Identification of HLA-A2- or HLA-A24-Restricted CTL Epitopes Possibly Useful for Glypican-3-Specific Immunotherapy of Hepatocellular Carcinoma

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Abstract

Purpose and Experimental Design: We previously reported that glypican-3 (GPC3) was overexpressed, specifically in hepatocellular carcinoma (HCC) and melanoma in humans, and it was useful as a novel tumor marker. We also reported that the preimmunization of BALB/c mice with dendritic cells pulsed with the H-2K^d-restricted mouse GPC3$_{298-306}$ (EYILSLEEL) peptide prevented the growth of tumor-expressing mouse GPC3. Because of similarities in the peptide binding motifs between H-2K^d and HLA-A24 (*A*2402), the GPC3$_{298-306}$ peptide therefore seemed to be useful for the immunotherapy of HLA-A24$^+$ patients with HCC and melanoma. In this report, we investigated whether the GPC3$_{298-306}$ peptide could induce GPC3-reactive CTLs from the peripheral blood mononuclear cells (PBMC) of HLA-A24 (*A*2402)$^+$ HCC patients. In addition, we used HLA-A2.1 (HHD) transgenic mice to identify the HLA-A2 (*A*0201)–restricted GPC3 epitopes to expand the applications of GPC3-based immunotherapy to the HLA-A2$^+$ HCC patients.

Results: We found that the GPC3$_{144-152}$ (FVGEFFTDV) peptide could induce peptide-reactive CTLs in HLA-A2.1 (HHD) transgenic mice without inducing autoimmunity. In five out of eight HLA-A2$^+$ GPC3$^+$ HCC patients, the GPC3$_{144-152}$ peptide-reactive CTLs were generated from PBMCs by *in vitro* stimulation with the peptide and the GPC3$_{298-306}$ peptide-reactive CTLs were also generated from PBMCs in four of six HLA-A24$^+$ GPC3$^+$ HCC patients. The inoculation of these CTLs reduced the human HCC tumor mass implanted into nonobese diabetic/severe combined immunodeficiency mice.

Conclusion: Our study raises the possibility that these GPC3 peptides may therefore be applicable to cancer immunotherapy for a large number of HCC patients.

Hepatocellular carcinoma (HCC) is now spreading rapidly, especially in Asian and Western countries. It is clear that patients with hepatitis B or C–based liver cirrhosis are at high risk for developing HCC (1), and patients with hepatitis

treated surgically or by other therapies are also at high risk for recurrence (2). Furthermore, the liver function of these patients is often very poor, so further treatment for recurrence is often restricted. As a result, the prognosis of HCC remains poor, and new therapies for the prevention of cancer development and recurrence, i.e., adjuvant therapy, is urgently needed. As for melanoma, the age-adjusted incidence rates have been increasing in most fair-skinned populations in recent decades (3). In 2005, it is estimated that 59,580 Americans will be diagnosed to have melanoma, and 7,770 will die from the disease (4).

We and others previously reported that glypican-3 (GPC3) was overexpressed in most types of HCC (5–9) and melanoma in humans (8), and we also previously reported that an H-2K^d-restricted antigenic peptide, the mouse GPC3$_{298-306}$ (EYILSLEEL) peptide, could be recognized by mouse CD8$^+$ CTLs. In addition, these CTLs rejected tumor expressing mouse GPC3 both *in vitro* and *in vivo* (10). Because the structural motifs of peptides bound to HLA-A24 (*A*2402) and mouse H-2K^d are similar, we investigated whether the GPC3 peptide was also useful as a cancer immunotherapy modality for HLA-A24$^+$ HCC patients. The gene frequency of HLA-A24 (*A*2402) is relatively high in Asian populations, especially in the Japanese, whereas it is low in Caucasians. On the other hand, The gene frequency of

HLA-A2 (*A*0201*) is high among various ethic groups, including both Asians and Caucasians (11). Therefore, it is suggested that the HLA-A2-restricted and GPC3-derived CTL epitopes might be very useful for the immunotherapy of many patients with HCC and melanoma all over the world. In the present study, we identified human GPC3-derived CTL epitopes restricted by HLA-A2 using HLA-A2.1 (HHD) transgenic mice (Tgm) and examined whether these HLA-A2 or HLA-A24-restricted epitope peptides could induce GPC3-reactive CTLs from peripheral blood mononuclear cells (PBMC) of patients with HCC.

Materials and Methods

Mouse. HLA-A2.1 (HHD) Tgm; H-2D$^{b-/-}$β2m$^{-/-}$ double knockout mice introduced with human β2m-HLA-A2.1 (α1 α2)-H-2D^b (α3 transmembrane cytoplasmic) (HHD) monochain construct gene were generated in the Department SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, France (12, 13) and kindly provided by Dr. F.A. Lemonnier. Nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) female mice at 6 weeks of age were purchased from CLEA Japan (Tokyo, Japan).

Patients, blood samples, and cell lines. Blood samples from patients with HCC were obtained during routine diagnostic procedures after obtaining a formal agreement signed by the patients in Kumamoto University Hospital from April to September 2005. Human liver cancer cell lines, SK-Hep-1 and T2-A0201 (a TAP-deficient and HLA-A*0201-positive cell line; refs. 14, 15), were provided by Kyogo Ito of Kurume University. Human liver cancer cell lines HepG2 and HuH-7 endogenously expressing GPC3, and GPC3$^-$ colon cancer cell line SW620, were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). C1R-A*2402 (an *HLA-A*2402* transfectant of C1R cells expressing a trace amount of HLA class I molecule; ref. 15) were generous gifts from Dr. Masafumi Takiguchi. The expression of HLA-A2 and HLA-A24 in these cell lines were examined using flow cytometry with an anti-HLA-A2 monoclonal antibody (mAb), BB7.2 and anti-HLA-A24 mAb (One Lambda, Inc., Canoga Park, CA), respectively, in order to select target cell lines for CTL assays. The origins and HLA genotypes of these cell lines have been described elsewhere (16, 17). These cells were maintained *in vitro* in RPMI 1640 or DMEM supplemented with 10% FCS.

Induction of GPC3-reactive mouse CTLs and IFN-γ enzyme-linked immunospot assay. Human GPC3-derived peptides (purity >90%) sharing the amino acid sequences with mouse GPC3 and carrying

binding motifs for *HLA-A*0201*-encoded molecules, were identified using BIMAS software (BioInformatics and Molecular Analysis Section, Center for Information Technology, NIH, Bethesda, MD) and we purchased a total of nine peptides carrying HLA-A2 (*A*0201*) binding motifs (Table 1) from Biologica (Tokyo, Japan). The immunizations of mice with peptides were done as previously described (7). In brief, bone marrow (BM) cells (2 × 10^6) from HLA-A2.1 (HHD) Tgm were cultured in RPMI 1640 supplemented with 10% FCS, together with granulocyte macrophage colony-stimulating factor (5 ng/mL) and 2ME (0.8 ng/mL) for 7 days in 10-cm plastic dishes, and these BM-dendritic cells (DC) were pulsed with the mixture of GPC3 peptides carrying HLA-A2 binding motifs (1 μmol/L for each peptide) at 37°C for 2 hours. We primed the HLA-A2.1 (HHD) Tgm with this syngeneic BM-DC vaccine (5 × 10^5/mouse) into the peritoneal cavity once a week for two weeks. Seven days after the last immunization, the spleens were collected and CD4$^-$ spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any nonspecific IFN-γ production by CD4$^+$ spleen cells cocultured with the BM-DC. The CD4$^-$ spleen cells (2 × 10^6/well) were stimulated with syngeneic BM-DC (2 × 10^5/well) pulsed with each peptide *in vitro*. Then, 6 days later, the frequency of cells producing IFN-γ/2 × 10^4 CD4$^-$ spleen cells upon stimulation with syngeneic BM-DC (1 × 10^4/well), pulsed with or without each peptide, was assayed in an enzyme-linked immunospot (ELISPOT) assay as previously described (18).

Induction of GPC3-reactive human CTLs. We isolated PBMCs from the heparinized blood of HLA-A24$^+$ and/or HLA-A2$^+$ Japanese patients with HCC or healthy donors by means of Ficoll-Conray density gradient centrifugation, and peripheral monocyte-derived DCs were generated as described previously (19, 20). CD8$^+$ T cells were isolated using CD8 microbeads (Miltenyi Biotec) from the PBMC of the same donors, and thereafter, peptide-reactive CD8$^+$ CTLs were generated (19, 20). Five days after the last stimulation, the cytotoxic activities of the CTLs were measured by a ^{51}Cr release assay.

CTL responses against cancer cell lines. CTLs were cocultured with each cancer cell line as a target cell (5 × 10^3/well) at the indicated effector/target ratio and ^{51}Cr release assay was done as described (21). The blocking of HLA-class I or HLA-DR, was done as follows. Before the coculture of CTLs with a cancer cell line in a ^{51}Cr release assay or ELISPOT assay, target cancer cells were incubated for 1 hour with 10 μg/mL anti–class I mAb W6/32 or 10 μg/mL anti–HLA-DR mAb, H-DR-1, and then the effects of mAbs on either the cytotoxic activity or production of IFN-γ by CTLs were examined as reported previously (22).

Histologic and immunohistochemical analysis. Immunohistochemical staining of CD8 or CD4 in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the GPC3$_{144-152}$ peptides and the staining of

Table 1. GPC3-derived peptides conserved between human and mouse GPC3 and predicted to be bound to HLA-A2 (*A*0201*)

A2-binding peptide	Position	Subsequence residue listing	HLA-A2 binding score*
GPC3A2-1	44-52	RLQPGLKWV	879
GPC3A2-2	102-110	FLIIQNAAV	319
GPC3A2-3	144-152	FVGEFFTDV	828
GPC3A2-4	155-163	YILGSDINV	162
GPC3A2-5	169-177	ELFDSLFPV	1055
GPC3A2-6	254-268	RMLTRMWYC	1259
GPC3A2-7	281-289	VMQGCMAGV	196
GPC3A2-8	326-334	TIHDSIQYV	496
GPC3A2-9	522-560	FLAELAYDL	402

*Binding scores were estimated by using BIMAS software (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform).

apoptotic cells with terminal deoxynucleotidyl transferase–mediated nick end labeling methods (ApopTag fluorescein *in situ* apoptosis detection kits; Serologicals Corporation, Norcross, GA) in tumor specimens of patients with HCC were done as described previously (23, 24). In addition, immunohistochemical staining of HLA-class I in HCC tumor tissue specimens were done by using anti-HLA-class I mAb, EMR 8-5.[5]

Detection by ELISA of the serum-soluble GPC3 protein. Detection of the serum-soluble GPC3 protein was done by an indirect ELISA using the rabbit anti-GPC3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (7). We used recombinant human GPC3 protein (R&D Systems Inc., Minneapolis, MN) as a standard, and the presence of >106 ng/mL of serum GPC3 protein was considered to be positive.

Transfer of CTLs to the NOD/SCID mice implanted with a human HCC cell line. The transfer of GPC3-reactive CTLs to the immunodeficient mice implanted with a human HCC cell line was done as described previously (7). Briefly, we s.c. inoculated SK-Hep1/GPC3 cells (1×10^7) positive for both HLA-A2 and HLA-A24 at the right flank of NOD/SCID mice. When the diameter of these tumors reached 5×5 mm on day 9 after tumor inoculation into mice, we intravenously injected the mixture of GPC3 peptide-reactive CTL lines or irrelevant HIV peptides; HLA-A2-restricted SLYNTYATL peptide and HLA-A24-restricted RYLRDQQLL peptide, stimulated CD8$^+$ T cells (3×10^6) established from four HLA-A24-positive or two HLA-A2-positive HCC patients, or saline alone. T cells were i.v. injected one more times on day 14. The CD8$^+$ T cells stimulated with HLA-A24-restricted GPC3$_{298-306}$ peptide or HIV (RYLRDQQLL) peptide and derived from two independent HLA-A24$^+$ HCC patients were mixed, and injected into three NOD/SCID mice on day 9, and the mixture of peptide-stimulated CD8$^+$ T cells from two other HLA-A24$^+$ HCC patients distinct from the T cell donors at the first injection, were injected into the mice on day 14. The HLA-A2-restricted peptide-stimulated CD8$^+$ T cells from one HLA-A2$^+$ HCC patient were also injected into a NOD/SCID mouse on day 9, followed by the injection on day 14 with the peptide-stimulated CD8$^+$ T cells derived from another HLA-A2$^+$ HCC patient.

Statistical analysis. The two-tailed Student's *t* test was used to evaluate the statistical significance of differences in the data obtained by ELISPOT assay. The statistical significance of the differences in several factors between patients showing a successful CTL induction and other patients was assessed by a χ^2 test. $P < 0.05$ was considered to be significant. Statistical analyses were made using the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

Results

Identification of HLA-A2-restricted CTL epitopes by using HLA-A2.1 (HHD) Tgm. To identify HLA-A2-restricted epitopes by using HLA-A2.1 (HHD) Tgm, we selected nine kinds of peptides having amino acid sequences conserved between human and mouse GPC3 and having high predicted binding scores to HLA-A2 (*A*0201*; Table 1). CD4$^-$ spleen cells from HLA-A2.1 (HHD) Tgm immunized i.p. twice with BM-DCs pulsed with the mixture of these nine peptides were again stimulated *in vitro* with BM-DCs pulsed with each peptide, and we found that CD4$^-$ spleen cells stimulated *in vitro* with the GPC3$_{144-152}$ peptide produced the largest amount of IFN-γ in a peptide-specific manner in ELISPOT assays. These CD4$^-$ spleen cells (2×10^4/well), showed 36 ± 2.85 spot counts/well, in response to the BM-DCs pulsed with the GPC3$_{144-152}$ peptide,

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[5] T. Torigoe, et al. Immunohistochemical analysis of HLA class I expression in tumor tissues revealed unusually high frequency of down-regulation in breast cancer tissues submitted.

whereas they showed 23 ± 1.84 spot counts/well in the presence of BM-DCs without peptide loading ($P < 0.005$) indicating that about $(36-23) / 2 \times 10^4 = 0.065\%$ of CD4$^-$ spleen cells were reactive to the GPC3 peptide. When we used syngeneic BM-DCs pulsed with a HLA-A2-binding HIV-derived peptide; SLYNTYATL as a control, no significant response (8.84 ± 1.73) was observed. The summation of the diameter of the IFN-γ ELISPOT observed in CD4$^-$ spleen cells stimulated with the GPC3$_{144-152}$ peptide pulsed BM-DCs was $1,878 \pm 131$ μm, that stimulated with the HIV-derived SLYNTYATL peptide pulsed BM-DCs was 437 ± 77 μm, and that observed in the presence of BM-DC without peptide loading was 762 ± 131 μm ($P < 0.001$). These assays were done thrice with similar results. As shown in Fig. 1B, the differences in the spot counts (left) or spot diameters (right) between stimulations with peptide pulsed BM-DC and BM-DC without peptide loading clearly revealed the GPC3$_{144-152}$ peptide-specific response of CD4$^-$ spleen cells. As for other peptides, no significant peptide-specific response was observed. These results suggest that the GPC3$_{144-152}$ peptide could be a CTL epitope peptide in HLA-A2.1 (HHD) Tgm, and we also expected this GPC3$_{144-152}$ peptide to be an epitope for human CTLs.

The immunization of the HLA-A2-restricted peptide, GPC3$_{144-152}$, did not induce autoimmunity in HLA-A2.1 (HHD) Tgm. It is well known that melanocyte-differentiation antigens such as MART-1 or gp100 are very useful for immunotherapy of melanoma patients, but they sometimes cause autoimmunity, such as vitiligo or uveitis, following vaccination. We previously reported that the immunization of the GPC3$_{298-306}$ peptide did not cause autoimmunity in BALB/c mouse (9). To investigate whether the immunization of mice with HLA-A2-restricted GPC3-derived peptides causes autoimmunity, the immunohistochemical staining of several organs with anti-CD4 and anti-CD8 mAb was done in HLA-A2.1 (HHD) Tgm immunized with a mixture of nine GPC3 peptides 7 days before the analysis. As shown in Fig. 2, we could not find any pathologic changes, such as lymphocyte infiltration or tissue destruction and repair in skin, lung, brain, heart, liver, and kidney of HLA-A2.1 (HHD) Tgm. The same result was also observed when mice were vaccinated with the GPC3$_{144-152}$ peptide alone ($n = 3$; data not shown). These results indicate that the GPC3$_{144-152}$ peptide-reactive CD8$^+$ CTLs do not attack the normal tissue specimens that we investigated.

Induction of GPC3-reactive CTLs from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. We evaluated the cytotoxic activity of CTLs that were induced with the GPC3$_{298-306}$ or GPC3$_{144-152}$ peptide from PBMCs isolated from HCC patients. PBMCs were isolated from HCC patients positive for HLA-A24 and/or HLA-A2, and CD8$^+$ T cells sorted from the PBMCs were cocultured with autologous monocyte-derived DCs pulsed with each peptide as described in Materials and Methods. CTLs from PBMCs of HLA-A2$^+$ HCC patients stimulated with the GPC3$_{144-152}$ peptide or CTLs from PBMCs of HLA-A24$^+$ HCC patients stimulated with the GPC3$_{298-306}$ peptide exhibited cytotoxicity against peptide-pulsed target cells. The representative data of CTLs restricted by HLA-A2 or HLA-A24 were shown in Fig. 3A. The CTLs induced from PBMCs of patient A2-8 showed cytotoxic activity to T2-A0201 cells (HLA-A2+) pulsed with the GPC3$_{144-152}$ peptide, but not to T2-A0201 cells without peptide loading by ^{51}Cr release assay. The CTLs induced from PBMCs of patient A24-12 exhibited cytotoxic

A

Day -21   Day -14   Day -7   Day -0

BM-DC
induction
peptide pulsed
BM-DC i.p.
peptide pulsed
BM-DC i.p.
Isolation of
spleen cells

Day 0   Day 6

ELISPOT assay

in vitro re-stimulation
CD4⁻ spleen cells + BM-CDs
pulsed with each peptide

B

Δ IFN-γ ELISPOT counts

0   5   10   15

GPC3A2-1
GPC3A2-2
GPC3A2-3
GPC3A2-4
GPC3A2-5
GPC3A2-6
GPC3A2-7
GPC3A2-8
GPC3A2-9

Δ Diameter of IFN-γ ELISPOT (mm)

0   500   1000   1500

GPC3A2-1
GPC3A2-2
GPC3A2-3
GPC3A2-4
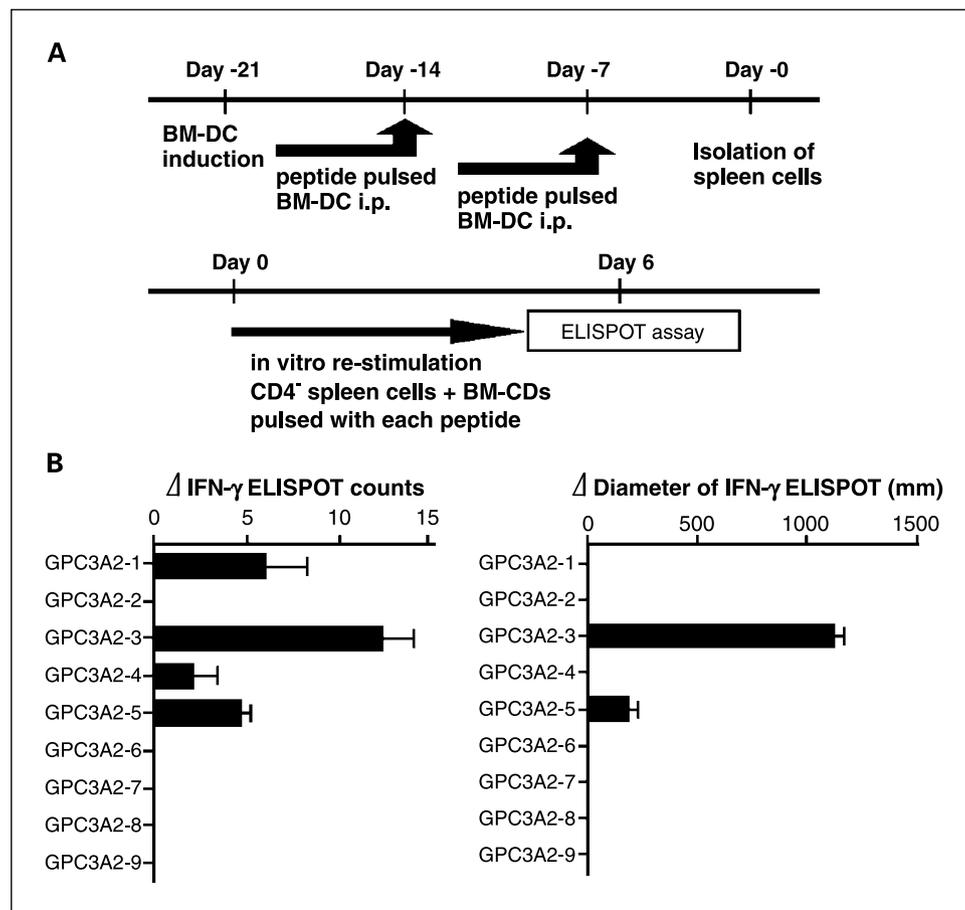GPC3A2-5
GPC3A2-6
GPC3A2-7
GPC3A2-8
GPC3A2-9

Fig. 1. Identification of HLA-A2-restricted CTL epitopes of GPC3 by using HLA-A2.1 (HHD) Tgm. *A,* protocol for identification of GPC3-derived and HLA-A2-restricted CTL epitopes. We primed the HLA-A2.1 (HHD) Tgm with BM-DCs (5×10^5) pulsed with the mixture of GPC3-derived peptides carrying HLA-A2 (*A*0201*) binding motif into the peritoneal cavity once a week for two weeks. Seven days after the last DC vaccination, spleens were collected and CD4⁻ spleen cells (2×10^6/well) were stimulated with syngeneic BM-DCs (2×10^5/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured CD4⁻ spleen cells as responder cells in ELISPOT assay to evaluate GPC3-specific response of CTLs. *B, bar graph,* IFN-γ ELISPOT counts/2×10^4 CD4⁻ spleen cells cocultured with peptide pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*left*). *Bar graph,* summation of IFN-γ ELISPOT diameters/2×10^4 CD4⁻ spleen cells cocultured with peptide-pulsed BM-DCs subtracted with those cocultured with BM-DCs without peptide loading (*right*). *Columns,* mean of triplicate assays; *bars,* SE. All assays were done thrice with similar results.

activity to the C1R-A*2402 cells (HLA-A24+) pulsed with the GPC3$_{298-306}$ peptide, but not to C1R-A*2402 cells without peptide loading. These results indicate that these CTLs had peptide-specific cytotoxicity. Other CTLs induced from the nine patients A2-1, A2-2, A2-3, A2-4, A24-1, A24-3, A24-4, A24-6, and A24-7 similarly exhibited peptide-specific cytotoxicity against peptide-pulsed target cells (data not shown).

Furthermore, we used GPC3 transfectants, SK-Hep1/GPC3 (GPC3+, HLA-A2+, HLA-A24+) or SW620/GPC3 (GPC3+, HLA-A2+, HLA-A24+) as target cells and examined whether we could find GPC3-specific cytotoxic activity of CTLs. As shown in Fig. 3B, the CTLs induced from PBMCs of patient A2-3 by stimulation with the GPC3$_{144-152}$ peptide showed specific cytotoxicity against SK-Hep1/GPC3, but not against GPC3-negative SK-Hep1. Similarly, the GPC3$_{298-306}$ peptide–induced CTLs showed specific cytotoxicity against SW620/GPC3 in

patient A24-7 or against SK-Hep1/GPC3 in patient A24-12, but not against SK-Hep1 or SW620, respectively, which did not endogenously express GPC3. These findings indicate that these peptides can be processed naturally in cancer cells, and the peptides in the context of HLA-A2 or HLA-A24 can be expressed on the cell surface of cancer cells to be recognized by the CTLs.

When we think about the application of GPC3 to cancer immunotherapy, the most important point is that these GPC3-reactive CTLs can exhibit specific cytotoxicity to the tumors endogenously expressing GPC3. We thus investigated whether these CTLs could kill human HCC cell lines expressing both endogenous GPC3 and the restriction HLA class I molecules. As shown in Fig. 3C, we could generate GPC3-reactive CTLs by stimulation with the GPC3$_{144-152}$ peptide and these CTLs exhibited cytotoxic activity to HepG2 (GPC3+, HLA-A2+, and

spleen   skin   lung   brain   heart   liver   kidney

CD4

CD8

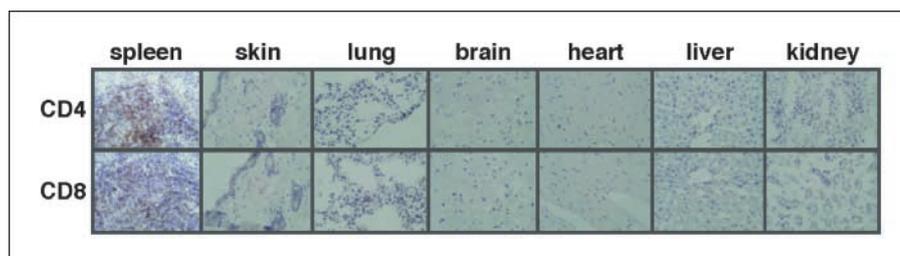**Fig. 2.** Immunohistochemical staining with anti-CD4 or anti-CD8 mAb in tissue specimens of HLA-A2.1 (HHD) Tgm immunized with the GPC3 $_{144-152}$ peptides. These tissue specimens were removed and analyzed 7 days after the second DC vaccination (original magnification, ×200).
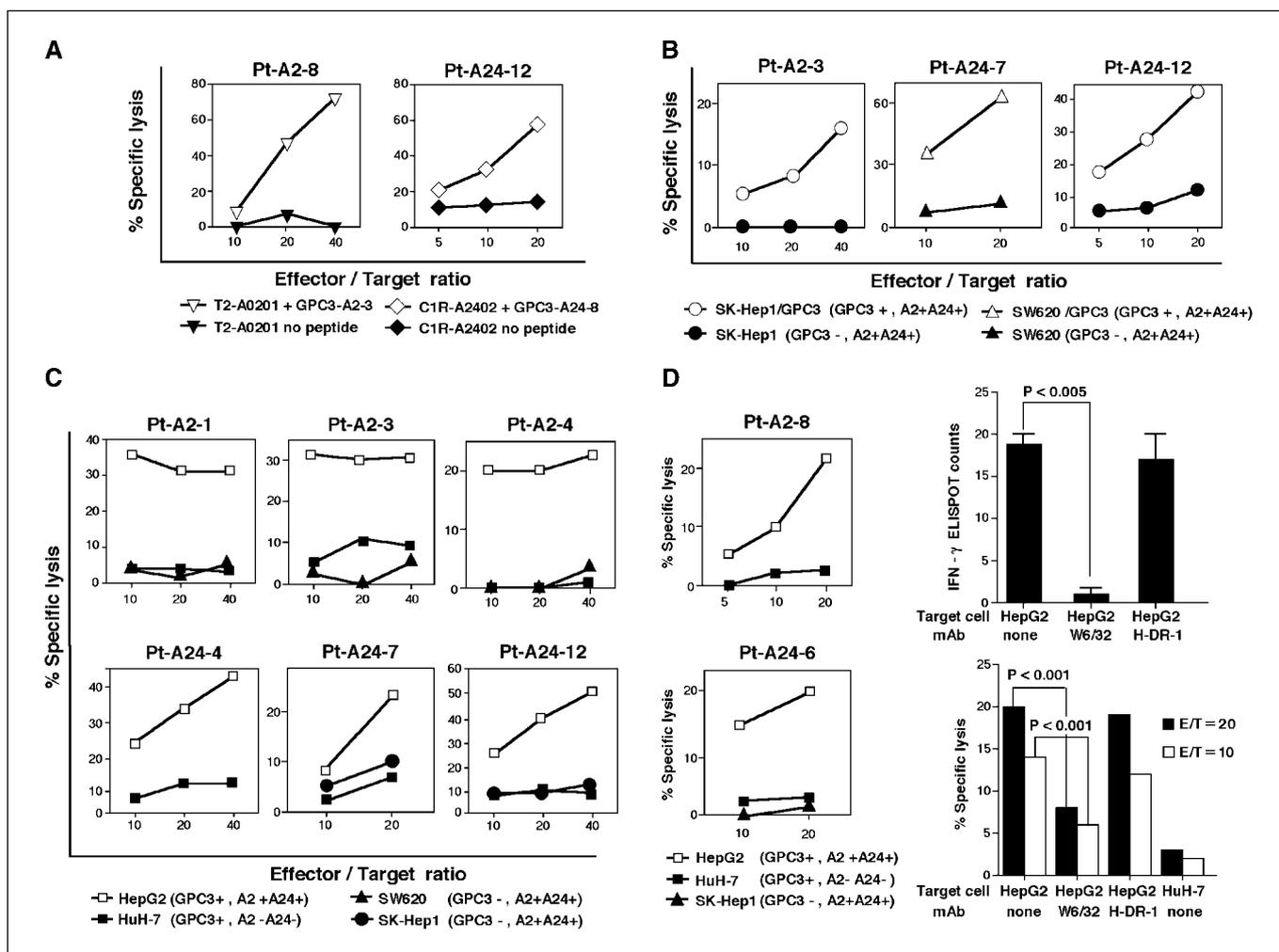
Fig. 3. CTL induction from PBMCs of HLA-A2- or HLA-A24-positive HCC patients. *A* and *B*, GPC3 peptide-reactive CTLs were generated from CD8^{+} T cells of HLA-A2^{+} and/or HLA-A24^{+} HCC patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the GPC3$_{144-152}$ or GPC3$_{298-306}$ peptide, the CTLs were subjected to a standard ^{51}Cr release assay at the indicated effector/target ratio. Their cytotoxicity against the GPC3$_{298-306}$ peptide pulsed C1R-A2402 cells or T2-A0201 cells, and each unpulsed cells (*A*), or GPC3$^-$ HLA-A2[+], HLA-A24[+] HCC cell line SK-Hep-1, GPC3$^-$ HLA-A2[+], HLA-A24[+] colon cancer cell line SW620, and those cell lines transfected with the human *GPC3* gene; SK-Hep-1/GPC3 or SW620/GPC3 (*B*) were examined by a ^{51}Cr release assay. *C* and *D*, GPC3$^+$ HLA-A2[+], HLA-A24[+] HCC cell line HepG2, GPC3$^+$ HLA-A2$^-$, HLA-A24$^-$ HCC cell line HuH-7, and GPC3$^-$ tumor cell lines SW620 and SK-Hep1 were used as target cells (*left*). *Points,* percentage of specific lysis calculated based on the mean values of a triplicate assay. *D,* inhibition of cytotoxicity by anti-HLA class I mAb (*right*). After the target HepG2 cells were incubated with anti-HLA class I mAb (W6/32, IgG$_{2a}$) or anti-HLA DR mAb (H-DR-1, IgG$_{2a}$), respectively, for 1 hour, the CTLs generated from PBMCs of patient A2-8 by stimulation with GPC3$_{144-152}$ peptide (*top*) or CTLs generated from patient A24-6 using the GPC3$_{298-306}$ peptide (*bottom*) were added. IFN-γ production (*top*; IFN-γ ELISPOT assay) and cytotoxicity (*bottom*; ^{51}Cr release assay) were markedly inhibited by W6/32, but not by H-DR-1.

HLA-A24+), but not to HuH-7 (GPC3+, HLA-A2−, and HLA-A24−) or SW620 (GPC3$^-$, HLA-A2+, and HLA-A24+) in patients A2-1, A2-3, and A2-4. Similarly, we could generate GPC3-reactive CTLs by stimulation of PBMCs with the GPC3$_{298-306}$ peptide and these CTLs exhibited cytotoxic activity to HepG2, but not to HuH-7 or SK-Hep-1 (GPC3$^-$, HLA-A2+, HLA-A24+) in patients A24-4, A24-7, and A24-12.

In an HLA-class I blocking experiment, anti-HLA class I mAb W6/32 markedly inhibited the IFN-γ production stimulated with HepG2 cells in ELISPOT assay of CTLs generated from patient A2-8 by stimulation with the GPC3$_{144-152}$ peptide (Fig. 3D, top), and inhibited cytotoxic activity against HepG2 cells in ^{51}Cr release assay of CTLs generated from patient A24-6 by stimulation with the GPC3$_{298-306}$ peptide (Fig. 3D, bottom), but anti-HLA-DR mAb, H-DR-1 did not inhibit the response of CTLs. These results clearly indicate that these CTLs recognized HepG2 in a HLA-class I–restricted manner.

As shown in Table 2, we could induce GPC3-reactive CTLs from PBMCs in ~50% of either the HLA-A2− or HLA-A24-positive HCC patients. In patients A2-6, A24-5, A24-9, and A24-11 who did not express GPC3 in tumor tissues, GPC3-reactive CTLs could not be induced from their PBMCs. Among eight HLA-A2-positive HCC patients who expressed GPC3 in HCC tissue or produced soluble GPC3 in sera, patients A2-1, A2-2, A2-3, A2-4, A2-6, A2-7, A2-9, and A2-10, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (50%). In patient A2-6, GPC3 was detected only in the serum but not in HCC tumor tissue. It was thought to be possible that the majority of GPC3 protein was secreted away in this type of HCC cell as described previously (7). Among six HLA-A24-positive patients who expressed GPC3 in tumor tissue, patients A24-1, A24-2, A24-3, A24-6, A24-10, and A24-12, GPC3-reactive CTLs could be generated from the PBMCs of only four patients (67%). We also examined whether it was possible to

induce GPC3-specific CTLs from PBMCs isolated from healthy donors (each HLA type, *n* = 3), but we failed to generate GPC3-specific and HLA-A2- or HLA-A24-restricted CTLs even though PBMCs were stimulated with the peptides thrice *in vitro* (data not shown). These results suggest that GPC3-reactive CTLs could only be induced in patients who expressed GPC3 in tumor tissue, thus, indicating the existence of GPC3-reactive CTL precursors in patients with GPC3⁺ HCC. We also examined whether GPC3-reactive CTLs could be generated more frequently from PBMCs isolated from HCC patients positive for serum-soluble GPC3. As shown in Table 2, the presence of serum-soluble GPC3 did not correlate statistically with the successful induction of GPC3-reactive CTLs. As a result, we could not observe the enhancement of CTL induction efficiency via possible antigen presentation of soluble serum GPC3 through HLA-class II pathways to CD4⁺ T cells or cross-presentation through the HLA class I pathway to CD8⁺ T cells (25, 26) in patients positive for serum GPC3.

Inoculation of the GPC3 peptide-induced CTLs reduced growth of a GPC3⁺ human HCC tumor cell line implanted into NOD/SCID mouse. To investigate the effects of GPC3 peptide-reactive CTL inoculation into the mice implanted with the GPC3⁺ human HCC cell line, we s.c. inoculated SK-Hep1/GPC3

cell lines positive for both HLA-A2 and HLA-A24 into NOD/SCID mice, and i.v. injected the mixture of CTLs generated from several HCC patients positive for HLA-A2 or HLA-A24 into mice implanted with SK-Hep1/GPC3 when the diameter of these tumors reached 5 × 5 mm in size as described in Materials and Methods. The CTLs injected into mice were prepared by stimulating peripheral blood CD8⁺ T cells with HLA-A2- or HLA-A24-restricted GPC3-epitope peptides or control-irrelevant HIV peptides as described in Materials and Methods. The tumor sizes of four individual mice in each group (Fig. 4A) and mean ± SD of tumor sizes in each group (Fig. 4B) were evaluated. After 5 days from the second inoculation of GPC3 peptide-reactive CTLs, the tumor size of SK-Hep1/GPC3 was apparently reduced in comparison to the size of tumor mass implanted into NOD/SCID mice injected with control T cells or saline alone (*P* < 0.01). These results clearly indicate the efficacy of adoptive GPC3 peptide-reactive CTL transfer therapy for GPC3⁺ tumor in mice.

Discussion

In this article, we identified HLA-A24-restricted or HLA-A2-restricted GPC3 CTL epitope peptides, and found that

Table 2. Expression of GPC3 in HCC tissue, quantification of serum-soluble GPC3, and GPC3-specific CTL induction in HCC patients

	Age	Gender	State of tumor*	GPC3 expression[†]	Serum GPC3[‡]	HLA expression[§]	CTL induction[‖]
HLA-A2 (*A*0201) − positive patients							
Pt-A2-1	80	F	IIIa	+	+	+	+
Pt-A2-2	72	M	II	+	+	+	+
Pt-A2-3	67	F	II	ND	+	ND	+
Pt-A2-4	54	M	I	+	−	+	+
Pt-A2-5	57	M	I	ND	−	ND	−
Pt-A2-6	66	M	I	−	+	−	−
Pt-A2-7	54	M	IIIa	+	−	+	−
Pt-A2-8	73	M	II	ND	−	ND	+
Pt-A2-9	68	F	IIIa	+	−	+	−
Pt-A2-10	54	M	II	+	+	+	−
HLA-A24 (*A*2402)-positive patients							
Pt-A24-1	60	M	IVa	+	+	+	+
Pt-A24-2	57	M	IVa	+	+	+	−
Pt-A24-3	75	F	IIIa	+	+	+	+
Pt-A24-4	59	M	IIIa	ND	−	ND	+
Pt-A24-5	52	M	IVb	−	−	+	−
Pt-A24-6	65	M	I	ND	+	ND	+
Pt-A24-7	61	M	I	ND	−	ND	+
Pt-A24-8	74	M	II	ND	−	ND	−
Pt-A24-9	59	M	IVb	−	−	−	−
Pt-A24-10	69	M	IVa	+	+	+	−
Pt-A24-11	72	M	II	−	−	+	−
Pt-A24-12	61	M	IIIa	+	+	+	+

Abbreviations: F, female; M, male; ND, not determined.
*Tumor-node-metastasis classification.
[†]Positive (+) or negative (−) staining of tumor cells in contrast with peritumor normal tissue as background staining.
[‡]Serum levels >106 ng/mL were evaluated as positive.
[§]Immunohistochemical staining of the membrane of tumor cells was evaluated as positive.
[‖]Specific lytic activity (≧20%) at E:T ratio = 20 against HepG2 target cells was evaluated as positive by ⁵¹Cr release assay.

Fig. 4. Marked inhibition of growth of a GPC3-transfected human HCC cancer cell line, SK-Hep1/GPC3, engrafted into NOD/SCID mice after adoptive transfer of human CTLs induced by the GPC3 peptides. *A,* when tumor size reached 25 mm^2 on day 9 after s.c. tumor implantation, human CTLs (3×10^6) reactive to HLA-A2-restricted (■) GPC3 peptide and generated from one HLA-A2$^+$ donor, or those reactive to HLA-A24-restricted (□) GPC3 peptide and pooled from two HLA-A24$^+$ donors were i.v. inoculated. On day 14, the inoculation of CTLs generated from the donors distinct from those at the first injection was repeated. The control CD8$^+$ T cells stimulated with irrelevant HLA-A2-restricted (▲) or HLA-A24-restricted (△) HIV peptides were also injected into mice as a control. Tumor volumes in NOD/SCID mice given twice on days 9 and 14 with GPC3 epitope peptide–induced CTL lines ($n = 4$), control CD8$^+$ T cells ($n = 4$), or saline alone ($n = 4$). Tumor size was expressed in square millimeters. *B, points,* mean tumor sizes in each group of mice; *bars,* ±SD ($n = 4$). Statistical significance was evaluated using *t* test.
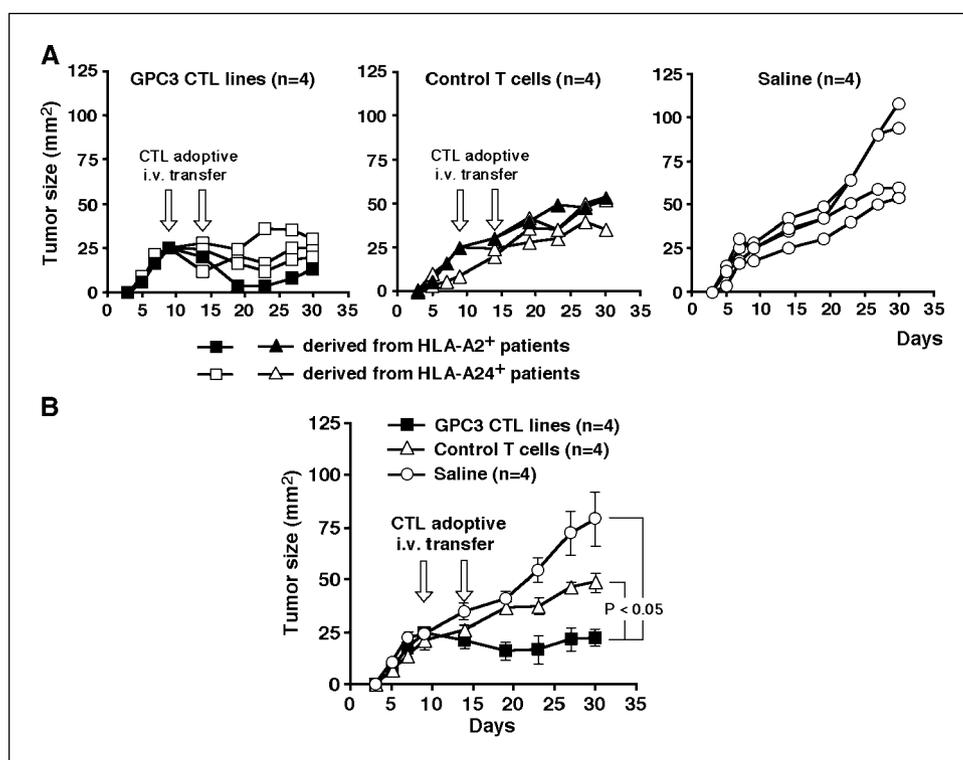
GPC3-reactive CTLs could be generated from PBMCs stimulated with these peptides in ∼50% of HCC patients. Vaccination based on these peptides did not induce autoimmunity in HLA-A2.1 (HHD) Tgm of a B57Bl/6 background. We previously identified the GPC3$_{298-306}$ peptide to be a CTL epitope in BALB/c mouse, and we expected that this GPC3 peptide might also be present in human CTL in a HLA-A24-restricted manner. As expected, we could generate HLA-A24-restricted and the GPC3$_{298-306}$ peptide-reactive human CTLs in this study. As a result, BALB/c mice may be useful for identifying HLA-A24-restricted CTL epitopes. HLA-A2.1 (HHD) Tgm was reported to be a versatile animal model for the preclinical evaluation of peptide-based immunotherapy (12, 13). We could also find its usefulness for the identification of HLA-A2-restricted antigenic epitope in this study.

In this study, we wanted to identify the most effective major CTL epitopes derived from GPC3. As a result, we used BM-DCs derived from HLA-A2.1 (HHD) Tgm and pulsed BM-DCs with the mixture of GPC3 peptides for the vaccination of mice. Some of the peptides tested stimulated the weak response of CTLs in an ELISPOT assay, and these peptides might also be useful for future analysis. It was recently reported that peptides having a weak affinity to MHC, which could not be predicted by a BIMAS system, could induce peptide-reactive CTLs with a cytotoxic activity (27). To search for more peptides that can be applicable for immunotherapy, it may be necessary to check these minor CTL epitopes in the future. In this study, the GPC3-derived peptides predicted to have high binding affinity to HLA-A2 molecules and having the amino acid sequences conserved between human and mouse GPCs were selected for the analysis. When we analyzed the amino acid sequence of human GPC3 protein, all of the top 28 human GPC3 peptides having high binding scores (>100) to HLA-A2 molecules shared

the same amino acid sequences with mouse GPC3. Therefore, it is unlikely that we excluded many candidates of human GPC3-derived and HLA-A2-restricted CTL epitopes from the analysis by selecting the peptides having amino acid sequences shared between human and mouse GPC3. Furthermore, we have to consider the differences in the T cell repertoire in mice and humans. Thereby, we may miss GPC3 peptides recognized by human CTLs but not by mouse CTLs.

Considering ideal immunogenic target molecules for cancer immunotherapy, it is important to select a tumor antigen that could not be lost by tumor cells through immunoediting (28, 29). Recently, Capurro et al. reported that GPC3 is involved in the carcinogenesis and proliferation of HCC via regulation of noncanonical Wnt signals (30). Therefore, it may be possible that tumor cells cannot lose the GPC3 expression in order to continue to grow. Furthermore, according to an immunohistochemical analysis of the expression of HLA-class I molecules using newly developed specific mAb, EMR 8-5,[5] we found that almost all HCC cells expressed HLA-class I as far as we could examine (Table 2). For these reasons, we think that GPC3 is a very useful candidate as a target tumor antigen for the immunotherapy of HCC. We and others previously reported that the expression of GPC3 in HCC was detected from an early stage and the quantification of the soluble GPC3 protein in sera was useful for a diagnosis of HCC at an early stage (5, 7). As a result, GPC3-based immunotherapy might be able to prevent the appearance of HCC in patients with hepatitis B or C–based liver cirrhosis.

In this study, we found that it is possible to induce GPC3-reactive CTLs by the stimulation of PBMCs with the two major GPC3 epitopes *in vitro* in 50% of the HCC patients having an appropriate *HLA-class I* allele. However, it is necessary to investigate more patients to estimate the probability of a

successful induction of GPC3-reactive CTLs in HCC patients. We intended to know whether there was any correlation between successful induction of GPC3 peptide-reactive CTLs and prognosis or CTL infiltration into tumor tissue of these patients, therefore, we investigated the seven index cases; patients A2-10, A24-1, A24-2, A24-4, A24-9, A24-11, and A24-12, to see whether there was any correlation between successful induction of GPC3 peptide-reactive CTLs and prognosis or CTL infiltration into the tumor tissue of these patients. In three patients, A24-1, A24-4, and A24-12, who could generate GPC3 peptide-reactive CTLs, patient A24-12 recurred at 6 months after operation. In four patients, A2-10, A24-2, A24-9, and A24-11, who failed to induce GPC3-peptide-reactive CTLs, patient A24-9, whose HCC did not express GPC3, recurred at 6 months after operation, and patient A24-2 recurred at 3 months after operation and died 3 months after recurrence. These three recurred patients had extremely strong tumor invasion to the vasculature. Therefore, it was difficult to evaluate the correlation between the positive CTL response and clinical improvement at the present stage, and we have to increase the number of patients investigated and to do further statistical analyses on these relationships. In patients who could be examined for the infiltration of CD8-positive cells into their tumor specimens and for the existence of terminal deoxynucleotidyl transferase–mediated nick end labeling–positive cells in tumor tissue, patients A2-10, A24-1, A24-2, and A24-9, there was no strong correlation between the positive GPC3 peptide-reactive CTL response and for the existence of CD8-positive or terminal deoxynucleotidyl transferase–mediated nick end labeling–positive cells in the tumor tissues (data not shown). As shown in Fig. 4, we observed a regression of the tumor masses in NOD/SCID mice implanted with SK-Hep1/GPC3 and transferred i.v. with the GPC3 peptide-reactive CTLs in comparison to the mice injected with control CD8$^+$ T cells or saline alone. Although the regression of tumor growth was observed for 2 weeks after the second transfer of CTLs, the tumors began to enlarge again after that period. We thought it was important to continue the transfer of CTLs again and again to obtain continuous regression of the GPC3-expressing tumor. These data suggest that the adoptive i.v. transfer of GPC3-reactive CTLs into mice bearing GPC3$^+$ tumors was useful to inhibit tumor growth in the mouse tumor model.

In addition, it is most important to confirm the usefulness of GPC3-specific *in vivo* cancer immunotherapy in patients with HCC. Investigation of the presence of GPC3-specific CTLs in patients with melanoma are also eagerly awaited. We previously reported that DC differentiated *in vitro* from mouse embryonic stem cells transfected with the mouse *GPC3* gene (24, 31) induced protective immunity against mouse melanoma cell line B16 F10 (32). We are now preparing a translational study of GPC3-based immunotherapy to reduce the risk of recurrence in HCC patients treated surgically. We will try to use the GPC3 epitope peptides identified in this study first, whereas in the second phase, we will make a trial of the peptide-pulsed DC vaccine. We expect that GPC3-based immunotherapy may be a novel treatment strategy that could potentially help to prevent the appearance, advance, and/or recurrence of HCC and melanoma.

Acknowledgments

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Identification of HLA-A2- or HLA-A24-Restricted CTL Epitopes Possibly Useful for Glypican-3-Specific Immunotherapy of Hepatocellular Carcinoma

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