

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.

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 GPC3₂₉₈₋₃₀₆ (EYILSLEEL) and HLA-A2 (A*0201)-restricted GPC3₁₄₄₋₁₅₂ (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.

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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

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 (1×10⁶/well) were cultured in 96-well flat-bottomed plates for the ELISPOT assay (BD Bioscience) with HLA-A2-restricted GPC3₄₄₋₅₂ (A2-1) (RLQPGLKWV), GPC3₁₄₄₋₁₅₂ (A2-3) (FVGEFFTDV), GPC3₁₅₅₋₁₆₃ (A2-4) (YILGSDINV) and HLA-A24-restricted GPC3₂₉₈₋₃₀₆ (A24-8) (EYILSLEEL) (10 μ M) 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 (3×10⁵ 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, TNF α (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 μ M 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 1.5×10⁴ peptide-pulsed DCs, 3×10⁵ 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 (1×10⁴ 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)] × 100.

ELISA for the detection of anti-GPC3 IgG antibodies. Recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) was diluted in 10 × Block Ace (Dainippon Pharmaceutical, Osaka) to a final concentration of 1 μ g/ml, dispensed into 96-well plates (100 μ l/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 μ l, 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 H₂SO₄, 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.

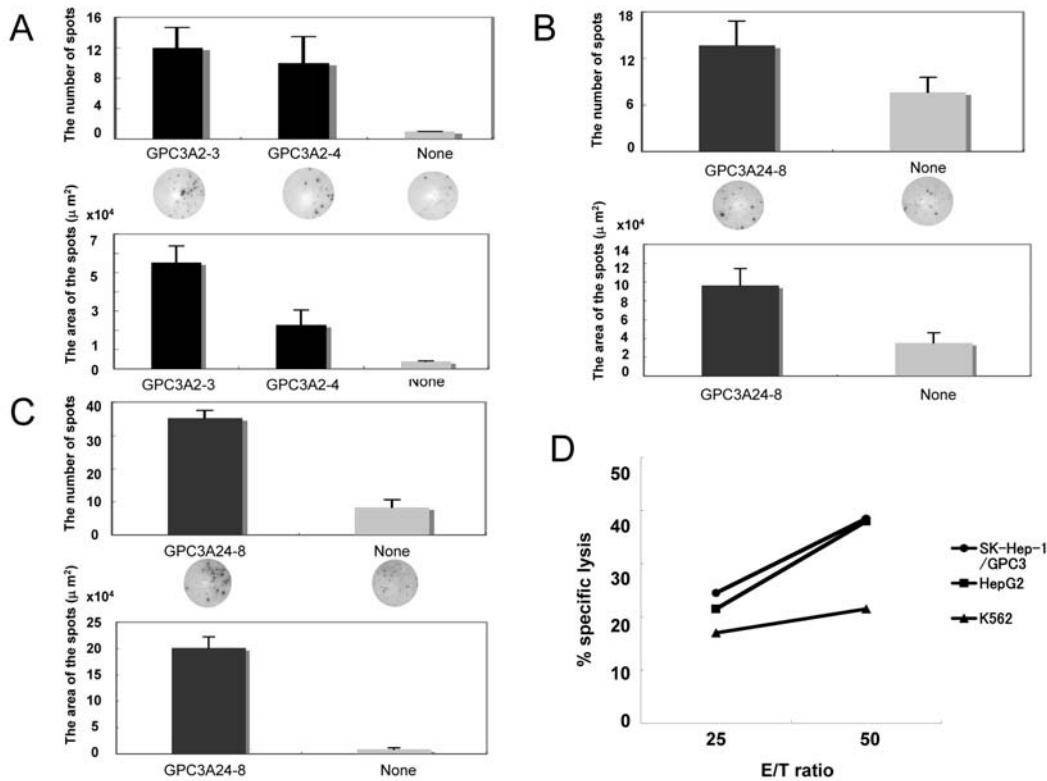


Figure 1. Frequency of GPC3-peptide-specific CTLs in the PBMCs of HLA-A2⁺ or HLA-A24⁺ CH and LC patients and the cytotoxicity of the CTLs induced by stimulation with the GPC3 (A2-3) peptide. GPC3-specific CD8⁺ T cells were detected in the chronic hepatitis [(A), HLA-A2⁺ CH4 patient; (B), HLA-A24⁺ CH5 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-class I⁻ 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 \pm 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⁺/A24⁺. 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-class I⁻ 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/RLQPGLKVV			GPC3 A2-3/FVGEFFTDV			GPC3 A2-4/YILGSDINV			GPC3 A24-8/RYLSEEL			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)	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	NT	NT	0.0±0.0	0.0±0.0	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	NT	NT	0.0±0.0	0.0±0.0	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	NT	NT	8.0±1.7	8045.0±1849.1	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	NT	NT	1.0±0.0	3853.0±375.2	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	0.5±0.0	354.0±0.0	0.5±0.0	354.0±0.0
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	NT	NT	4.3±0.5	2098.3±2166.5	4.3±0.5	2098.3±2166.5
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	NT	NT	2.0±3.4	2826.0±4894.7	2.0±3.4	2826.0±4894.7
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	NT	NT	14.0±8.0	41331.0±31472.6	14.0±8.0	41331.0±31472.6
LC4 (A*2402)	NT	NT	NT	NT	NT	NT	35.3±2.3	200882.0±21210.9	8.3±2.3	8714.0±2855.5	3.0±0.0	7065.0±3996.5	3.0±0.0	7065.0±3996.5
LC5 (A*2402)	NT	NT	NT	NT	NT	NT								

^aCH, chronic hepatitis; ^bLC, liver cirrhosis. ^cWe show values higher than the value for 'No peptide' by a bold font. ^dNT, not tested.

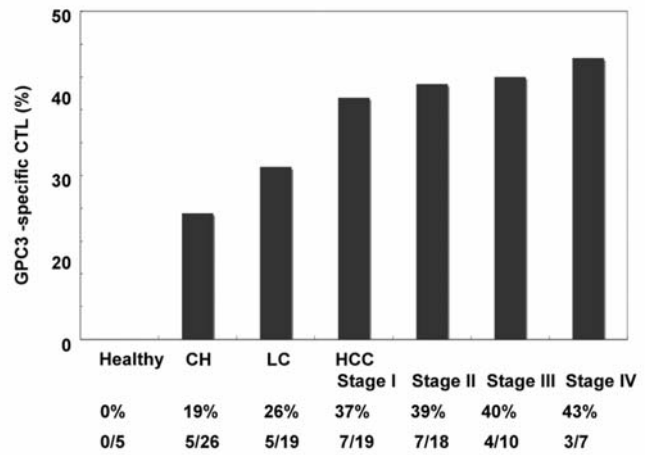


Figure 2. Frequency of HLA-A2⁺ or HLA-A24⁺ CH, LC and HCC patients positive for GPC3-peptide-specific CTLs in the PBMCs. GPC3-peptide-specific CTLs were detected in 19 and 26% of the patients with CH and LC, respectively. In the HCC patients, the percentage of these CTLs tended to increase with increasing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

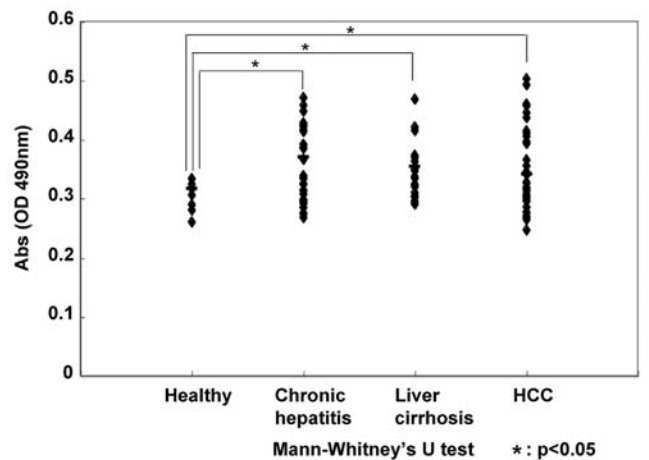


Figure 3. Plasma titers of anti-GPC3 IgG in the CH, LC and HCC patients. Anti-GPC3 IgG was detected by ELISA using recombinant GPC3 protein. A significantly higher titer of IgG to GPC3 was observed in the CH ($p<0.05$), LC ($p<0.05$) and HCC patients ($p<0.05$) as compared with that in healthy donors.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



SPANDIDOS PUBLICATIONS 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 (\pm SD)	Positive (n=0) mean (\pm SD)	Negative (n=28) mean (\pm SD)	Positive (n=5) mean (\pm SD)	Negative (n=19) mean (\pm SD)	Positive (n=5) mean (\pm SD)	Negative (n=33) mean (\pm SD)	Positive (n=21) mean (\pm SD)
Age	31.2 \pm 7.1	-	61.6 \pm 11.2	60.6 \pm 12.9	67.3 \pm 10.1	71.0 \pm 2.7	65.8 \pm 7.9	64.0 \pm 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 \pm 18.9	9.6 \pm 7.3	21.2 \pm 25.4	8.8 \pm 7.7	26335.1 \pm 143782.5	1431.5 \pm 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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