines and normal tissues. (A) Various C57BL/6 and BALB/c mouse-derived cancer cell lines showed strong expression of *SPARC* except EL4, A20 and RL male 1. (B) Mouse *SPARC* gene was expressed in the normal tissues including ovary, spinal cord, brain and uterus by the RT-PCR analysis. (C) SPARC was not detected in the normal tissues by immunohistochemical staining.

Histological and immunohistochemical analysis. Immunohistochemical detections of SPARC was done as described previously.[21] We purchased Human, Normal Organs, and Cancers, Tissue Array, BC4 (SuperBioChips Laboratories, Seoul, Korea) for immunohistochemical analysis. Immunohistochemical staining of CD8 or CD4 was done as described previously.[28]

***In vivo* tumor prevention model.** BMDC (5×10^5 cells/body) loaded with or without SPARC-$4_{225-234}$ peptide or phosphate-buffered saline (PBS) were transferred i.p. into BALB/c mice ($n = 8$, each group) twice on days –14 and –7, and N2C cells (3×10^4/body) were challenged s.c. into the shaved back region on day 0. The tumor sizes were determined biweekly using a caliper square, and the tumor volume (mm^3) was calculated as long diameter × squared short diameter.

Statistical analysis. We analyzed all data with the StatView statistical program for Macintosh (SAS Institute, Cary, NC, USA) and evaluated the statistical significance with an unpaired Student's *t*-test. $P < 0.05$ was considered significant. The percentage of overall survival rate was calculated by the Kaplan–Meier method, and statistical significance was evaluated with the Wilcoxon rank sum test.

Results

Expression of *SPARC* mRNA in mouse cancer cell lines and normal tissues. We examined the expression level of *SPARC* mRNA using reverse transcription polymerase chain reaction (RT-PCR). The mouse fibroblast cell line NIH/3T3 and various mouse cancer cell lines, including melanoma (B16, B16F1 and B16F10), fibrosarcoma (MCA) and lung cancer (3LL) originated from C57BL/6 mice, and sarcoma (BALB/3T3 and MethA), colon cancer (colon26) and breast cancer (N2C) originated from BALB/c mice, showed strong expression of *SPARC* (Fig. 1A). C57BL/6 mouse leukemia/lymphoma cell line, EL4, and BALB/c mouse lymphoma cell line, A20 and RL male 1, did not express *SPARC*. Although *SPARC* mRNA was expressed in the ovary, spinal cord, brain and uterus, the expression levels observed in these tissues were lower than those observed in cancer cell lines (Fig. 1B). Moreover, normal tissues including ovary, spinal cord, brain and uterus did not express SPARC at protein level investigated by the immunohistochemical analysis (Fig. 1C).

Identification of the SPARC-derived and H2-K^d-restricted CTL epitopes in BALB/c mice. Structural motifs of peptides bound to human HLA-A24 (*A*2402*) and mouse H2-K^d are similar. The amino acid sequences of human and mouse SPARC have a 92% homology.[4] Thereby, we searched for SPARC-derived and H2-K^d- or HLA-A24 (*A*2402*)-restricted peptides of which amino acid sequences were completely shared between human and mouse SPARC, and prepared four different synthetic peptides (Table 1). CD4$^-$ spleen cells isolated from BALB/c mice immunized twice with BMDC pulsed with mixture of these four peptides were stimulated *in vitro* with BMDC pulsed with each peptide for 5–6 days. Subsequently, we collected these CD4$^-$ T cells from the culture and we found that CD4$^-$ T cells stimulated with the SPARC-$1_{143-151}$ (DYIGPCKYI) or SPARC-$4_{225-234}$ (MYIFPVHWQF) peptides produced a large amount of IFN-γ in a peptide-specific manner in ELISPOT assays (Fig. 2A). Moreover, we tested cytotoxic activities of these cells directed against target cells by standard 6-h ^{51}Cr release assays. CTL induced by SPARC-$1_{143-151}$ or SPARC-$4_{225-234}$ peptides showed specific cytotoxicity against T2K^d (H2-K^d+, TAP negative) cells pulsed with each SPARC peptide but not against T2K^d cells unpulsed with SPARC peptide (Fig. 2B). In addition, those CTL had cytotoxic activities directed against MethA (SPARC$^+$, H-2^d) but not against RL male 1 (SPARC$^-$, H-2^d). These findings suggest that these SPARC-$1_{143-151}$ and SPARC-$4_{225-234}$ peptides had the capacity to induce the H2-K^d-restricted peptide-reactive CTL and that the CTL killed the tumor naturally expressing both SPARC and H2-K^d.

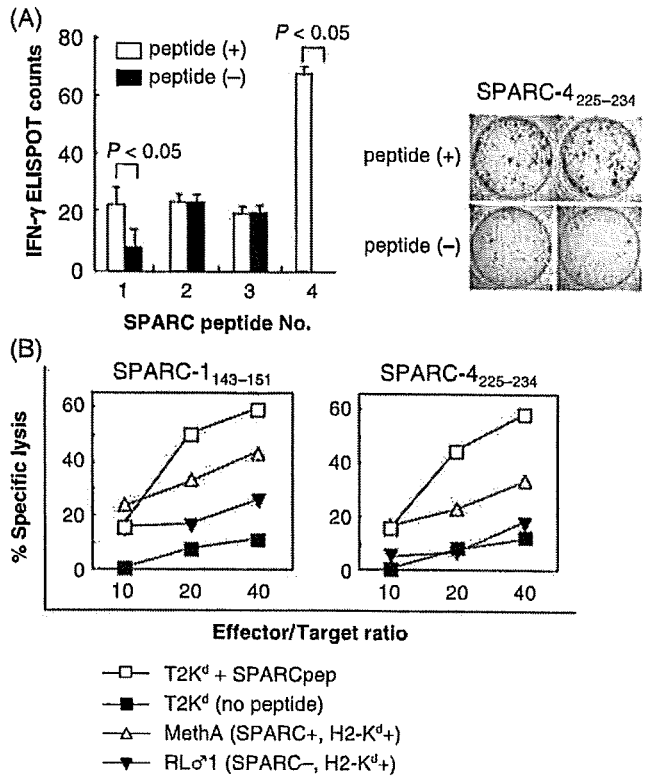

Fig. 2. Identification of the SPARC-derived and H2-K^d-restricted cytotoxic T lymphocyte (CTL) epitopes in BALB/c mice. We immunized the BALB/c mice with syngeneic bone marrow-derived dendritic cells (BMDC) (5×10^5/mouse) pulsed with the mixture of candidate peptides *in vivo* once a week for 2 weeks. Seven days after the last immunization, CD4$^-$ spleen cells isolated from immunized mice (2×10^6/well) were stimulated with BMDC (2×10^5/well) pulsed with each peptide *in vitro*. (A) Six days after the stimulation *in vitro*, the CTL-producing γ-interferon (IFN-γ) in response to the peptide-pulsed BMDC were detected by an enzyme-linked immunospot (ELISPOT) assay. (B) Five days after the stimulation *in vitro* under the same conditions, cytotoxic activities of these cells directed against indicated target cells was tested with standard ^{51}Cr release assays. We found that CD4$^-$ spleen cells stimulated with the SPARC-$1_{143-151}$ or SPARC-$4_{225-234}$ peptide produced a large amount of IFN-γ, and had cytotoxic activities directed against both H2-K^d and SPARC-expressing MethA tumor cell line or T2K^d pulsed with each peptide, but not to SPARC-negative RL male 1 and peptide-unpulsed T2K^d. These assays were done twice with similar results.

Immunization of SPARC-$4_{225-234}$ peptide did not induce the autoimmune diseases in BALB/c mice. To investigate whether the immunization of mice with the SPARC-derived H2-K^d-restricted peptide causes autoimmune diseases, the immunohistochemical staining of several important organs with anti-CD4 and anti-CD8 monoclonal antibody was performed in BALB/c mice immunized with BMDC pulsed with a mixture of four SPARC peptides once a week for 2 weeks. Tissue specimens of these mice were removed and analyzed 7 days after the second dendritic cell (DC) vaccination. As shown in Fig. 3, we could not find any pathological changes, such as lymphocyte infiltration or tissue destruction, in brain, heart, lung, liver, kidney, uterus, ovary and spinal cord of BALB/c mice. Although SPARC was expressed in spinal cord and brain by RT-PCR, the BALB/c mice immunized with BMDC pulsed with SPARC-$4_{225-234}$ peptide did not show any neurological disorders such as paralysis or abnormal behavior. No sign of autoimmune diseases

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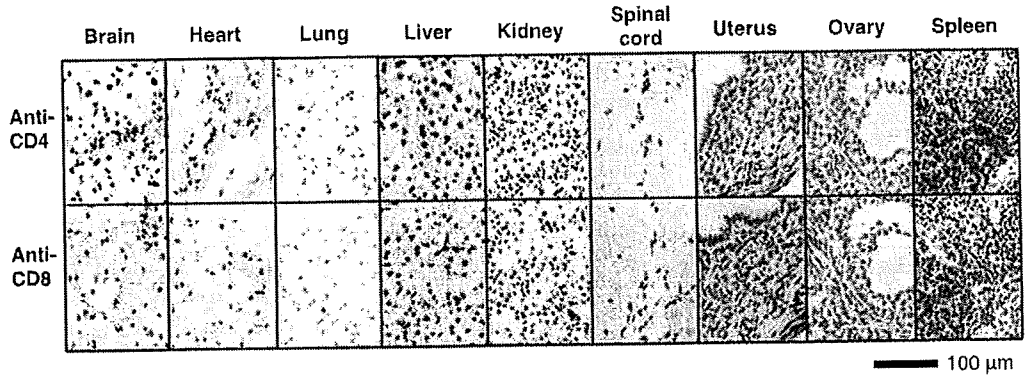

Fig. 3. Immunohistochemical staining with anti-CD4 or anti-CD8 monoclonal antibody in tissue specimens of BALB/c mice twice immunized with BMDC pulsed with the mixture of H2-K^d-restricted SPARC-derived peptides twice. These tissue specimens were removed and analyzed 7 days after the second dendritic cells (DC) vaccination (original magnification, ×200).

such as weight loss, diarrhea and skin abnormalities was observed (data not shown). These results indicate that the immunization of mice with SPARC-$4_{225-234}$ peptide does not induce autoimmune diseases.

Inhibition of tumor growth in BALB/c mice by vaccination of SPARC-$4_{225-234}$ peptide-pulsed BMDC. We investigated whether the immunization of mice with the SPARC epitope peptide inhibit the growth of tumor expressing SPARC *in vivo* or not. The BALB/c mice were injected i.p. twice at 7-day intervals with SPARC-$4_{225-234}$ peptide-pulsed BMDC, peptide-unpulsed BMDC or PBS ($n = 8$, each group). During the vaccination period, none of the treated mice showed any abnormalities. Subcutaneous inoculation of N2C cells (3×10^4) into the right flank was given 7 days after the last vaccination. Growth curves of N2C tumor mass are shown in Fig. 4(A,B). The N2C tumor appeared 25 days after the inoculation in the PBS-injected group. Measurement of tumor size was continued until 62 days after inoculation of the tumor cells when one mouse in the PBS-injected group died. Mean tumor size (1024.8 ± 1820.7 mm^3) on day 62 observed in the mouse group inoculated with SPARC-$4_{225-234}$ peptide-pulsed BMDC was significantly smaller (5343.6 ± 3117.2 mm^3, $P < 0.01$) than that observed in the mice inoculated with peptide-unpulsed BMDC and in those injected with PBS (6623.1 ± 3883.9 mm^3, $P < 0.01$). Complete tumor rejection was observed in four out of the eight mice in the SPARC-$4_{225-234}$ peptide-pulsed BMDC group. Although one out of the eight mice in the peptide-unpulsed BMDC group also completely rejected the tumor growth, there was no statistical significance in difference of tumor growth between the mice inoculated with peptide-unpulsed BMDC and those injected with PBS ($P = 0.48$). All mice injected with PBS died within 125 days after inoculation of the tumor cells. Mice inoculated with the SPARC-$4_{225-234}$ peptide-pulsed BMDC lived significantly longer than the mice of the other two groups (Fig. 4C). No significant abnormalities including neurological disorders were observed in four tumor-free mice in the SPARC-$4_{225-234}$ peptide-pulsed BMDC group for over 150 days after vaccination. This experiment was repeated twice with similar results. These findings indicate that vaccination of mice with BMDC pulsed with the SPARC epitopes resulted in significant inhibition of tumor growth and prolongation of overall survival *in vivo*.

Discussion

In this study, we demonstrated that: (i) *SPARC* was overexpressed in mouse cancer cell lines; (ii) the CTL induced by the SPARC-derived peptides were reactive to tumor overexpressing SPARC; and (iii) the immunization of BALB/c mice with SPARC peptide-pulsed BMDC protected mice from tumor growth and induced prolonged survival without causing autoimmune diseases.

Although immunohistochemical staining of these tissues with anti-SPARC antibody was negative (Fig. 1C), *SPARC* mRNA was expressed in several important normal tissues including brain and spinal cord using RT-PCR. Therefore, we needed to investigate whether induction of SPARC-specific CTL could induce antitumor immunity without causing autoimmune diseases in the mouse model system. One of the reasons for lack of autoimmune diseases might be that the H2-K^d-positive SPARC epitope peptide complexes are more densely expressed on tumor cells in comparison to those expressed on normal tissues including brain and spinal cord, if any. These possibilities must be evaluated in a future study.

The HLA-A24 is positive in 60% of the Japanese population (95% of whom are genotypically *A*2402*), 20% of Caucasians and 12% of Africans.[29] It is important especially for the Japanese to identify HLA-A24-restricted CTL epitope peptides. Structural motifs of peptides bound to human HLA-A24 and BALB/c mouse H2-K^d are similar,[30–32] and the amino acid sequences of human and mouse SPARC protein exhibit a 92% homology.[33] SPARC-derived and H2-K^d-restricted CTL epitopes identified in BALB/c mice may well be applicable to induce human HLA-A24-restricted CTL. Therefore, in this study, we used BALB/c mice, and searched for SPARC-derived peptides having amino acid sequences shared between mouse and human SPARC. We could identify the SPARC-derived and H2-K^d-restricted CTL epitopes. According to these findings, we tried to induce the HLA-A24-restricted human CTL reactive to these peptides by stimulating peripheral blood mononuclear cells (PBMC) of healthy donors or various cancer patients *in vitro* with the peptides. In a preliminary study, these SPARC peptide-specific and HLA-A24-restricted human CTL were also generated *in vitro* (unpublished observation, 2008).

We observed the inhibitory effect of immunization of mice with BMDC pulsed with SPARC-derived epitope peptide on growth of the inoculated N2C tumor cell line in BALB/c mice. N2C expressing SPARC is a mouse breast cancer cell line originating from Her-2/neu transgenic BALB/c mice.[34] It was reported that SPARC was overexpressed in human breast cancers, and associated with poor prognosis or invasive phenotype type.[35–38] N2C tumors grew as solid nests forming lobules embedded in dense, well-vascularized, connective tissue and surrounded by the stromal septa.[34] The stromal cells in N2C tumors also expressed SPARC. N2C tumor grown in SPARC