Table I. The affinity of Lengsin-derived peptides for HLA-A*0201 molecules.

Peptide (position)	Amino acid length	Peptide sequence	Binding score[a]	% MFI increase[b]
Lengsin(149-158)	10	LMPELSTFRV	2030	79
Lengsin(206-215)	10	FIYDFCIFGV	7699	65
Lengsin(270-279)	10	FLPEFGISSA	215	75
Lengsin(347-355)	9	GLLKHSAAL	79	44
HIV-gag(77-85)	9	SLYNTYATL	103	66

[a]Binding scores were estimated by using BIMAS software (http://www-bimas.cit.nih.gov/molbio/hla_bind/). [b]Percent MFI increase of HLA-A*0201 molecules on T2 cells. Percent MFI increase = (MFI with sample peptide - MFI without peptide)/MFI without peptide x 100. See the HLA-A*0201-binding assay in Materials and methods.

In the current study, we attempted to identify Lengsin-derived CTL epitope via a reverse immunology approach. We studied four Lengsin-derived peptides with the HLA-A*0201 binding motif selected by HLA-peptide binding predictions of the Bioinformatics and Molecular Analysis Section (BIMAS) program and evaluated their ability to provoke peptide-specific CTL responses in HLA-A*0201 transgenic (HHD) mice, and then we induced and generated peptide-specific CTL clones from peripheral blood lymphocytes of HLA-A*0201$^+$ healthy donors. We report here that the Lengsin(270-279) (FLPEFGISSA)-specific human CTL clones specifically recognize peptide-pulsed T2 cells, COS-7 transfectants expressing HLA-A*0201 and Lengsin, and HLA-A*0201$^+$/Lengsin$^+$ tumor cells in an HLA-A*0201-restricted manner.

Materials and methods

Animals. HLA-A*0201-transgenic HHD mice were described previously (9). H-2D$^{b-/-}$ β_2m$^{-/-}$ double knockout mice introduced with human β_2m-HLA-A2.1 (α1α2)-H-2D^b (α3 transmembrane cytoplasmic) (HHD) monochain construct gene were generated at the Département SIDA-Rétrovirus, Unité d'Immunité Cellulaire Antivirale, Institut Pasteur, France and kindly provided by Dr F.A. Lemonnier. The mice were kept under specific pathogen-free conditions. Mouse experiments were approved by the Animal Research Committee of National Cancer Center Hospital East.

Peptides and plasmids. Human Lengsin-derived peptides (purity >90%) sharing the amino acid sequences with mouse Lengsin and carrying binding motifs for HLA-A*0201-encoded molecules, were identified using HLA-peptide binding predictions of the Bioinformatics and Molecular Analysis Section (BIMAS) program (http://bismas.dcrt.nih.gov/molbio/hla_bind/index.html). We purchased a total of four Lengsin-derived peptides carrying HLA-A*0201 binding motifs (Table I) from GeneWorld (Tokyo, Japan). HIV-gag (77-85)(SLYNTYATL) peptide was used as an irrelevant peptide in murine and human CTL assays. Full-length Lengsin cDNA was obtained from a human lung carcinoma cell line and subcloned into pcDNA3.1 vector (Invitrogen) as described previously (16). Expression vector pcDNA3.1 containing the HLA-A*0201 cDNA was provided by Riken BRC (19). The HLA-A*0201 cDNA was subcloned into pIRES-puro vector (Clontech) for stable transfection.

Cell lines. Human liver cancer cell line SK-Hep-1 (HLA-A*0201$^+$/Lengsin$^-$) and simian COS-7 (HLA-A*0201$^-$/Lengsin$^-$) were obtained from the American Type Culture Collection (Manassas, VA, USA). Human lung carcinoma cell line 1-87 (HLA-A*0201$^-$/Lengsin$^+$) was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). T2 is a lymphoblastoid cell line that lacks TAP function and has HLA-A*0201 molecules that can be easily loaded with exogenous peptides. 1-87 and SK-Hep-1 were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco). T2 was cultured in RPMI supplemented with 10% heat-inactivated FBS. The 1-87-A0201 cell line (HLA-A*0201$^+$/Lengsin$^+$) was obtained by stable transfection of 1-87 with HLA-A*0201 cDNA, and the SK-Hep-1-Lengsin (HLA-A*0201$^+$/Lengsin$^+$) cell line was obtained by stable transfection of SK-Hep-1 with full-length Lengsin cDNA using FuGene® HD (Roche Applied Science). 1-87-A0201 and SK-Hep-1-Lengsin was cultured in DMEM supplemented with 10% heat-inactivated FBS containing 1 μg/ml puromycin (Sigma-Aldrich) and 1 mg/ml G418 (Gibco), respectively. In addition, COS-7 cells transiently expressing HLA-A*0201 and/or full-length Lengsin as the target cells were generated by cotransfection with pCDNA3.1 vector encoding these proteins using Lipofectamine™ 2000 (Invitrogen).

*HLA-A*0201-binding assay.* To determine the binding ability of the predicted peptides to HLA-A*0201 molecules, an *in vitro* cellular binding assay was performed as previously reported (20). Briefly, after incubation of T2 cells in culture medium at 26°C for 18 h, cells (2x10^5) were washed with PBS and suspended in 1 ml of Opti-MEM® (Invitrogen) with or without 100 μg of peptide, followed by incubation at 26°C for 3 h and then at 37°C for 3 h. After washing with PBS, HLA-A*0201 expression was measured by flow cytometry using FITC-conjugated HLA-A2-specific monoclonal antibody (mAb) (BB7.2; BioLegend), and mean fluorescence intensity (MFI) was recorded. Percent MFI increase was calculated as follows: Percent MFI increase = (MFI with the given peptide - MFI without peptide)/(MFI without peptide) x 100.

Induction of Lengsin-derived peptide-specific CTLs in HHD mice. In vivo immunization of mice and *in vitro* stimulation of primed spleen cells were performed as previously described (12). Briefly, bone marrow (BM) cells (2x10^6) from HHD mice

were cultured in RPMI-1640 supplemented with 10% FBS, together with granulocyte macrophage colony-stimulating factor (5 ng/ml) and 2-ME (0.8 ng/ml) for 7 days in 10-cm plastic dishes, and these bone-marrow-derived dendritic cells (BM-DCs) were pulsed with a mixture of the four Lengsin-derived peptides carrying HLA-A*0201 binding motifs (1 μM for each peptide) at 37°C for 2 h. We primed the HHD mice with this syngeneic BM-DC vaccine ($5x10^5$/mouse) into the peritoneal cavity once a week for two weeks. Seven days after the last immunization, the spleens were collected and CD4-negative spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any non-specific IFN-γ production by $CD4^+$ spleen cells cocultured with the BM-DCs. The CD4-negative spleen cells ($2x10^6$/well) were restimulated with syngeneic BM-DCs ($2x10^5$/well) pulsed once with the mixture of peptides *in vitro*. Six days later, the frequency of cells producing IFN-γ/$1x10^5$ CD4-negative spleen cells upon stimulation with syngeneic BM-DCs ($5x10^4$/well) pulsed with each peptide or the irrelevant peptide, was evaluated in an enzyme-linked immunospot (ELISPOT) assay as described below.

Generation of Lengsin peptide-specific $CD8^+$ T cell clones from human PBMCs. This study was approved by the Ethics Committee of the National Cancer Center, and conforms to the ethical guidelines of the Declaration of Helsinki (1995). CTL clones were generated using the methods described previously with some modifications (21). Blood samples were collected from HLA-A*0201^+ healthy donors, after informed consent was obtained. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Paque (GE Healthcare) density gradient centrifugation. PBMCs were cultured ($5x10^6$ cells/well) with 1 or 0.1 μg/ml Lengsin-derived peptide in AIM-V® medium supplemented with 10% human AB serum, 10 U/ml recombinant human interleukin (rhIL)-2 (Chiron, Emeryville, CA, USA) once a week for two or three weeks and supplemented with 10 U/ml rhIL-2 between stimulations. Seven days after the last stimulation, $CD8^+$ T cells were isolated using a CD8 T-cell isolation kit (Miltenyi Biotec) and plated at 3, 1 and 0.3 cells/well in 96-well round-bottom plates with 5 μg/ml PHA-P (Wako, Tokyo, Japan), 200 U/ml rhIL-2 and $8x10^4$ cells/well allogeneic irradiated (100 Gy) PBMCs. Proliferating T cell clones were screened for peptide-specific IFN-γ production by ELISPOT assay using T2 cells pulsed with or without the immunizing peptide.

Enzyme-linked immunosorbent spot (ELISPOT) assay. Specific IFN-γ secretion of murine and human CTLs in response to stimulator cells was assayed using the IFN-γ ELISPOT Kit (BD Biosciences) according to the manufacturer's instructions. Stimulator cells were pulsed with or without peptide for 1 h at 37°C and then washed thrice. Responder cells ($5x10^4$/well) were incubated with stimulator cells for 20 h. In blocking experiments, peptide-pulsed T2 or tumor cells were preincubated with HLA-A2-specific mAb (BB7.2) or isotype control mAb (mIgG2b; BioLegend) for 1 h. The resulting spots were counted using an ELIPHOTO counter (Minerva Tech, Tokyo, Japan).

Cytotoxicity assay. To measure cytolytic activity of the CTL clones, calcein AM release-based cytotoxic cell assay was performed as described previously (22,23). Briefly, $2x10^6$ target cells were labeled with green fluorescent probe calcein AM at 37°C for 30 min and washed thrice. Target cells were pulsed with or without peptide for 1 h at 37°C and washed thrice. Effector cells were incubated with $1x10^4$ target cells for 4 h at the indicated effector-to-target (E:T) ratios. The fluorescence emitted by target cells was measured using a Terascan system (Minerva Tech, Tokyo, Japan) before and after coincubation with effector cells. Percentage of specific lysis was determined as: (experimental release - spontaneous release)/(maximal release - spontaneous release) x 100.

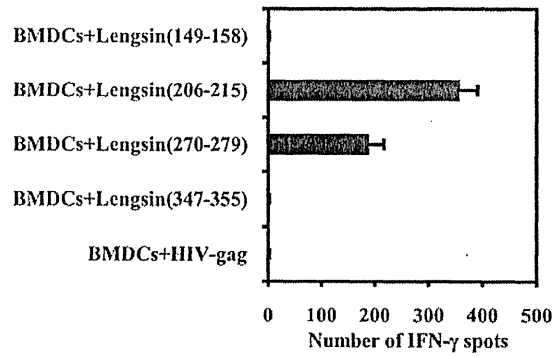

Figure 1. Immunogenicity of Lengsin-derived peptides in HHD mice. CD4-negative spleen cells from HHD mice vaccinated with a mixture of four peptides were restimulated once *in vitro* with BM-DCs loaded with the mixture of peptides. CTLs were tested for specificity of each peptide by IFN-γ ELISPOT assay. CTLs were stimulated with BM-DCs pulsed with 1 μM of each peptide or an irrelevant peptide (HIV-gag). Results are expressed as the mean ± SD. A representative of three experiments is shown.

Results

*Identification of Lengsin-derived peptide binding to HLA-A*0201 molecules.* As the candidates of HLA-A*0201-restricted and human Lengsin-derived CTL epitopes, we selected four peptides having high predicted HLA-A*0201-binding scores calculated using the BIMAS software program, and we evaluated their binding ability to HLA-A*0201 molecules (Table I). All four peptides were able to bind to HLA-A*0201 molecules.

Induction of CTL response against the Lengsin-derived peptides in HHD mice. To evaluate the immunogenic potential of the four predicted HLA-A*0201-binding peptides derived from Lengsin, we immunized HHD mice with BM-DCs pulsed with a mixture of the four peptides. The results of ELISPOT assays revealed that the CD4-negative spleen cells stimulated *in vitro* with BM-DCs efficiently produced IFN-γ when pulsed with the Lengsin(206-215) or Lengsin(270-279) but not with Lengsin(149-158), Lengsin(347-355), or HIV-gag peptides (Fig. 1). Similar results were obtained in three independent experiments. These results suggest that Lengsin(206-215) and Lengsin(270-279) have immunogenic potential and are able to induce peptide-specific CTLs in HHD mice.

Generation of Lengsin-peptide-specific CTL clones from human PBMCs. Next, we assessed the capacity of Lengsin(206-

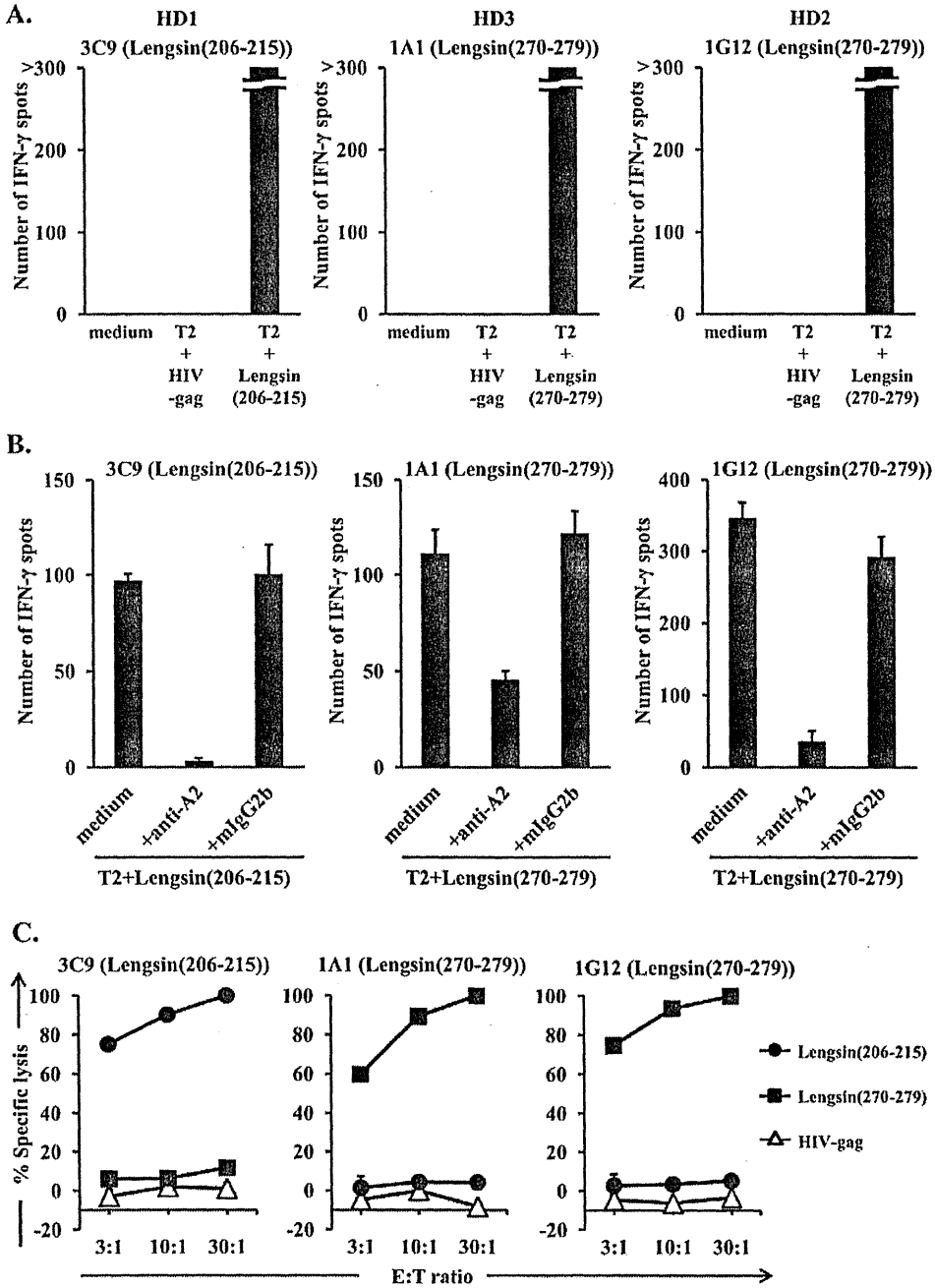

Figure 2. Recognition of peptide-pulsed T2 cells by human CTL clones. (A) Lengsin(206-215)-specific CTL clone 3C9 ($1x10^4$ cells) was stimulated with T2 cells pulsed with 1 μM Lengsin(206-215) or HIV-gag peptide. Lengsin(270-279)-specific CTL clones 1A1 and 1G12 ($1x10^4$ cells) were stimulated with T2 pulsed with 1 μM Lengsin(270-279) or HIV-gag peptide. IFN-γ-producing CTLs were detected by IFN-γ ELISPOT. (B) Blocking experiments were performed using HLA-A2-specific mAb (BB7.2) or isotype control mAb (mIgG2b). Each CTL clone ($1x10^3$ cells) was incubated with cognate peptide-pulsed T2 cells as stimulator cells. IFN-γ-producing CTLs were detected by IFN-γ ELISPOT. (C) The cytolytic activity of Lengsin(206-215)-specific CTL clone 3C9 or Lengsin(270-279)-specific CTL clones 1A1 and 1G12 was assessed by cytotoxicity assay against T2 cells pulsed with 1 μM Lengsin(206-215) (•), Lengsin(270-279) (■) or HIV-gag (△) peptide. Results are expressed as the mean ± SD.

215) or Lengsin(270-279) to generate peptide-specific CTLs *in vitro* from human PBMCs of HLA-A*0201$^+$ healthy donors. CTLs were induced by two or three weekly *in vitro* stimulations with the Lengsin(206-215) or Lengsin(270-279) and were subsequently cloned by limiting dilution. We obtained one CTL clone (clone 3C9 derived from healthy donor (HD)1), which was able to specifically recognize T2 cells pulsed with Lengsin(206-215) but not T2 cells pulsed with irrelevant HIV-gag peptide, and two CTL clones (clone 1A1 derived from HD3 and clone 1G12 derived from HD2), which were able to specifically recognize T2 cells pulsed with Lengsin(270-279) but not T2 cells pulsed with HIV-gag peptide, assessed by IFN-γ ELISPOT assay (Fig. 2A). These three CTL clones had a CD3$^+$CD8$^+$CD4$^-$ phenotype determined by flow cytometric

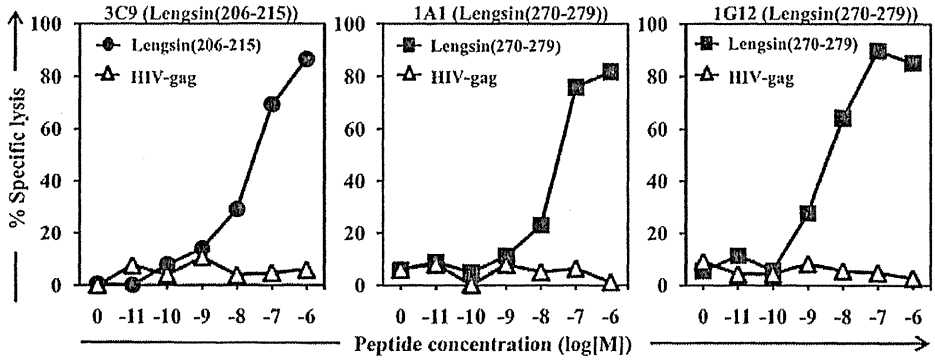

Figure 3. Functional avidity of CTL clones was determined by peptide titration experiments in cytotoxicity assay. Lengsin(206-215)-specific CTL clone 3C9 was incubated with titrated Lengsin(206-215) (●) or HIV-gag (△) peptide on T2 cells at an E:T ratio of 10:1. Lengsin(270-279)-specific CTL clones 1A1 and 1G12 were incubated with titrated Lengsin(270-279) (■) or HIV-gag (△) peptide on T2 cells at an E:T ratio of 10:1. A representative of two experiments is shown.

analysis (data not shown). In addition, IFN-γ production of CTL clones against cognate peptide-pulsed T2 was inhibited by HLA-A2-specific mAb, but not isotype control mAb, indicating that recognition by these CTL clones was HLA-A2 restricted (Fig. 2B). Moreover, we evaluated the cytolytic activity of these CTL clones against cognate peptide-pulsed T2 cells (Fig. 2C). Lengsin(206-215)-specific CTL clone 3C9 could specifically lyse Lengsin(206-215)-pulsed T2 cells but not Lengsin(270-279)- or HIV-gag peptide-pulsed T2 cells, and Lengsin(270-279)-specific CTL clones 1A1 and 1G12 could specifically lyse Lengsin(270-279)-pulsed T2 cells but not Lengsin(206-215)- or HIV-gag peptide-pulsed T2 cells. These results suggest that Lengsin(206-215) or Lengsin (270-279) can induce peptide-specific CTLs from human PBMCs, and Lengsin(206-215)- or Lengsin(270-279)-specific CTL clones specifically recognize cognate peptide-pulsed T2 cells in an HLA-A*0201-restricted manner.

Functional avidity of Lengsin-specific CTL clones. To evaluate the functional avidity of Lengsin(206-215)- or Lengsin(270-279)-specific CTL clones for cognate peptide-MHC ligands, peptide titration experiments were performed in cytotoxicity assay (Fig. 3). The peptide concentration required to obtain half-maximal lysis (EC50) by CTL clones 3C9, 1A1 and 1G12 was 11.0, 11.2 and 4.0 nM, respectively. These results suggest that Lengsin(206-215)-specific CTL clone 3C9 and Lengsin(270-279)-specific CTL clone 1A1 have approximately similar avidity, and Lengsin(270-279)-specific CTL clone 1G12 has relatively higher avidity than these two clones for cognate peptide-MHC ligands.

Lengsin(270-279)-specific CTL clone recognizes Lengsin-transfected target cells in the absence of peptide, but Lengsin (206-215)-specific CTL clone fails. Next, we evaluated the ability of Lengsin(206-215)- or Lengsin(270-279)-specific CTL clones to recognize HLA-A*0201$^+$/Lengsin$^+$ target cells. These CTL clones were incubated with COS-7 cells expressing HLA-A*0201 and/or Lengsin, SK-Hep-1-mock (HLA-A*0201$^+$/Lengsin$^-$) and SK-Hep-1-Lengsin (HLA-A*0201$^+$/Lengsin$^+$) in the presence or absence of cognate peptide (Fig. 4). Specific IFN-γ production of Lengsin(270-279)-specific CTL clones 1A1 and 1G12 was detectable against COS-7 cells expressing both HLA-A*0201 and Lengsin, but not the non-treated, and only HLA-A*0201 or Lengsin-cDNA-transfected COS-7 cells in the absence of cognate peptide. In contrast, IFN-γ production of Lengsin(206-215)-specific CTL clone 3C9 was not detectable against COS-7 cells expressing both HLA-A*0201 and Lengsin in the absence of cognate peptide (Fig. 4A). Furthermore, specific IFN-γ production of Lengsin(270-279)-specific CTL clones 1A1 and 1G12 was detectable against both HLA-A*0201 and Lengsin-positive cell line SK-Hep-1-Lengsin (HLA-A*0201$^+$/Lengsin$^+$), but not Lengsin-negative SK-Hep-1-mock (HLA-A*0201$^+$/Lengsin$^-$) in the absence of cognate peptide (Fig. 4B). The specific IFN-γ production was blocked by HLA-A2-specific mAb, but not the isotype control, suggesting that the observed production was HLA-A2 restricted (Fig. 4C). In contrast, IFN-γ production of Lengsin(206-215)-specific CTL clone 3C9 was not detectable against SK-Hep-1-Lengsin in the absence of cognate peptide (Fig. 4B). These results suggest that the Lengsin(270-279)-specific CTL clones can specifically recognize Lengsin-transfected HLA-A*0201$^+$ target cells; however, the Lengsin(206-215)-specific CTL clone is unable to recognize these target cells unless Lengsin(206-215) is exogenously added.

*Lengsin(270-279)-specific CTL clone also recognizes HLA-A*0201$^+$ lung carcinoma cells endogenously expressing Lengsin.* We previously reported that Lengsin was expressed in the lung carcinoma cell line 1-87 (16). In order to investigate whether Lengsin(270-279) is naturally processed and presented on the surface of lung carcinoma cells, we generated a 1-87-A0201 (HLA-A*0201$^+$/Lengsin$^+$) stable cell line as a target of both HLA-A*0201 and Lengsin-positive lung carcinoma cells. Lengsin(206-215)-specific CTL clone 3C9 or Lengsin(270-279)-specific CTL clone 1G12 was incubated with 1-87-mock (HLA-A*0201$^-$/Lengsin$^+$) or 1-87-A0201 (HLA-A*0201$^+$/Lengsin$^+$) in the presence or absence of cognate peptide. Lengsin(270-279)-specific CTL clone 1G12 was able to specifically recognize 1-87-A0201 (HLA-A*0201$^+$/Lengsin$^+$) but not the non-HLA-A*0201 cell line 1-87-mock (HLA-A*0201$^-$/Lengsin$^+$) in the absence of cognate peptide (Fig. 5A). The specific IFN-γ production was blocked by HLA-A2-specific mAb but not the isotype control mAb, suggesting that the observed IFN-γ production was HLA-A2 restricted

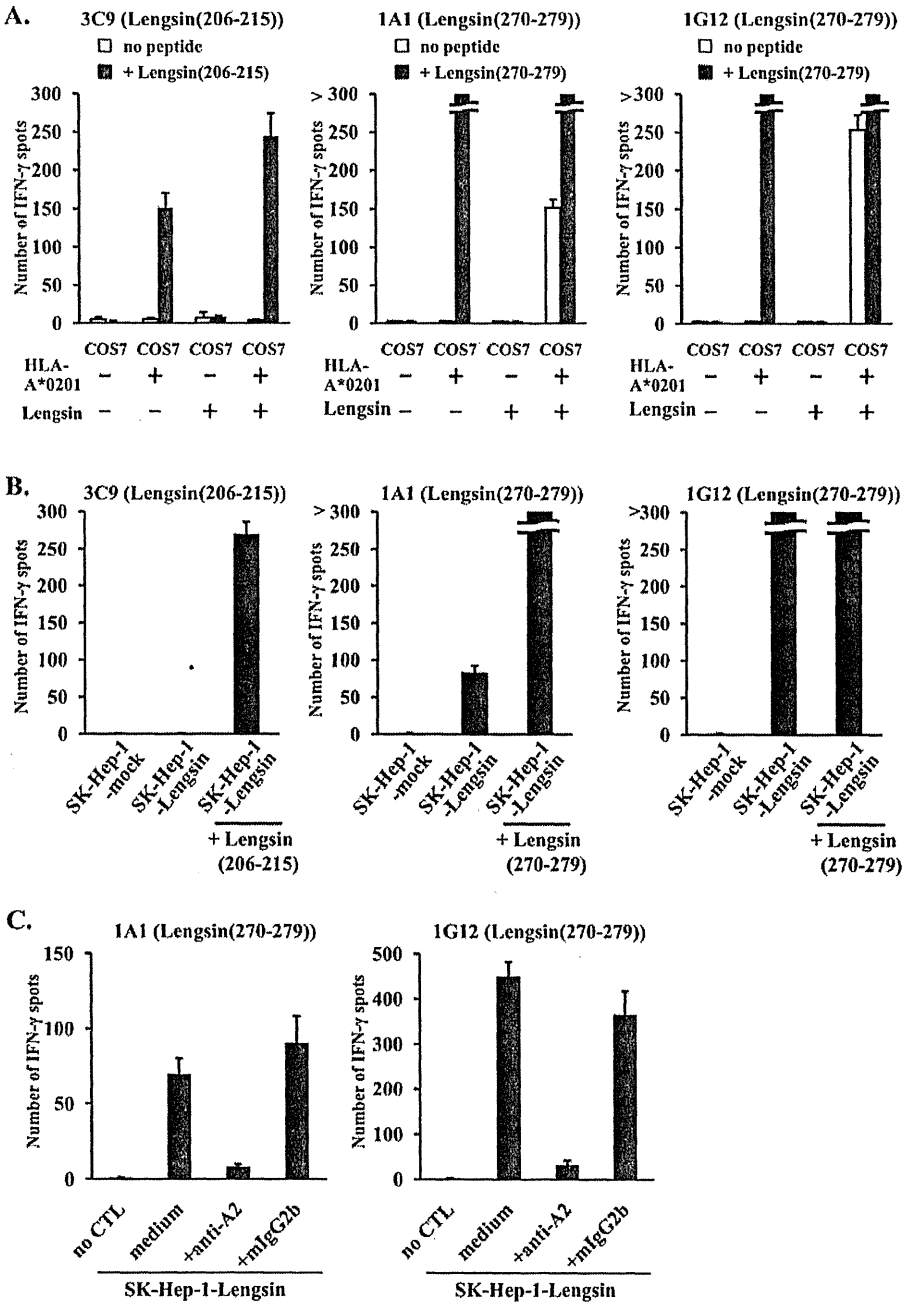

Figure 4. Recognition of Lengsin transfectant cells by Lengsin-specific CTL clones. IFN-γ-producing CTLs were detected by IFN-γ ELISPOT. (A) COS-7 cells were transiently transfected with plasmids encoding HLA-A*0201 and/or Lengsin. Forty-eight hours after transfection, Lengsin(206-215)-specific CTL clone 3C9 or Lengsin(270-279)-specific CTL clones 1A1 and 1G12 ($1x10^4$ cells) were incubated with each type of transfected cells pulsed (solid bars) or not (shaded bars) for 1 h with 1 μM indicated peptides. (B) Lengsin(206-215)-specific CTL clone 3C9 or Lengsin(270-279)-specific CTL clones 1A1 and 1G12 were incubated with SK-Hep-1-mock (HLA-A*0201$^+$/Lengsin$^-$) or SK-Hep-1-Lengsin (HLA-A*0201$^+$/Lengsin$^+$) pulsed or not for 1 h with 1 μM indicated peptides. (C) Blocking experiments were performed using HLA-A2-specific mAb (BB7.2) or isotype control mAb (mIgG2b). Lengsin(270-279)-specific CTL clones 1A1 and 1G12 ($1x10^4$ cells) were incubated with SK-Hep-1-Lengsin (HLA-A*0201$^+$/Lengsin$^+$) as stimulator cells. Results are expressed as the mean ± SD.

(Fig. 5B). In addition, Lengsin(270-279)-specific CTL clone 1G12 was able to specifically lyse 1-87-A0201 (HLA-A*0201$^+$/Lengsin$^+$) (Fig. 5C). In contrast, Lengsin(206-215)-specific CTL clone 3C9 was unable to recognize and lyse 1-87-A0201 (HLA-A*0201$^+$/Lengsin$^+$) in the absence of cognate peptide (Fig. 5A and C). These results suggest that the Lengsin(270-279)-specific CTL clone can recognize the HLA-A*0201$^+$ lung carcinoma cell line endogenously expressing Lengsin in an HLA-A*0201-restricted fashion; therefore, Lengsin(270-279) is naturally processed and presented by HLA-A*0201 molecules on the surface of lung carcinoma cells expressing Lengsin.

Discussion

In order to demonstrate that predicted candidate peptides are naturally presented peptides on tumor cells, it is necessary that

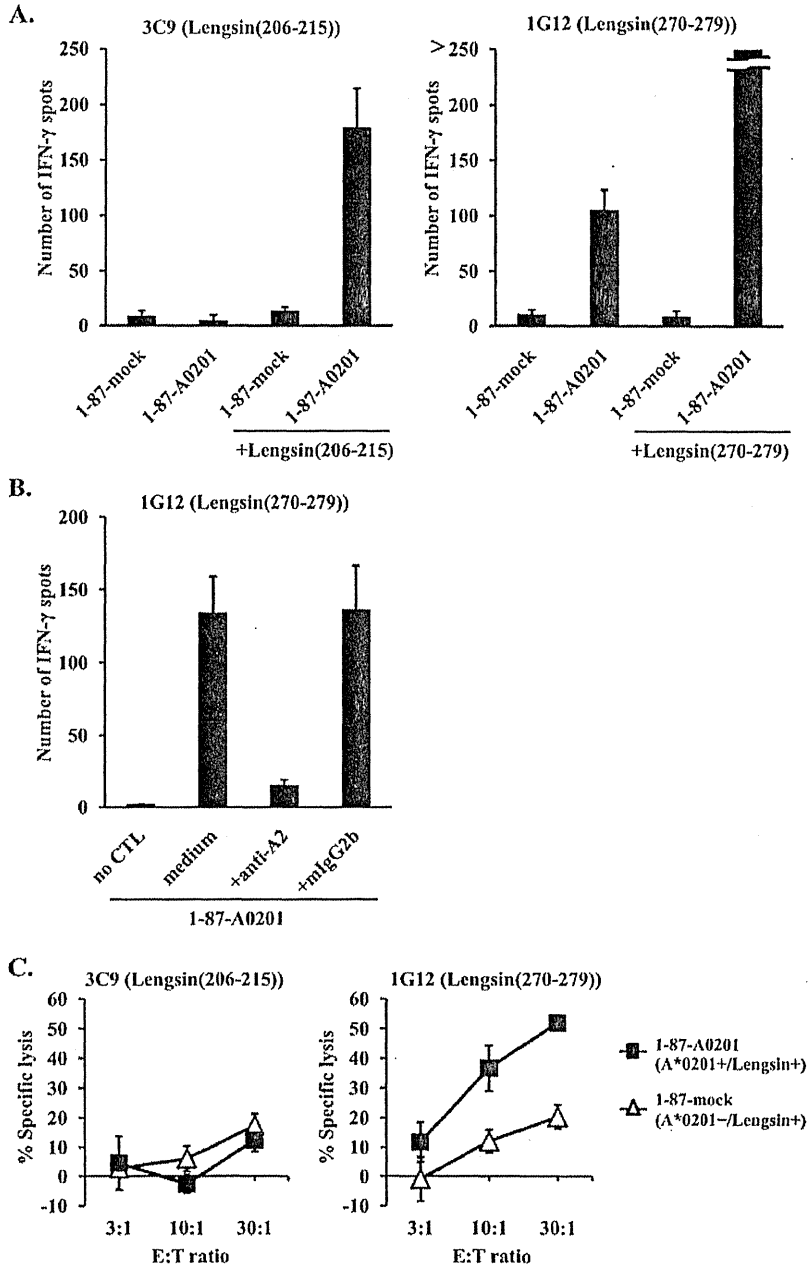

Figure 5. Recognition of lung carcinoma cells endogenously expressing Lengsin by Lengsin-specific CTL clones. (A) Lengsin(206-215)-specific CTL clone 3C9 or Lengsin(270-279)-specific CTL clone 1G12 ($5x10^5$ cells) was incubated with 1-87-mock (HLA-A*0201$^-$/Lengsin$^+$) or 1-87-A0201 (HLA-A*0201$^+$/Lengsin$^+$) pulsed or not for 1 h with 1 μM indicated peptides. IFN-γ-producing CTLs were detected by IFN-γ ELISPOT. (B) Blocking experiments were performed using HLA-A2-specific mAb (BB7.2) or isotype control mAb (mIgG2b). Lengsin(270-279)-specific CTL clone 1G12 ($5x10^5$ cells) was incubated with 1-87-A0201 (HLA-A*0201$^+$/Lengsin$^+$) as stimulator cells. IFN-γ-producing CTLs were detected by IFN-γ ELISPOT. (C) Specific lysis of lung carcinoma cell lines 1-87-mock (HLA-A*0201$^-$/Lengsin$^+$, △) and 1-87-A0201 (HLA-A*0201$^+$/Lengsin$^+$, ■) by Lengsin(206-215)-specific CTL clone 3C9 or Lengsin (270-279)-specific CTL clone 1G12. Results are expressed as the mean ± SD.

peptide-specific CTL clones or lines are induced by the peptides and the CTL clones or lines specifically recognize the tumor cells. In this study, Lengsin(206-215) and Lengsin(270-279) were able to induce peptide-specific CTLs in HHD mice and humans, indicating that these peptides could be immunogenic. Especially, we were able to establish each peptide-specific CTL clone from human PBMCs. The Lengsin(270-279)-specific CTL clones specifically recognized cognate peptide-pulsed T2 cells and HLA-A*0201$^+$ tumor cells endogenously expressing Lengsin, suggesting that Lengsin(270-279) is naturally processed and presented on the surface of Lengsin-expressing tumor cells in association with HLA-A*0201. Therefore, Lengsin(270-279) may be a new target for antigen-specific T-cell immunotherapy against lung cancer. However, the Lengsin(206-215)-specific CTL clone failed to recognize those tumor cells expressing Lengsin, although it showed efficient recognition against HLA-A*0201$^+$ target cells in the presence of exogenously added peptide.

CTLs induced by reverse immunology approaches often fail to recognize HLA-matched tumor targets expressing specific genes (24-27). Possible explanations with respect to each factor of the induced CTLs and the targeted tumor cells seem plausible.

With regard to the factor of induced CTLs, they might not have sufficient avidity to recognize a limited number of naturally presented peptides on the surface of tumor cells (28-30). Consequently, it is necessary to induce high-avidity tumor-reactive CTLs. Reported methods for inducing high-avidity CTLs include stimulation with low concentration of peptides (31,32), with three costimulatory molecules (B7-1, ICAM-1 and LFA-3) (33), cultured with IL-12 (34) or IL-15 (35), and using allogeneic PBMCs as a source of CTLs (36-38). Additionally, it is very important to demonstrate recognition of tumor cells by CTL clones but not bulk CTL populations in terms of specificity. The bulk CTL populations that were used may have contained distinct CTL clones, some of which were responsible for peptide recognition and others that accounted for the apparent tumor reactivity (39). In fact, it has been reported that hTERT(540-548) is controversial as to its status as a naturally processed and presented peptide due to different results depending on assessment of bulk CTL populations (11,40) or CTL clones (41-43). Therefore, it is also important to establish highly avid tumor-reactive CTL clones for evaluation of specific tumor reactivity of CTLs. Melanoma-associated antigen-specific high-avidity tumor-reactive CTL clones have been generated by CD107a-guided sorting (44). In our laboratory, we recently generated GPC3(144-152)-specific high-avidity tumor-reactive CTL clones by CD107a-guided sorting from PBMCs of patients vaccinated with GPC3(144-152) peptide (45). In this study, we evaluated tumor reactivity using CTL clones, but not bulk CTL populations. Lengsin(206-215)-specific high-avidity tumor-reactive CTL clones could possibly be induced and generated by further studies using the above methods.

With regard to the factor of targeted tumor cells, the predicted candidate peptides might not be processed and presented on the surface of tumor cells. In this study, Lengsin(206-215)-specific CTL clone 3C9 and Lengsin(270-279)-specific CTL clone 1A1 showed approximately similar avidity in peptide titration experiments. CTL clone 1A1 recognized target cells expressing Lengsin; however, CTL clone 3C9 failed. For this reason, Lengsin(206-215) might not be processed and presented on target cells. Peptides presented by MHC class I molecules are usually derived from intracellular proteins that are degraded by the proteasome (46). In contrast, by using the proteasome inhibitor lactacystin, it has happened that the expression of naturally processed peptide is sharply increased by a proteasome-independent mechanism, resulting in enhanced recognition by CTL clones (25,47-49). It would be interesting to examine whether Lengsin(206-215)- or Lengsin(270-279)-specific CTL clones recognize proteasome inhibitor-treated tumor cells.

As a CTL-independent approach, tandem mass spectrometry (MS) can provide direct identification of naturally presented peptides eluted from MHC class I molecules (50-52). Using tandem MS analysis, we can positively demonstrate whether Lengsin(206-215) or Lengsin(270-279) are naturally presented peptides on the surface of tumor cells. In addition, a new presented peptide derived from Lengsin might be detected.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Clinical Research from the Ministry of Health, Labour and Welfare, Japan. Human HLA-A*02011 cDNA (RDB no. 2866) was provided by RIKEN BRC through the National Bio-Resources Project of MEXT, Japan.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Morgan RA, Dudley ME, Wunderlich JR, *et al*: Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314: 126-129, 2006.
3. Johnson LA, Morgan RA, Dudley ME, *et al*: Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood 114: 535-546, 2009.
4. Robbins PF, Morgan RA, Feldman SA, *et al*: Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 29: 917-924, 2011.
5. Browning M and Krausa P: Genetic diversity of HLA-A2: evolutionary and functional significance. Immunol Today 17: 165-170, 1996.
6. Viatte S, Alves PM and Romero P: Reverse immunology approach for the identification of CD8 T-cell-defined antigens: advantages and hurdles. Immunol Cell Biol 84: 318-330, 2006.
7. van der Bruggen P, Bastin J, Gajewski T, *et al*: A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. Eur J Immunol 24: 3038-3043, 1994.
8. Celis E, Tsai V, Crimi C, *et al*: Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. Proc Natl Acad Sci USA 91: 2105-2109, 1994.
9. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA and Perarnau B: HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. J Exp Med 185: 2043-2051, 1997.
10. Firat H, Garcia-Pons F, Tourdot S, *et al*: H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies. Eur J Immunol 29: 3112-3121, 1999.
11. Minev B, Hipp J, Firat H, Schmidt JD, Langlade-Demoyen P and Zanetti M: Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. Proc Natl Acad Sci USA 97: 4796-4801, 2000.
12. Komori H, Nakatsura T, Senju S, *et al*: Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 12: 2689-2697, 2006.
13. Passoni L, Scardino A, Bertazzoli C, *et al*: ALK as a novel lymphoma-associated tumor antigen: identification of 2 HLA-A2.1-restricted CD8$^+$ T-cell epitopes. Blood 99: 2100-2106, 2002.
14. Hassainya Y, Garcia-Pons F, Kratzer R, *et al*: Identification of naturally processed HLA-A2--restricted proinsulin epitopes by reverse immunology. Diabetes 54: 2053-2059, 2005.
15. Alves PM, Faure O, Graff-Dubois S, *et al*: EphA2 as target of anticancer immunotherapy: identification of HLA-A*0201-restricted epitopes. Cancer Res 63: 8476-8480, 2003.
16. Nakatsugawa M, Hirohashi Y, Torigoe T, *et al*: Novel spliced form of a lens protein as a novel lung cancer antigen, Lengsin splicing variant 4. Cancer Sci 100: 1485-1493, 2009.
17. Lang RA: Apoptosis in mammalian eye development: lens morphogenesis, vascular regression and immune privilege. Cell Death Differ 4: 12-20, 1997.
18. Martin WD, Egan RM, Stevens JL and Woodward JG: Lens-specific expression of a major histocompatibility complex class I molecule disrupts normal lens development and induces cataracts in transgenic mice. Invest Ophthalmol Vis Sci 36: 1144-1154, 1995.

19. Akatsuka Y, Goldberg TA, Kondo E, *et al*: Efficient cloning and expression of HLA class I cDNA in human B-lymphoblastoid cell lines. Tissue Antigens 59: 502-511, 2002.
20. Hirohashi Y, Torigoe T, Maeda A, *et al*: An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. Clin Cancer Res 8: 1731-1739, 2002.
21. Tsukahara T, Kawaguchi S, Torigoe T, *et al*: HLA-A*0201-restricted CTL epitope of a novel osteosarcoma antigen, papilloma-virus binding factor. J Transl Med 7: 44, 2009.
22. Wang XM, Terasaki PI, Rankin GW Jr, Chia D, Zhong HP and Hardy S: A new microcellular cytotoxicity test based on calcein AM release. Hum Immunol 37: 264-270, 1993.
23. Kondo M, Sakuta K, Noguchi A, *et al*: Zoledronate facilitates large-scale ex vivo expansion of functional gammadelta T cells from cancer patients for use in adoptive immunotherapy. Cytotherapy 10: 842-856, 2008.
24. Tanzarella S, Fleischhauer K, van Endert P, Bordignon C and Traversari C: Characterization of antigenic peptide epitopes by reverse immunology: induction of cytotoxic T lymphocytes specific for exogenous peptide only. Int J Cancer 72: 912-915, 1997.
25. Noppen C, Levy F, Burri L, *et al*: Naturally processed and concealed HLA-A2.1-restricted epitopes from tumor-associated antigen tyrosinase-related protein-2. Int J Cancer 87: 241-246, 2000.
26. So T, Hanagiri T, Chapiro J, *et al*: Lack of tumor recognition by cytolytic T lymphocyte clones recognizing peptide 195-203 encoded by gene MAGE-A3 and presented by HLA-A24 molecules. Cancer Immunol Immunother 56: 259-269, 2007.
27. Parkhurst MR, Fitzgerald EB, Southwood S, Sette A, Rosenberg SA and Kawakami Y: Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma antigen tyrosinase-related protein 2 (TRP2). Cancer Res 58: 4895-4901, 1998.
28. Yee C, Savage PA, Lee PP, Davis MM and Greenberg PD: Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. J Immunol 162: 2227-2234, 1999.
29. Karbach J, Gnjatic S, Pauligk C, *et al*: Tumor-reactive CD8$^+$ T-cell clones in patients after NY-ESO-1 peptide vaccination. Int J Cancer 121: 2042-2048, 2007.
30. Wilde S, Sommermeyer D, Frankenberger B, *et al*: Dendritic cells pulsed with RNA encoding allogeneic MHC and antigen induce T cells with superior antitumor activity and higher TCR functional avidity. Blood 114: 2131-2139, 2009.
31. Alexander-Miller MA, Leggatt GR and Berzofsky JA: Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc Natl Acad Sci USA 93: 4102-4107, 1996.
32. Zeh HJ 3rd, Perry-Lalley D, Dudley ME, Rosenberg SA and Yang JC: High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. J Immunol 162: 989-994, 1999.
33. Hodge JW, Chakraborty M, Kudo-Saito C, Garnett CT and Schlom J: Multiple costimulatory modalities enhance CTL avidity. J Immunol 174: 5994-6004, 2005.
34. Xu S, Koski GK, Faries M, *et al*: Rapid high efficiency sensitization of CD8$^+$ T cells to tumor antigens by dendritic cells leads to enhanced functional avidity and direct tumor recognition through an IL-12-dependent mechanism. J Immunol 171: 2251-2261, 2003.
35. Oh S, Perera LP, Burke DS, Waldmann TA and Berzofsky JA: IL-15/IL-15Ralpha-mediated avidity maturation of memory CD8$^+$ T cells. Proc Natl Acad Sci USA 101: 15154-15159, 2004.
36. Sadovnikova E, Jopling LA, Soo KS and Stauss HJ: Generation of human tumor-reactive cytotoxic T cells against peptides presented by non-self HLA class I molecules. Eur J Immunol 28: 193-200, 1998.
37. Savage P, Gao L, Vento K, *et al*: Use of B cell-bound HLA-A2 class I monomers to generate high-avidity, allo-restricted CTLs against the leukemia-associated protein Wilms tumor antigen. Blood 103: 4613-4615, 2004.
38. Kronig H, Hofer K, Conrad H, *et al*: Allorestricted T lymphocytes with a high avidity T-cell receptor towards NY-ESO-1 have potent anti-tumor activity. Int J Cancer 125: 649-655, 2009.
39. Parkhurst MR, Riley JP, Igarashi T, Li Y, Robbins PF and Rosenberg SA: Immunization of patients with the hTERT:540-548 peptide induces peptide-reactive T lymphocytes that do not recognize tumors endogenously expressing telomerase. Clin Cancer Res 10: 4688-4698, 2004.
40. Vonderheide RH, Hahn WC, Schultze JL and Nadler LM: The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunity 10: 673-679, 1999.
41. Ayyoub M, Migliaccio M, Guillaume P, *et al*: Lack of tumor recognition by hTERT peptide 540-548-specific CD8(+) T cells from melanoma patients reveals inefficient antigen processing. Eur J Immunol 31: 2642-2651, 2001.
42. Purbhoo MA, Li Y, Sutton DH, *et al*: The HLA-A*0201-restricted hTERT(540-548) peptide is not detected on tumor cells by a CTL clone or a high-affinity T-cell receptor. Mol Cancer Ther 6: 2081-2091, 2007.
43. Chen DY, Vance BA, Thompson LB, Domchek SM and Vonderheide RH: Differential lysis of tumors by polyclonal T cell lines and T cell clones specific for hTERT. Cancer Biol Ther 6: 1991-1996, 2007.
44. Rubio V, Stuge TB, Singh N, Betts MR, Weber JS, Roederer M and Lee PP: Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. Nat Med 9: 1377-1382, 2003.
45. Yoshikawa T, Nakatsugawa M and Suzuki S: HLA-A2-restricted glypican-3 peptide-specific CTL clones induced by peptide vaccine show high avidity and antigen-specific killing activity against tumor cells. Cancer Sci 102: 918-925, 2011.
46. Rock KL and Goldberg AL: Degradation of cell proteins and the generation of MHC class I-presented peptides. Annu Rev Immunol 17: 739-779, 1999.
47. Wherry EJ, Golovina TN, Morrison SE, Sinnathamby G, McElhaugh MJ, Shockey DC and Eisenlohr LC: Re-evaluating the generation of a 'proteasome-independent' MHC class I-restricted CD8 T cell epitope. J Immunol 176: 2249-2261, 2006.
48. Valmori D, Gileadi U, Servis C, *et al*: Modulation of proteasomal activity required for the generation of a cytotoxic T lymphocyte-defined peptide derived from the tumor antigen MAGE-3. J Exp Med 189: 895-906, 1999.
49. Luckey CJ, King GM, Marto JA, *et al*: Proteasomes can either generate or destroy MHC class I epitopes: evidence for nonproteasomal epitope generation in the cytosol. J Immunol 161: 112-121, 1998.
50. Cox AL, Skipper J, Chen Y, *et al*: Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. Science 264: 716-719, 1994.
51. Schirle M, Keilholz W, Weber B, *et al*: Identification of tumor-associated MHC class I ligands by a novel T cell-independent approach. Eur J Immunol 30: 2216-2225, 2000.
52. Hirano N, Butler MO, Xia Z, *et al*: Identification of an immunogenic CD8$^+$ T-cell epitope derived from gamma-globin, a putative tumor-associated antigen for juvenile myelomonocytic leukemia. Blood 108: 2662-2668, 2006.

Cancer Science
The official journal of the Japanese Cancer Association JCA

Glypican-3 could be an effective target for immunotherapy combined with chemotherapy against ovarian clear cell carcinoma

Shiro Suzuki,[1,2] Toshiaki Yoshikawa,[1] Tomoya Hirosawa,[2,3] Kiyosumi Shibata,[2] Fumitaka Kikkawa,[2] Yoshiki Akatsuka[3,4] and Tetsuya Nakatsura[1,5]

[1]Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Chiba; [2]Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, Nagoya; [3]Division of Immunology, Aichi Cancer Center Research Institute, Nagoya; [4]Department of Hematology and Oncology, Fujita Health University, Aichi, Japan

(Received February 8, 2011/Revised June 2, 2011/Accepted June 5, 2011/Accepted manuscript online June 10, 2011/Article first published online July 8, 2011)

Glypican-3 (GPC3) is useful not only as a novel tumor marker, but also as an oncofetal antigen for immunotherapy. We recently established HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clones from hepatocellular carcinoma patients after $GPC3_{144-152}$ peptide vaccination. The present study was designed to evaluate the tumor reactivity of a HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone against ovarian clear cell carcinoma (CCC) cell lines. The $GPC3_{144-152}$ peptide-specific CTL clone could recognize HLA-A2-positive and GPC3-positive ovarian CCC cell lines on interferon (IFN)-γ enzyme-linked immunospot assay and showed cytotoxicity against KOC-7c cells. The CTL clone recognized naturally processed GPC3-derived peptide on ovarian CCC cells in a HLA class I-restricted manner. Moreover, we confirmed that the level of GPC3 expression was responsible for CTL recognition and that subtoxic-dose chemotherapy made tumor cells more susceptible to the cytotoxic effect of CTL. Thus, it might be possible to treat ovarian CCC patients by combining chemotherapy with immunotherapy. Our data suggest that GPC3 could be an effective target for immunotherapy against ovarian CCC. (*Cancer Sci* 2011; 102: 1622–1629)

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecological malignancy. Cytoreductive surgery and systemic combination chemotherapy with a platinum drug and a taxane represent the standard of care for EOC patients. Ovarian clear cell carcinoma (CCC) is the second most frequent subtype of EOC in Japan, although CCC represents 8–10% of all EOC in the United States.[1,2] Compared with other EOC subtypes, ovarian CCC is associated with a poorer prognosis and increased chemoresistance.[1,3] More efficient conventional therapies and novel strategies for effectively treating ovarian CCC are required.

Glypican-3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans that are attached to the cell surface via the glycosylphosphatidylinositol (GPI) anchor.[4] It is known as an oncofetal antigen specifically overexpressed in hepatocellular carcinoma (HCC).[5] Previous studies have shown that GPC3 was also overexpressed in other malignant tumors, such as melanoma, Wilms' tumor, hepatoblastoma, yolk sac tumor, ovarian CCC and lung squamous cell carcinoma.[6–10]

We previously identified the HLA-A24-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptides, both of which can induce GPC3-reactive cytotoxic T cells (CTL).[11] Recently, HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clones were established from HCC patients after $GPC3_{144-152}$ peptide vaccination in our laboratory.[12] Although CTL reactivity against HCC cell lines was analyzed using these CTL clones, other GPC3-positive tumor cell lines have not been studied. Therefore, we examined the reactivity of a HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone against ovarian CCC cell lines, and whether subtoxic-dose chemotherapy sensitizes ovarian CCC cells to lysis of $GPC3_{144-152}$ peptide-specific CTL.

Materials and Methods

$GPC3_{144-152}$ peptide-specific CTL clone and cell lines. We established the HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone from the PBMC of HCC patients vaccinated with $GPC3_{144-152}$ (FVGEFFTDV) peptide by single-cell sorting using CD107a antibody. The established CTL clone was tested for avidity by using $GPC3_{144-152}$ peptide-pulsed T2 targets with a range of peptide concentrations, starting at 10^{-6} M and decreasing by log steps to 10^{-14} M. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the avidity of the CTL clone and was rounded to the nearest log. This CTL clone had high avidity CTL (10^{-11} M) and could recognize HCC cell lines expressing GPC3 in a HLA-class-I-restricted manner.[12] Two human ovarian CCC cell lines, KOC-7c (HLA-A*0201/A*3101) and TOV-21G (HLA-A*1101/A*2601), and two human HCC cell lines, HepG2 (HLA-A*0201/A*2402) and SK-Hep-1 (HLA-A*0201/A*2402), were used in the present study. They were conserved in our laboratory. TOV-21G.A2 acquires expression of HLA-A2 following transfection with an HLA-A2 expression plasmid.[13] TOV-21G.A24 was similarly transfected with an HLA-A24 expression plasmid. SK-Hep-1.hG acquires expression of human GPC3 following transfection with a human GPC3 expression plasmid. SK-Hep-1.vec cell line transfected with an empty vector was used as a control. To study the effect of silencing GPC3, KOC-7c GPC3-shRNA and Neg-shRNA (control shRNA) were established by short hairpin RNA knockdown technology as described previously.[14] These cells were maintained in RPMI 1640 or DMEM medium (Sigma, St Louis, MO, USA) supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2.

RNA preparation and quantitative real-time PCR (qRT-PCR). Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. GPC3 gene expression levels were analyzed by qRT-PCR assays using the following primers generated according to the indicated reference sequences: sense, 5′-GAGCCAGTGGTCAGTCAAAT-3′ and antisense, 5′-CTTCATCATCACCGCAGTC-3′. Amplification reactions were carried out in 96-well plates in 25 μL reaction volume using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). All reactions were

[5]To whom correspondence should be addressed.
E-mail: tnakatsu@east.ncc.go.jp

doi: 10.1111/j.1349-7006.2011.02003.x

performed in technical triplicate using an ABI 7500 Fast Real-Time PCR System. Relative expression of the GPC3 gene to the endogenous control gene, β-actin, was calculated using the comparative C_T method. β-actin qRT-PCR primer sequences were: sense, 5′-TCCATCATGAAGTGTGACGT-3′ and antisense, 5′-GAGCAATGATCTTGATCTTCAT-3′.

Flow cytometry analysis and cell sorting. Flow cytometry (FCM) was performed to quantify the expression of GPC3 and Fas on the cell surface using the following antibodies: primary anti-GPC3 (clone 1G12; BioMosaics, Burlington, VT, USA); Alexa Fluor 488 conjugated second Ab (Invitrogen); phycoerythrin (PE)-conjugated anti-Fas (clone DX2; BioLegend, San Diego, CA, USA); FITC-conjugated anti-HLA-A2 (clone BB7.2; MBL, Nagoya, Japan); and FITC-conjugated mouse IgG2b isotype control (clone 3D12; MBL).

The FCM data was acquired using the FACSCanto II system (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Mean fluorescence intensity (MFI) of GPC3 staining was calculated as follows: MFI ratio = MFI with the anti-GPC3 Ab/MFI with the secondary Ab. MFI of HLA-A2 staining was similarly calculated (MFI ratio = MFI with the anti-HLA-A2 Ab/MFI with isotype control Ab).

Cell sorting was performed using the FACSAria II cell sorter (BD Biosciences) to isolate $GPC3^+$ and $GPC3^-$ cells from KOC-7c cells. We purified KOC-7c GPC3 high or low cells with the top or bottom 10% of GPC3 expression, respectively.

Response of $GPC3_{144-152}$ peptide-specific CTL clone against cancer cell lines. $GPC3_{144-152}$ peptide-specific CTL clone cells were co-cultured with each cancer cell line as target cells at the indicated effector/target (E/T) ratio and cytotoxicity assay or IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed. Blocking of HLA class I was done as follows. Before coculturing the CTL clone with a cancer cell line in an assay, the target cancer cells were incubated for 1 h with anti-HLA class I mAb (clone W6/32; BioLegend), or isotype control IgG2a mAb, and then the effects of Ab on CTL clone activity was examined.

IFN-γ ELISPOT analysis. ELISPOT assay for detecting antigen-specific IFN-γ-producing T cells was performed using the ELISPOT kit (BD Biosciences). The spots were automatically counted and analyzed with the Eliphoto system (Minerva Tech, Tokyo, Japan).

Cytotoxicity assay. The cytotoxic capacity was analyzed with the Terascan VPC system (Minerva Tech). The CTL clone was used for effector cells. Target cells were labeled in calcein-AM solution for 30 min at 37°C. The labeled cells were then co-cultured with effector cells for 4–6 h. Fluorescence intensity was measured before and after the 4–6 h culture, and specific cytotoxic activity was calculated as previously described.[12]

Cold inhibition assay. Calcein AM-labeled target cells were cultured with effector cells in a 96-well plate with cold target cells. T2 target cells, which were prepulsed with either HIV_{19-27} peptide or $GPC3_{144-152}$ peptide, were used as cold target cells.

CD107a degranulation assay. $GPC3_{144-152}$ peptide-specific CTL clone cells were incubated with cancer cell lines at a 2:1 ratio for 4 h at 37°C. APC-conjugated CD107a-specific mAb (clone H4A3; BD Biosciences) were present during the incubation period; after incubation, cells were stained with additional PE-conjugated anti-CD8 mAb (clone HIT8a; BioLegend) and analyzed by FCM.

Growth inhibition assay. Growth inhibition was evaluated by a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) colorimetric assay using a Cell Counting Kit (Dojindo, Kumamoto, Japan). Cells (5×10^3) were seeded into 96-well plates in 100 μL of culture medium for 24 h prior to drug exposure, and then treated with various concentrations of paclitaxel (PTX) or cisplatin (CDDP) for 18 or 48 h. Cell viability was determined colorimetrically by optical density at 450 nm wavelength using a microplate reader (Bio-Rad, Hercules, CA, USA). The percentage of cell survival for each drug concentration was calculated as: (absorbance of test wells/absorbance of control wells) × 100.

Apoptosis analysis. The Annexin V-FITC Apoptosis Detection Kit (BioVision, Mountain View, CA, USA) was used to determine apoptosis after treatment with PTX or CDDP. After treatment with the chemodrug, floating and adhering cells were collected via trypsinization and centrifuged. The supernatant was removed and resuspended in 500 μL of binding buffer to which 5 μL of Annexin-V-FITC and propidium iodido (PI) was added. The cells were incubated at room temperature for 5 min in the dark and assessed by FCM.

Statistical analysis. Univariate regression analysis was used to evaluate the correlation between GPC3 expression and GPC3-specific CTL recognition. Mann–Whitney *U*-test and Kruskal–Wallis test followed by Scheffe's *post hoc* test were used to detect differences between groups. For all statistical tests, differences were considered significant at $P < 0.05$.

Results

HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone recognizes ovarian CCC cell lines. To ascertain whether the HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone recognizes ovarian CCC cell lines expressing HLA-A2 and GPC3, we first evaluated the expression of GPC3 on cancer cell lines. We used KOC-7c and HLA-A0201 gene stable transfectant TOV-21G.A2 and two human HCC cell lines for the target cells. As positive controls, we used two HCC cell lines. SK-Hep-1.hG cells were an established stable GPC3-expressing cell line. As we performed qRT-PCR and FCM of GPC3 in these cell lines, GPC3 expression in ovarian CCC cell lines was less than that in HCC cell lines. Representative data of relative mRNA expression (ratio to KOC-7c) and MFI ratio are shown (Fig. 1A). The CTL response generally correlates with the numbers and density of MHC/antigen peptide complex on the target cells. Accordingly, we also evaluated HLA-A2 expression on the cell surface in cancer cell lines with FCM analysis (Fig. 1B). IFN-γ production of the CTL clone was detected against two ovarian CCC cell lines (Fig. 1C). In Figure 1C, we used TOV-21G.A24 as a negative control. Furthermore, we determined whether efficient $GPC3_{144-152}$ peptide-specific CTL clone recognition was correlated with GPC3 expression levels. We found that CTL clone recognition was correlated with the relative GPC3 mRNA expression and GPC3 MFI ratio in the cell lines ($r^2 = 0.995$ and 0.935, respectively) (Fig. 1D,E). In addition, we also analyzed whether CTL reactivity is correlated with not only GPC3 expression but also the expression of HLA-A2. The correlation between HLA-A2 expression levels on FCM analysis and CTL clone recognition (IFN-γ production or CD107a degranulation) was insufficient in the cell lines (data not shown). Although HLA-A2 expression on the cell surface in TOV-21G.A2 was moderately low, that in three other cell lines was sufficient on FCM analysis. TOV-21G.A2 cells have low expression of not only HLA-A2 but also GPC3. Therefore the GPC3 expression level is more important than the HLA-A2 expression level on $GPC3_{144-152}$ peptide-specific CTL clone reactivity.

$GPC3_{144-152}$ peptide-specific CTL clone lyses ovarian CCC cell lines. We detected GPC3-specific CTL responses by a CD107a degranulation assay. GPC3-specific CTL responses against TOV-21G.A2 and KOC-7c cells exhibited 2.79% and 5.42% CD107a staining, respectively, approximately 1.8- and 3.4-fold increases compared with the SK-Hep-1.vec as a negative control (Fig. 2A). CD107a degranulation was also correlated with the

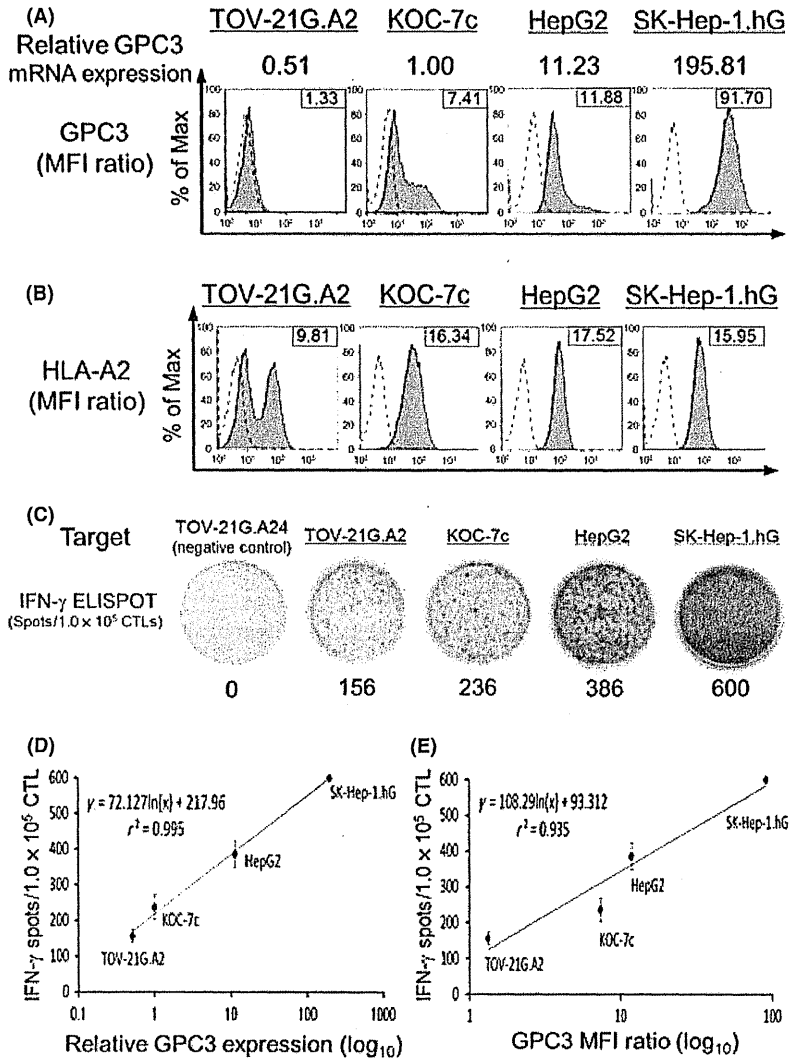

Fig. 1. HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone recognizes ovarian clear cell carcinoma (CCC) cell lines. (A) Expression of GPC3 on cancer cell lines. We used two human ovarian CCC cell lines (TOV-21G.A2 and KOC-7c) and two human HCC cell lines. We performed qRT-PCR and flow cytometry analysis (dashed line, secondary Ab stained control; gray-filled area, GPC3 staining). Numbers in the histograms correspond to the ratio of mean fluorescence intensity (MFI) of GPC3 staining, calculated as: MFI ratio = (MFI with the anti-GPC3 Ab)/(MFI with the secondary Ab). Representative data of relative GPC3 mRNA expression (ratio to KOC-7c) and GPC3 MFI ratio are shown. GPC3 expression in ovarian CCC cell lines was less than in HCC cell lines. (B) Expression of HLA-A2 on cancer cell lines. Numbers in histograms correspond to the ratio of MFI of HLA-A2 staining, calculated as: MFI ratio = (MFI with the anti-HLA-A2 Ab)/(MFI with isotype control Ab). (C) Representative results of IFN-γ ELISPOT analysis are shown. Effector/target ratio = 2. TOV-21G.A24 cells were used as a negative control. (D) IFN-γ production of a $GPC3_{144-152}$ peptide-specific CTL clone was correlated with relative GPC3 mRNA expression (r^2 = 0.995). (E) Similarly, $GPC3_{144-152}$ peptide-specific CTL clone recognition was correlated with the GPC3 MFI ratio (r^2 = 0.935).

relative GPC3 mRNA expression and GPC3 MFI ratio in the cell lines (r^2 = 0.978 and 0.865, respectively) (Fig. 2B). The $GPC3_{144-152}$ peptide-specific CTL clone was further tested for its capacity to kill ovarian CCC cell lines, by a calcein-AM-based cytotoxicity assay. SK-Hep-1.vec cells were used for a negative control. The CTL clone displayed mild, but clear, specific cytotoxicity against KOC-7c cells (Fig. 2C). However, GPC3-specific cytotoxicity was insufficient against TOV-21G.A2 cells compared with TOV-21G.A24 cells (data not shown). In both ovarian CCC cell lines, Fas expression on the cell surface was sufficiently similarly to that of the HCC cell lines on FCM analysis (Fig. 2D).

HLA class I specificity was confirmed by the blockade of reactivity against ovarian CCC cell line KOC-7c. HLA class I-restricted activity was demonstrated by blocking of IFN-γ release and lysis of the $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c after pretreatment with a HLA class I-specific mAb (W6/32) or mouse IgG2a isotype control, respectively, for 1 h. This reactivity could be inhibited by anti-HLA class I mAb but not by isotype control (Fig. 3). These results clearly indicate that the CTL clone recognized KOC-7c in a HLA class I-restricted manner.

Effect of GPC3 silencing using shRNA on the response of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c cells. To verify the GPC3 antigen-specific response of the CTL clone against ovarian CCC cell lines, we examined GPC3 knockdown on the GPC3-positive cell line KOC-7c. KOC-7c GPC3-shRNA was established using shRNA knockdown technology. The GPC3 expression of KOC-7c was obviously decreased by GPC3 shRNA on qRT-PCR. We examined the IFN-γ production and

doi: 10.1111/j.1349-7006.2011.02003.x

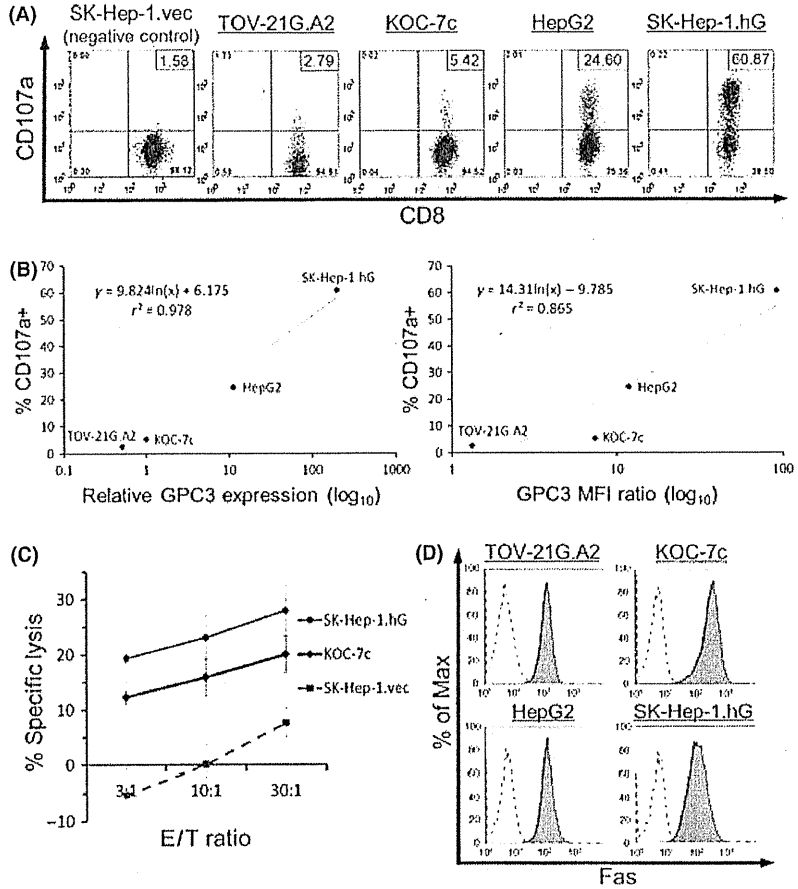

Fig. 2. $GPC3_{144-152}$ peptide-specific CTL clone lyses ovarian clear cell carcinoma (CCC) cell lines. (A) CD107a degranulation assay. Representative data are shown. GPC3-specific CTL responses against TOV-21G.A2 and KOC-7c cells exhibited 2.79% and 5.42% CD107a staining, respectively. (B) CD107a degranulation was correlated with relative GPC3 mRNA expression and GPC3 mean fluorescence intensity (MFI) ratio in cell lines (r^2 = 0.978 and 0.865, respectively). (C) Cytotoxicity (4 h) assay was performed at three effector/target ratios. We used SK-Hep-1.hG as a positive control. SK-Hep-1.vec cells were used as a negative control. The CTL clone showed specific cytotoxicity against KOC-7c cells. Data represent the mean ± SD. (D) Flow cytometry analysis of Fas expression on cancer cell lines. In all cell lines, Fas expression was sufficient (dashed line, unlabelled control; gray-filled area, PE-Fas staining).

lysis of the CTL clone against KOC-7c GPC3-shRNA and KOC-7c GPC3 Neg-shRNA cells. IFN-γ production was significantly decreased by GPC3 shRNA ($P = 0.004$) (Fig. 4A). GPC3-specific cytotoxicity was reduced against KOC-7c GPC3-shRNA cells compared with KOC-7c Neg-shRNA cells (Fig. 4B). These results indicate that HLA-A2-restricted $GPC3_{144-152}$ peptide could be processed naturally by ovarian CCC cells, and the peptides in the context of HLA-A2 could be expressed on the surface of ovarian CCC cells.

Level of GPC3 expression on the cell surface is related to $GPC3_{144-152}$ peptide-specific CTL clone recognition. To confirm that the level of GPC3 expression on the cell surface is responsible for CTL recognition, KOC-7c GPC3 high and low cells were sorted by FACSAria II (Fig. 5A). As shown in Figure 5B, KOC-7c GPC3 high cells expressed higher mRNA of GPC3 than GPC3 low cells. Figure 5C shows the IFN-γ release of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c wild type, GPC3 high and GPC3 low cells. There were significant differences in IFN-γ production between the three populations ($P < 0.001$). GPC3-specific cytotoxicity was increased against KOC-7c GPC3 high cells compared with GPC3 low cells in a cytotoxicity assay without cold target cells. In a cold target inhibition assay, cytotoxicity against KOC-7c GPC3 high cells was suppressed by the addition of $GPC3_{144-152}$ peptide-pulsed T2 cells but not by the addition of HIV_{19-27} peptide-pulsed T2 cells, even though cytotoxicity against KOC-7c GPC3 low cells was not changed by T2 pulsed with either $GPC3_{144-152}$ or HIV_{19-27} peptide (Fig. 5D).

Chemotherapy sensitizes KOC-7c cells to the cytotoxic effect of $GPC3_{144-152}$ peptide-specific CTL clone. Taxane plus platinum combination chemotherapy is generally considered to be the "gold standard" regimen for treatment of EOC. As PTX and CDDP have different mechanisms of action, we chose these two agents to investigate whether they sensitize ovarian CCC cells to GPC3-specific lysis. To evaluate the subtoxic dose of each drug, we assessed growth inhibition and apoptosis assays by FCM using Annexin V and PI staining. Growth-inhibitory effects were observed for treatment with either PTX or CDDP alone in a time- and dose-dependent manner. We calculated the 25% inhibitory concentration (IC25) of each drug as the minimum cytotoxic condition and regarded lower values as the subtoxic dose. The IC25 values of PTX and CDDP for 18 h were 22.8 ng/mL and 6.2 μg/mL, respectively (Fig. 6A). Exposure of CTL clone or KOC-7c cells to PTX (10 ng/mL) or CDDP (1 μg/mL) for 18 h had no significant cytotoxic effect, as determined by apoptosis assay. In other words, cell viability in untreated and PTX- and CDDP-treated groups of CTL clone or KOC-7c cells exceeded 95% in all cases (Fig. 6B). These

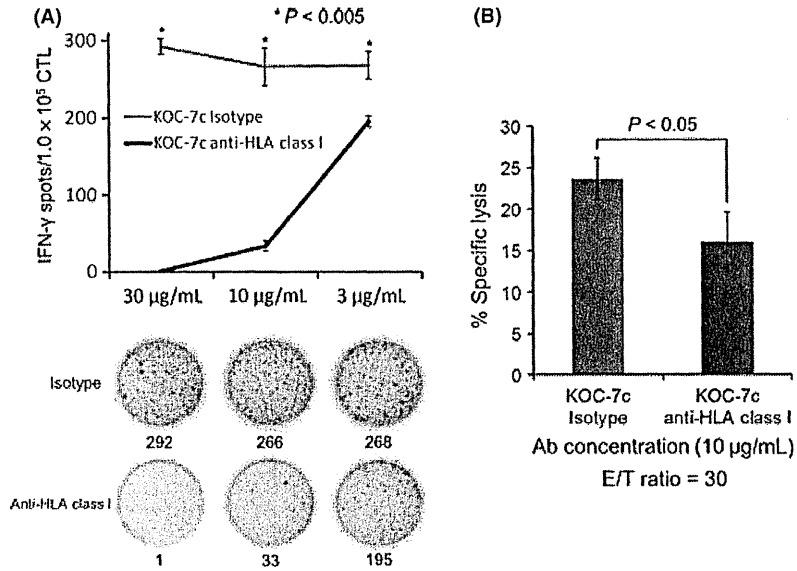

Fig. 3. Analysis of HLA class I restriction. (A) Inhibition of IFN-γ production by anti-HLA class I mAb. Effector/target ratio = 2. Data represent the mean ± SD of six wells. IFN-γ production of the CTL clone was markedly inhibited by anti-HLA class I mAb compared with that by isotype control in a concentration-dependent manner (*$P < 0.005$). (B) Inhibition of cytotoxicity by anti-HLA class I mAb. Effector/target (E/T) ratio = 30. Ab concentration = 10 μg/mL. Data represent the mean ± SD from the 4 h cytotoxicity assay. Cytotoxicity could be inhibited by anti-HLA class I mAb but not by isotype control ($P < 0.05$).

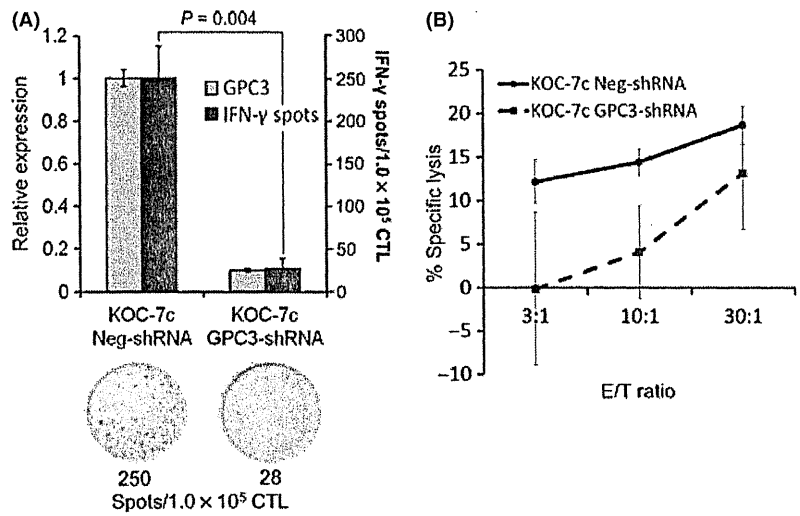

Fig. 4. Effect of GPC3 silencing using shRNA on the response of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c cells. (A) GPC3 expression of KOC-7c was obviously decreased by GPC3 shRNA on qRT-PCR. IFN-γ production was significantly decreased by GPC3 shRNA ($P = 0.004$). Data represent the mean ± SD. Effector/target (E/T) ratio = 2. (B) KOC-7c GPC3-shRNA cells were less cytolytic than KOC-7c Neg-shRNA cells. Data represent the mean ± SD from the 4 h cytotoxicity assay.

conditions excluded direct cytoxic effects of the compounds and effects as a subtoxic dose. In contrast, PTX (10 ng/mL) or CDDP (1 μg/mL) for 48 h showed mild cytotoxicity (basal levels of apoptosis >5%), and PTX (1 μg/mL) or CDDP (10 μg/mL) for 18 h induced substantial cell death (data not shown). KOC-7c cells were exposed to the subtoxic dose of each drug for 18 h and then examined by cytotoxicity assay. Pretreatment of KOC-7c cells with PTX (10 ng/mL) or CDDP (1 μg/mL) significantly increased CTL-mediated cytotoxicity of target cells (Fig. 6C). In all experiments, the level of spontaneous calcein release of target cells treated with chemotherapeutic agents was similar to that of untreated cells.

Discussion

Ovarian CCC has a poor prognosis due to low sensitivity to conventional chemotherapy.[1,3] To improve the prognosis, strategies are needed to efficiently kill all cancer cells by surgery and chemotherapy, as well as to stimulate the immune response to keep residual tumor cells in check. Thus, effective novel treatment strategies combined with surgery and chemotherapy are needed for treating ovarian CCC. Cancer vaccines are an attractive approach because of their low toxicity.

In previous studies, GPC3 was overexpressed in several malignant tumors, including ovarian CCC.[6–10] GPC3 is useful

doi: 10.1111/j.1349-7006.2011.02003.x

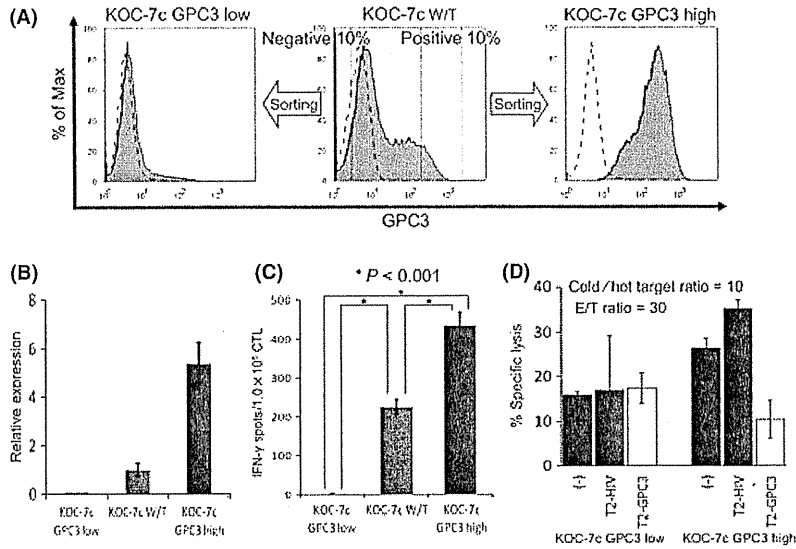

Fig. 5. The level of GPC3 expression on the cell surface is responsible for CTL recognition. (A) KOC-7c GPC3 high and GPC3 low cells were sorted as described in the Materials and Methods. (B) Relative GPC3 mRNA expression (ratio to KOC-7c wild type) is shown. Data represent the mean ± SD. (C) IFN-γ production of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c wild type, GPC3 high and GPC3 low cells. There were significant differences between the three populations (*$P < 0.001$). Mean ± SD of six wells is shown. (D) Cold target inhibition assay of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c GPC3 high and GPC3 low cells. Effector/target (E/T) ratio = 30. T2 was prepulsed with either HIV_{19-27} peptide or $GPC3_{144-152}$ peptide and then used as cold target cells. Cold/hot target ratio = 10. Cytotoxicity of the CTL clone against KOC-7c GPC3 high cells was inhibited by the addition of $GPC3_{144-152}$ peptide-pulsed T2 cells but not by the addition of HIV_{19-27} peptide-pulsed T2 cells. In contrast, cytotoxicity against the KOC-7c GPC3 low cells was not suppressed by T2 pulsed with either $GPC3_{144-152}$ or HIV_{19-27} peptide. Data represent the mean ± SD from the 4 h cytotoxicity assay.

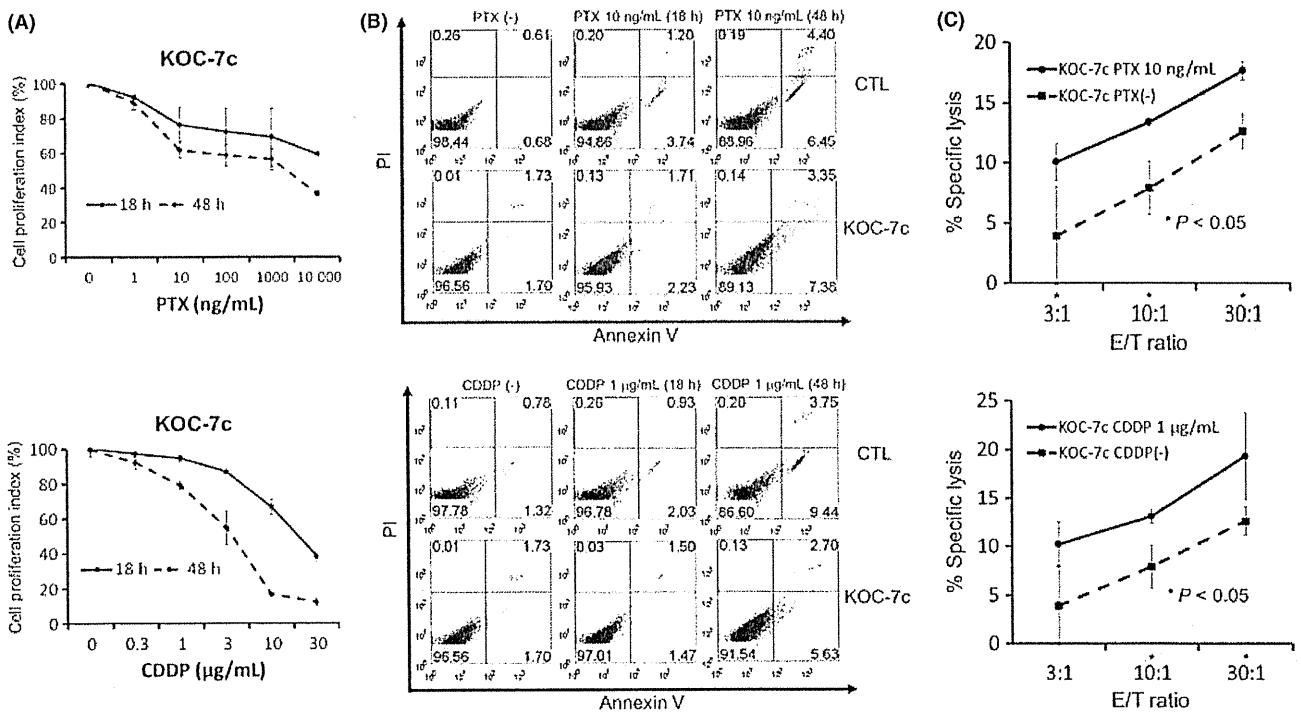

Fig. 6. Subtoxic-dose chemotherapy sensitizes KOC-7c cells to the cytotoxic effect of the $GPC3_{144-152}$ peptide-specific CTL clone. We used two agents (paclitaxel [PTX] and cisplatin [CDDP]) to investigate whether they sensitize ovarian clear cell carcinoma (CCC) cells to GPC3-specific lysis. (A) Growth-inhibitory effects were observed for treatment with each drug alone in a time- and dose-dependent manner. Data represent the mean ± SD. (B) Apoptosis analysis by flow cytometry analysis. Representative data are shown. The numbers in each quadrant represent the percentage of cells in the quadrant. Exposure of CTL clone or KOC-7c cells to PTX (10 ng/mL) or CDDP (1 μg/mL) for 18 h had no significant cytotoxic effect. By contrast, PTX (10 ng/mL) or CDDP (1 μg/mL) for 48 h showed mild cytotoxicity. (C) KOC-7c cells were pretreated with the subtoxic dose of each drug for 18 h and then a cytotoxicity assay (4 h) was performed. Pretreatment of KOC-7c cells with PTX (10 ng/mL) or CDDP (1 μg/mL) significantly increased CTL-mediated cytotoxicity of target cells (*$P < 0.05$). Data represent the mean ± SD.

as a novel biomarker and oncofetal antigen for immunotherapy.[15–22] However, association of ovarian CCC with CTL recognition has not been performed, hindering the selection of appropriate candidates for GPC3-specific immunotherapy. We recently established HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clones.[12] In the present study, we analyzed the IFN-γ production and cytotoxicity of an established CTL clone against ovarian CCC cell lines expressing HLA-A0201 and GPC3. The $GPC3_{144-152}$ peptide-specific CTL clone could recognize HLA-A2-positive and GPC3-positive ovarian CCC cell lines, suggesting that ovarian CCC present endogenously processed $GPC3_{144-152}$ peptide. Even though the CTL clones recognized two ovarian CCC cell lines on the IFN-γ ELISPOT assay, they showed inefficient lysis against TOV-21G.A2 cells. This was not due to a low expression level of HLA-A2 molecules on the cell surface, because the tumor cells were lysed after being pulsed with the antigenic peptide (data not shown). We also confirmed that the level of antigen expression is important in GPC3-specific CTL recognition of malignant cells. Therefore, low-level expression of GPC3 on tumor cells might be insufficient for triggering CTL-mediated killing.

Recent clinical studies have reported high rates of objective clinical response when cancer vaccines are combined with chemotherapy in patients with various cancers.[23–27] To evaluate the feasibility of chemoimmunotherapy for ovarian CCC, we investigated the cytotoxic effect of subtoxic-dose PTX or CDDP combined with $GPC3_{144-152}$ peptide-specific CTL clone in the human ovarian CCC cell line KOC-7c. We found that chemotherapy made ovarian CCC cells more susceptible to the cytotoxic effect of the $GPC3_{144-152}$ peptide-specific CTL clone. Chemotherapeutic drugs generally suppress the immune function, and each drug has a different level of immune suppression. Therefore, combination therapy requires an optimal dose that does not suppress peptide-induced immune activation. Importantly, the synergistic cytotoxic effect remained when both CTL and tumor cells were pretreated with PTX or CDDP under identical conditions (data not shown). However, high-dose chemotherapy has been shown to be toxic and the synergistic effect increased slightly more compared with the subtoxic dose, therefore limiting its potential therapeutic usefulness *in vitro*. The mechanism of improvement in immunotherapy with chemotherapy remains unclear, but the two possible types of mechanism are: systemic factors and local tumor microenvironment factors. For example, possible systemic effects include the elimination of cells with immunosuppressive activity such as regulatory T cells[28] and myeloid-derived suppressor cells,[29] or improved cross-presentation of tumor antigens. Examples of possible local effects include the disruption of tumor stroma that results in improved penetration of CTL into the tumor site, increased permeability of tumor cells to CTL-derived granzymes via upregulation of mannose-6-phosphate (M6P) receptors on the surface of tumor cells,[30] increased expression of tumor-associated antigens by tumor cells or upregulation of Fas (and other death receptors) on tumor cells, or FasL on CTL, etc.[31,32] We performed experiments to address the change in permeability for GrzB and the expression of M6P receptors in KOC-7c cells pretreated with PTX or CDDP. However, both drugs had no significant effect on the expression of M6P receptors. Moreover, we could not confirm the mechanism through an increase in permeability to GrzB in CCC cell line KOC-7c cells. Paclitaxel is known to upregulate the expression of Fas on the surface of tumor cells, resulting in an increase in Fas–FasL interaction.[33] However, Fas expression was sufficient in ovarian CCC cell lines without chemotherapy, and both drugs had no significant effect on Fas expression. The threshold for Fas-induced apoptosis in ovarian CCC is high and/or Fas signaling in CCC is altered through unknown mechanisms. In addition, both drugs had no significant effect on GPC3 expression under subtoxic-dose conditions (data not shown).

In conclusion, the present study suggests that GPC3 could become an effective target for HLA-A2-restricted peptide vaccine therapy against ovarian CCC. Moreover, our data suggest the possibility of treating ovarian CCC patients by combining standard chemotherapy with relatively non-toxic and highly specific immunotherapy. We will clarify the mechanisms of this phenomenon in our next study.

Acknowledgments

This work was supported in part by Grants-in-Aid for Research on Hepatitis and for Clinical Research from the Ministry of Health, Labour and Welfare, Japan.

Disclosure Statement

The authors have no conflict of interest.

References

1 Sugiyama T, Kamura T, Kigawa J *et al.* Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. *Cancer* 2000; **88**: 2584–9.
2 Kennedy AW, Biscotti CV, Hart WR, Webster KD. Ovarian clear cell adenocarcinoma. *Gynecol Oncol* 1989; **32**: 342–9.
3 Recio FO, Piver MS, Hempling RE, Driscoll DL. Lack of improved survival plus increase in thromboembolic complications in patients with clear cell carcinoma of the ovary treated with platinum versus nonplatinum-based chemotherapy. *Cancer* 1996; **78**: 2157–63.
4 Filmus J, Selleck SB. Glypicans: proteoglycans with a surprise. *J Clin Invest* 2001; **108**: 497–501.
5 Nakatsura T, Yoshitake Y, Senju S *et al.* Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. *Biochem Biophys Res Commun* 2003; **306**: 16–25.
6 Nakatsura T, Kageshita T, Ito S *et al.* Identification of glypican-3 as a novel tumor marker for melanoma. *Clin Cancer Res* 2004; **10**: 6612–21.
7 Saikali Z, Sinnett D. Expression of glypican 3 (GPC3) in embryonal tumors. *Int J Cancer* 2000; **89**: 418–22.
8 Toretsky JA, Zitomersky NL, Eskenazi AE *et al.* Glypican-3 expression in Wilms tumor and hepatoblastoma. *J Pediatr Hematol Oncol* 2001; **23**: 496–9.
9 Maeda D, Ota S, Takazawa Y *et al.* Glypican-3 expression in clear cell adenocarcinoma of the ovary. *Mod Pathol* 2009; **22**: 824–32.
10 Aviel-Ronen S, Lau SK, Pintilie M *et al.* Glypican-3 is overexpressed in lung squamous cell carcinoma, but not in adenocarcinoma. *Mod Pathol* 2008; **21**: 817–25.
11 Komori H, Nakatsura T, Senju S *et al.* Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. *Clin Cancer Res* 2006; **12**: 2689–97.
12 Yoshikawa T, Nakatsugawa M, Suzuki S *et al.* HLA-A2-restricted glypican-3 peptide-specific CTL clones induced by peptide vaccine show high avidity and antigen-specific killing activity against tumor cells. *Cancer Sci* 2011; **102**: 918–25.
13 Akatsuka Y, Goldberg TA, Kondo E *et al.* Efficient cloning and expression of HLA class I cDNA in human B-lymphoblastoid cell lines. *Tissue Antigens* 2002; **59**: 502–11.
14 Sakurai M, Shibata K, Umezu T *et al.* Growth-suppressing function of glypican-3 (GPC3) via insulin like growth factor II (IGF-II) signaling pathway in ovarian clear cell carcinoma cells. *Gynecol Oncol* 2010; **119**: 332–6.
15 Shirakawa H, Suzuki H, Shimomura M *et al.* Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. *Cancer Sci* 2009; **100**: 1403–7.
16 Shirakawa H, Kuronuma T, Nishimura Y *et al.* Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer. *Int J Oncol* 2009; **34**: 649–56.
17 Ikuta Y, Nakatsura T, Kageshita T *et al.* Highly sensitive detection of melanoma at an early stage based on the increased serum secreted protein

doi: 10.1111/j.1349-7006.2011.02003.x

acidic and rich in cysteine and glypican-3 levels. *Clin Cancer Res* 2005; **11**: 8079–88.
18 Nakatsura T, Nishimura Y. Usefulness of the novel oncofetal antigen glypican-3 for diagnosis of hepatocellular carcinoma and melanoma. *BioDrugs* 2005; **19**: 71–7.
19 Hayashi E, Motomura Y, Shirakawa H *et al.* Detection of glypican-3-specific CTLs in chronic hepatitis and liver cirrhosis. *Oncol Rep* 2009; **22**: 149–54.
20 Motomura Y, Ikuta Y, Kuronuma T *et al.* HLA-A2 and -A24-restricted glypican-3-derived peptide vaccine induces specific CTLs: preclinical study using mice. *Int J Oncol* 2008; **32**: 985–90.
21 Motomura Y, Senju S, Nakatsura T *et al.* Embryonic stem cell-derived dendritic cells expressing glypican-3, a recently identified oncofetal antigen, induce protective immunity against highly metastatic mouse melanoma, B16-F10. *Cancer Res* 2006; **66**: 2414–22.
22 Nakatsura T, Komori H, Kubo T *et al.* Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. *Clin Cancer Res* 2004; **10**: 8630–40.
23 Gribben JG, Ryan DP, Boyajian R *et al.* Unexpected association between induction of immunity to the universal tumor antigen CYP1B1 and response to next therapy. *Clin Cancer Res* 2005; **11**: 4430–6.
24 Antonia SJ, Mirza N, Fricke I *et al.* Combination of p53 cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer. *Clin Cancer Res* 2006; **12**: 878–87.
25 Arlen PM, Gulley JL, Parker C *et al.* A randomized phase II study of concurrent docetaxel plus vaccine versus vaccine alone in metastatic androgen-independent prostate cancer. *Clin Cancer Res* 2006; **12**: 1260–9.
26 Schlom J, Arlen PM, Gulley JL. Cancer vaccines: moving beyond current paradigms. *Clin Cancer Res* 2007; **13**: 3776–82.
27 Wheeler CJ, Black KL, Liu G *et al.* Vaccination elicits correlated immune and clinical responses in glioblastoma multiforme patients. *Cancer Res* 2008; **68**: 5955–64.
28 Vicari AP, Luu R, Zhang N *et al.* Paclitaxel reduces regulatory T cell numbers and inhibitory function and enhances the anti-tumor effects of the TLR9 agonist PF-3512676 in the mouse. *Cancer Immunol Immunother* 2009; **58**: 615–28.
29 Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009; **9**: 162–74.
30 Ramakrishnan R, Assudani D, Nagaraj S *et al.* Chemotherapy enhances tumor cell susceptibility to CTL-mediated killing during cancer immunotherapy in mice. *J Clin Invest* 2010; **120**: 1111–24.
31 Bergmann-Leitner ES, Abrams SI. Differential role of Fas/Fas ligand interactions in cytolysis of primary and metastatic colon carcinoma cell lines by human antigen-specific CD8+ CTL. *J Immunol* 2000; **164**: 4941–54.
32 Frost PJ, Butterfield LH, Dissette VB, Economou JS, Bonavida B. Immunosensitization of melanoma tumor cells to non-MHC Fas-mediated killing by MART-1-specific CTL cultures. *J Immunol* 2001; **166**: 3564–73.
33 Srivastava RK, Sasaki CY, Hardwick JM, Longo DL. Bcl-2-mediated drug resistance: inhibition of apoptosis by blocking nuclear factor of activated T lymphocytes (NFAT)-induced Fas ligand transcription. *J Exp Med* 1999; **190**: 253–65.

Cancer Science
The official journal of the Japanese Cancer Association JCA

HLA-A2-restricted glypican-3 peptide-specific CTL clones induced by peptide vaccine show high avidity and antigen-specific killing activity against tumor cells

Toshiaki Yoshikawa,[1,2] Munehide Nakatsugawa,[1] Shiro Suzuki,[1] Hirofumi Shirakawa,[1] Daisuke Nobuoka,[1] Noriko Sakemura,[1] Yutaka Motomura,[1] Yukie Tanaka,[3] Shin-Ichi Hayashi[2] and Tetsuya Nakatsura[1,4]

[1]Section for Cancer Immunotherapy, Investigative Treatment Division, Research Center for Innovative Oncology, National Cancer Center Hospital East, Kashiwa; [2]Division of Immunology, Department of Molecular and Cellular Biology, School of Life Science, Faculty of Medicine, Tottori University, Yonago; [3]Division of Hematology, Saitama Medical Center, Jichi Medical University, Saitama, Japan

(Received December 6, 2010/Revised January 13, 2011/Accepted January 13, 2011/Accepted manuscript online February 1, 2011/Article first published online March 4, 2011)

Glypican-3 (GPC3) is an onco-fetal antigen that is overexpressed in human hepatocellular carcinoma (HCC), and is only expressed in the placenta and embryonic liver among normal tissues. Previously, we identified an HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide that can induce GPC3-reactive CTLs without inducing autoimmunity in HLA-A2 transgenic mice. In this study, we carried out a phase I clinical trial of HLA-A2-restricted $GPC3_{144-152}$ peptide vaccine in 14 patients with advanced HCC. Immunological responses were analyzed by *ex vivo* γ-interferon enzyme-linked immunospot assay. The frequency of $GPC3_{144-152}$ peptide-specific CTLs after vaccination (mean, 96; range, 5–441) was significantly larger than that before vaccination (mean, 6.5; range, 0–43) ($P < 0.01$). An increase in the $GPC3_{144-152}$ peptide-specific CTL frequency was observed in 12 (86%) of 14 patients after vaccination. Additionally, there was a significant correlation between the maximum value of $GPC3_{144-152}$ peptide-specific CTLs after vaccination and the dose of the peptide injected ($P = 0.0166$, $r = 0.665$). Moreover, we established several $GPC3_{144-152}$ peptide-specific CTL clones from PBMCs of patients vaccinated with $GPC3_{144-152}$ peptide by single cell sorting using Dextramer and CD107a antibody. These CTL clones had high avidity (the recognition efficiency showing 50% cytotoxicity was 10^{-10} or 10^{-11} M) and could recognize HCC cell lines expressing GPC3 in an HLA-class I-restricted manner. These results suggest that $GPC3_{144-152}$ peptide vaccine can induce high avidity CTLs capable of killing HCC cells expressing GPC3. This trial was registered with University Hospital Medical Information Network number 000001395. (*Cancer Sci* 2011; 102: 918–925)

In peptide-based vaccine trials, occasional marked clinical regressions of melanoma have been observed after peptide vaccination; however, tumor regressions have not correlated well with T cell responses measured in peripheral blood lymphocytes.[1–3] This may be because the clinical response to a vaccine was unrelated to the immune response to that vaccine or due to inadequate immune response monitoring. Moreover, vaccination with synthetic peptides has occasionally induced ineffective CTL responses due to various mechanisms.[4–9] When evaluating T cell response to peptide vaccines, it is important to confirm that the peptide is presented naturally on cancer cells and that responding CTLs lyse human cancer cells.

Glypican-3 (GPC3) is specifically overexpressed in human hepatocellular carcinoma (HCC).[10] The expression of GPC3 was correlated with a poor prognosis in HCC patients.[11] Moreover, GPC3 is useful not only as a novel tumor marker, but also as a target antigen for immunotherapy in several studies with mice.[12–14] We identified HLA-A*24:02-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptides, both of which can induce GPC3-reactive CTLs without inducing autoimmunity,[15] and reported a preclinical study using a mouse model with a view to designing an optimal schedule for the clinical trials of a GPC3-derived peptide vaccine and showed dose-dependency in the immunizing effect of the peptide vaccine.[16]

In this study, we completed the phase I clinical trial of a GPC3-derived peptide vaccine for 30 patients with advanced HCC (manuscript in preparation). Among them, 16 patients had the *HLA-A24* gene and 14 had the *HLA-A2* gene. Here, we describe the immunological evaluation of HLA-A2-restricted $GPC3_{144-152}$ peptide vaccine in a phase I trial involving 14 patients. We highlight three important points: (i) HLA-A2-restricted $GPC3_{144-152}$ peptide is immunogenic in advanced HCC patients; (ii) dose-dependent effects of $GPC3_{144-152}$ peptide vaccine; and (iii) establishment of CTL clones showing not only high avidity but also natural antigen-specific killing activity against HCC cells.

Materials and Methods

Patients. Fourteen patients with advanced HCC were injected with HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide vaccine at the National Cancer Center Hospital East (Kashiwa, Japan). *HLA-A2* gene-positive status was determined by genomic DNA typing tests (Mitsubishi Chemical Medience, Tokyo, Japan). All patients gave written informed consent before entering the study. The profiles of the 14 patients are summarized in Table 1. This study was approved by the Ethics Committee of the National Cancer Center, and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Treatment protocol. Vaccinations with GMP grade peptide, $GPC3_{144-152}$ (FVGEFFTDV) (American Peptide Co., Sunnyvale, CA, USA) emulsified with incomplete Freund's adjuvant (Montanide ISA-51 VG; Seppic, Paris, France) were carried out intradermally three times at 14-day intervals. Five incremental dose levels at 0.3, 1, 3, 10, and 30 mg/body were planned for the peptide administration.

Preparation of PBMCs. Peripheral blood (30 mL) was obtained from each patient at times designated in the protocol (before the first vaccination and 2 weeks after each vaccination) and centrifuged using a Ficoll–Paque gradient.

[4]To whom correspondence should be addressed.
E-mail: tnakatsu@east.ncc.go.jp

doi: 10.1111/j.1349-7006.2011.01896.x

Table 1. Summary of profiles of 14 patients with advanced human hepatocellular carcinoma who participated in this study, with their clinical and immunological responses before and after vaccination with HLA-A2-restricted $GPC3_{144-152}$ peptide

Pt.	HLA	Age (years)	Sex	Stage	Dose of peptide (mg)	Clinical response†	GPC3-specific CTLs‡		
							Pre	Post	Change
A2-1	A*02:06/A*02:07	67	M	IV	0.3	SD	43	40	–
A2-2	A*02:01	62	M	IIIA	0.3	PD	0	18	+
A2-3	A*02:01	55	M	IIIA	0.3	SD	1	10	+
A2-4	A*02:01	68	F	IIIC	1.0	SD	16	15	–
A2-5	A*02:01	72	M	IIIA	1.0	SD	16	101	+
A2-6	A*02:01/A*02:06	62	M	II	1.0	PD	0	23	+
A2-7	A*02:01	67	F	IV	3.0	SD	0	23	+
A2-8	A*02:01	58	M	IIIA	3.0	SD	0	101	+
A2-9	A*02:01	52	M	IV	10.0	SD	1	100	+
A2-10	A*02:01	70	M	IV	10.0	PD	0	5	+
A2-11	A*02:01	68	M	II	10.0	PD	1	125	+
A2-12	A*02:07	75	F	IV	30.0	PR	11	196	+
A2-13	A*02:06	52	M	IV	30.0	PD	2	151	+
A2-14	A*02:01	67	M	IV	30.0	PD	0	441	+

†The clinical response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines. ‡Peripheral blood was taken from each patient before and after vaccination, and glypican-3 (GPC3)-specific CTLs were measured by *ex vivo* γ-interferon enzyme-linked immunospot assay. F, female; M, male; PD, progressive disease; PR, partial response; Pt., patient; SD, stable disease; +, increase; –, decrease.

***Ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) analysis.** ELISPOT assay for the detection of antigen-specific IFN-γ producing T cells was carried out using the BD ELISPOT kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's protocols. In brief, non-cultured PBMCs (5×10^5 cells/well) were added to plates in the presence of 10 μg/mL peptide antigens and incubated for 20 h at 37°C, 5% CO_2. The GPC3 antigen was HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide. The PBMCs with HLA-A2-restricted HIV_{19-27} (TLNAWVKVV) peptide were used as a negative control. The spots were automatically counted using the Eliphoto system (Minerva Tech, Tokyo, Japan).

Cell lines. The human liver cancer cell line HepG2 ($GPC3^+$, HLA-A*02:01/A*24:02), SK-Hep-1 ($GPC3^-$, HLA-A*02:01/A*24:02), the human melanoma cell line 526mel ($GPC3^+$, HLA-A*02:01), and the human colon cancer cell line SW620 ($GPC3^-$, HLA-A*02:01/A*24:02) were used as target cells. T2 (HLA-A*02:01, TAP^-) was pulsed with $GPC3_{144-152}$ peptide or HIV_{19-27} peptide at room temperature for 1 h. They were conserved in our laboratory.

Induction of $GPC3_{144-152}$ peptide-specific CTLs from PBMCs. The PBMCs were cultured (2×10^6 cells/well) with 10 μg/mL $GPC3_{144-152}$ peptide in AIM-V medium supplemented with 10% human AB serum, recombinant human interleukin (IL)-2 for 14 days.

Dextramer staining and flow cytometry analysis. The PBMCs were stained with HLA-A*02:01 Dextramer-RPE ($GPC3_{144-152}$ [FVGEFFTDV], HIV_{19-27} [TLNAWVKVV]; Immudex, Copenhagen, Denmark) for 10 min at room temperature and anti-CD8-FITC (ProImmune, Oxford, UK) for 20 min at 4°C. Flow cytometry analysis was carried out using FACSAria cell sorter (BD Bioscience).

CD107a staining and flow cytometry analysis. $CD8^+$ T cells were isolated using human CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from PBMCs stimulated with $GPC3_{144-152}$ peptide for 14 days. $CD8^+$ T cells were incubated with T2 pulsed with $GPC3_{144-152}$ or HIV_{19-27} peptide and HepG2 at a 2:1 ratio for 3.5 h at 37°C. CD107a-specific antibodies (BD Bioscience) were included during the incubation period.

Generation of CTL clones. $CD8^+$ GPC3 Dextramer$^+$ or $CD107a^+$ cells were sorted using a FACSAria cell sorter and seeded in a 96-well plate (1 cell/well) and stimulated by the addition of irradiated (100 Gy) allogeneic PBMCs (8×10^4 cells/well) as feeder cells, in AIM-V medium supplemented with 10% human AB serum, IL-2 (200 U/mL), and phytohemagglutinin-P (PHA) (5 μg/mL) for 14–21 days.

Response of CTL clones against cancer cell lines. The CTL clones were cocultured with each cancer cell line as a target cell at the indicated effector/target (E/T) ratio, and cytotoxicity assay or IFN-γ ELISPOT assay was carried out. Blocking of HLA-class I or HLA-A2 was carried out as previously described.[15]

Cytotoxicity assay. Cytotoxic activity against target cells was analyzed using the Terascan VPC system (Minerva Tech). Target cells were labeled with calcein AM (Dojindo, Kumamoto, Japan) solution for 30 min at 37°C. The labeled cells were then incubated with effector cells for 4–6 h. Fluorescence intensity was measured before and after the 4–6 h culture, and specific cytotoxic activity was calculated using the following formula: % cytotoxicity = {1– [(average fluorescence of the sample wells – average fluorescence of the maximal release control wells)/(average fluorescence of the minimal release control wells – average fluorescence of the maximal release control wells)]} × 100%.

Determination of recognition efficiency. Calcein AM-labeled T2 target cells were pulsed with a range of peptide concentrations, starting at 10^{-6} M and decreasing by log steps to 10^{-14} M. The CTL clones were incubated with T2 target cells at a 10:1 E/T ratio for 4 h. For each CTL clone, % cytotoxicity was plotted against each peptide concentration. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the recognition efficiency of that clone.

Cold inhibition assay. Calcein AM-labeled target cells were cultured with effector cells in a 96-well plate with cold target cells. T2 target cells, which were prepulsed with either HIV_{19-27} peptide or $GPC3_{144-152}$ peptide, were used as cold target cells.

RNA interference. Small interfering RNAs specific for human GPC3 were chemically synthesized double-strand RNAs (Invitrogen, Carlsbad, CA, USA). A non-silencing siRNA, AllStras Neg. Control siRNA, was obtained from Qiagen (Valencia, CA, USA). The GPC3-specific siRNA sequence used in this study was: 5′-GGAGGCUCUGGUGAUGGAAUGAUAA-3′. Synthetic siRNA duplexes were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocols.

Statistical analysis. Student's *t*-test was used to determine statistically significant differences between the two groups.

Correlation between the frequency of GPC3-specific CTLs and the dose of the peptide injected was analyzed using Spearman's rank correlation coefficient. Data from the ELISPOT assay using siRNA were statistically analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was set as $P < 0.05$.

Results

Analysis of $GPC3_{144–152}$ peptide-specific CTLs in PBMCs of vaccinated patients. To analyze immune responses in the 14 patients vaccinated with $GPC3_{144–152}$ peptide, we evaluated the $GPC3_{144–152}$ peptide-specific immune responses by *ex vivo* IFN-γ ELISPOT assay. The representative data of patient A2-12 on changes in the frequency of $GPC3_{144–152}$ peptide-specific CTLs before and after vaccination are shown in Figure 1(a). The frequencies of $GPC3_{144–152}$ peptide-specific CTLs were 11 and 196 of 5×10^5 PBMCs at pre- and post-vaccination, respectively. The results of the comparison of the frequency of $GPC3_{144–152}$ peptide-specific CTLs before vaccination and after vaccination in all patients are shown in Table 1 and Figure 1(b). $GPC3_{144–152}$ peptide-specific CTLs were clearly detected in four and 14 of the 14 patients at pre- and post-vaccination, respectively. The frequency of $GPC3_{144–152}$ peptide-specific CTLs after vaccination (mean, 96; range, 5–441) was significantly larger than that before vaccination (mean, 6.5; range, 0–43) ($P < 0.01$). An increase in $GPC3_{144–152}$ peptide-specific CTLs was found in 12 (86%) of the 14 patients, except in two cases (patients A2-1 and A2-4). These results suggest that $GPC3_{144–152}$ peptide vaccination can induce an increase in $GPC3_{144–152}$ peptide-specific CTLs in HCC patients. Moreover, we compared the frequency of $GPC3_{144–152}$ peptide-specific CTLs after vaccination for each dose of peptide injected. We found that the maximum value of $GPC3_{144–152}$ peptide-specific CTLs after vaccination was significantly correlated with the dose of the peptide injected ($P = 0.0166$, $r = 0.665$) (Fig. 1c).

Establishment of $GPC3_{144–152}$ peptide-specific CTL clones by three different methods. To further investigate the ability of $GPC3_{144–152}$ peptide-specific CTLs induced by peptide vaccination to recognize an antigen, we established CTL clones from PBMCs of three vaccinated patients (patients A2-8, A2-9, and A2-14) by three different methods (Fig. 2). A representative clone from each patient is shown. In patient A2-9 (Fig. 2a), the frequency of $GPC3_{144–152}$ peptide-specific CTLs was 50 of 5×10^5 PBMCs 1 month after the third vaccination, as determined by *ex vivo* ELISPOT assay, and 14 days after the *in vitro* stimulation with $GPC3_{144–152}$ peptide, Dextramer assay was carried out. The population of $CD8^+$ GPC3 $Dextramer^+$ cells was 2.6% of all stimulated cells. These cells were sorted to a single cell in each well of a 96-well plate. Twenty-one days after cell sorting, peptide specificity was examined by Dextramer assay. The established CTL clone was $CD8^+$ GPC3 $Dextramer^+$ cells (99.7%) which did not react with HIV Dextramer as a negative control (Fig. 2a).

We next attempted to sort from small populations of $GPC3_{144–152}$ peptide-specific CTLs without *in vitro* culture. In patient A2-14 (Fig. 2b), the frequency of $GPC3_{144–152}$ peptide-specific CTLs was 329 of 5×10^5 PBMCs 2 weeks after the third vaccination, as determined by *ex vivo* ELISPOT assay; $CD8^+$ GPC3 $Dextramer^+$ cells could be clearly detected in 0.1% of PBMCs. $CD8^+$ GPC3 $Dextramer^+$ cells were directly sorted to a single cell from PBMCs without *in vitro* stimulation. The established CTL clone was $CD8^+$ GPC3 $Dextramer^+$ cells (99.9%) which did not react with HIV-Dextramer (Fig. 2b).

Finally, to establish high avidity and tumor-reactive CTLs from a heterogeneous population, we attempted to sort the population of $CD8^+$ T cells which mobilized CD107a in response to naturally GPC3-expressing HepG2 cells. In the PBMCs from

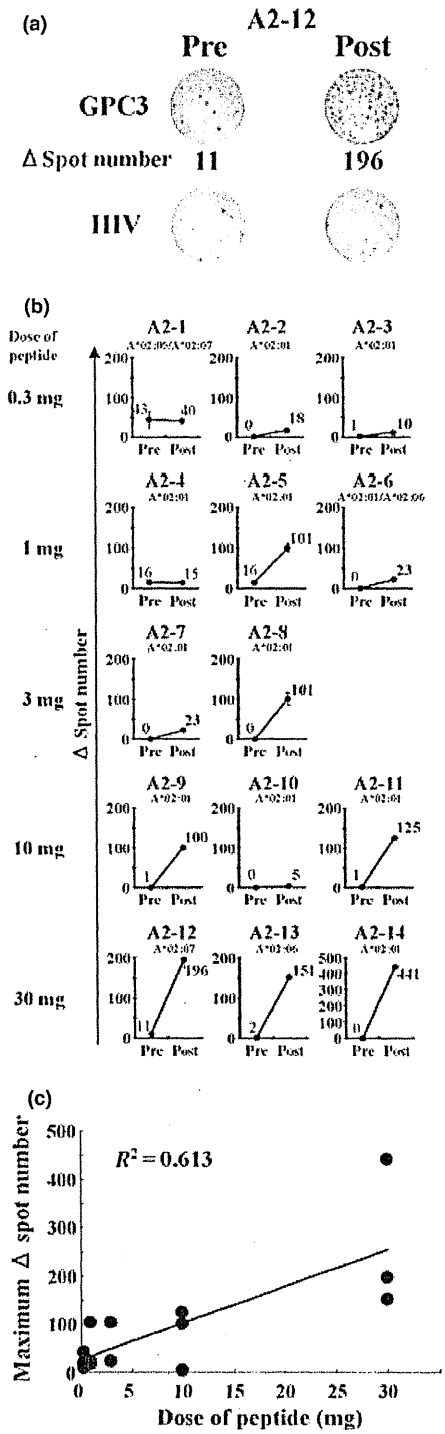

Fig. 1. Changes in the frequency of $GPC3_{144–152}$ peptide-specific CTLs before and after vaccination. Direct *ex vivo* γ-interferon enzyme-linked immunospot assay of PBMCs (5×10^5) was carried out. The Δ spot number indicates the number of $GPC3_{144–152}$ peptide-specific CTLs calculated by subtracting the spot number in a well of $HIV_{19–27}$ peptide. (a) Representative result showing the frequency of $GPC3_{144–152}$ peptide-specific CTLs pre- and post-vaccination. (b) Changes in the frequency of $GPC3_{144–152}$ peptide-specific CTLs before and after vaccination in all patients (A2-1–14). An increase in $GPC3_{144–152}$ peptide-specific CTLs was observed in 12 (86%) of 14 patients. (c) The maximum number of $GPC3_{144–152}$ peptide-specific CTLs after vaccination was significantly correlated with the dose of the peptide injected ($P = 0.0166$, $r = 0.665$).

doi: 10.1111/j.1349-7006.2011.01896.x

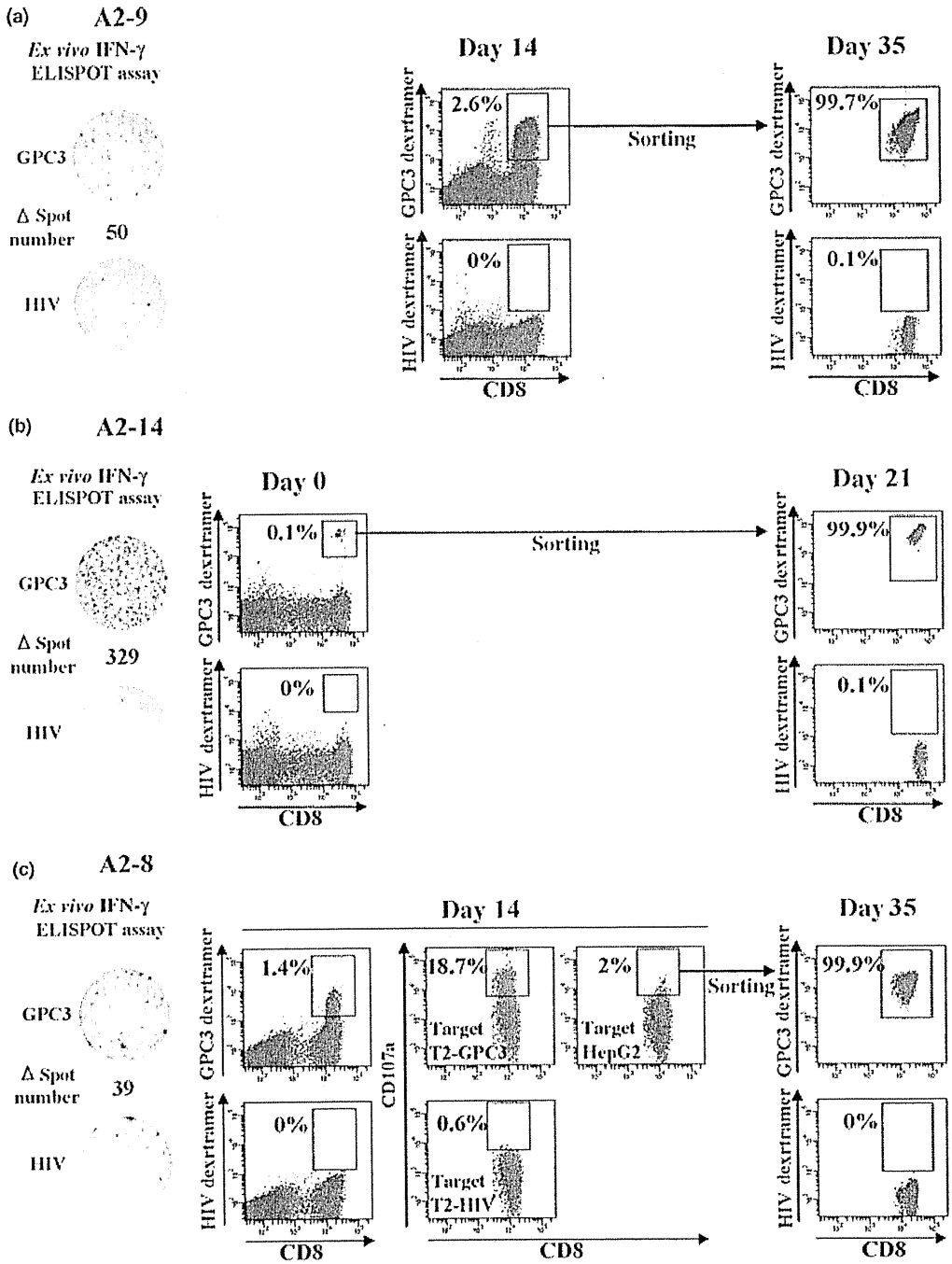

Fig. 2. Establishment of $GPC3_{144–152}$ peptide-specific CTL clones by three different methods. Left panels show the frequency of $GPC3_{144–152}$ peptide-specific CTLs in the PBMCs used, as established by *ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. (a) The PBMCs of patient A2-9 were stimulated with $GPC3_{144–152}$ peptide *in vitro* for 14 days. The population of $CD8^+$ GPC3 $Dextramer^+$ cells was sorted to a single cell. (b) $CD8^+$ GPC3 $Dextramer^+$ cells were directly sorted to a single cell from PBMCs of patient A2-14 without *in vitro* stimulation. (c) The PBMCs of patient A2-8 were stimulated with $GPC3_{144–152}$ peptide *in vitro* for 14 days. $CD8^+$ $CD107a^+$ cells that reacted against HepG2 were sorted to a single cell. Right panels show Dextramer analysis of the established clones 21 days after cell sorting.

patient A2-8 (Fig. 2c), the frequency of $GPC3_{144–152}$ peptide-specific CTLs was 39 of 5×10^5 PBMCs 1.5 months after the third vaccination, as determined by *ex vivo* ELISPOT assay, which were stimulated with $GPC3_{144–152}$ peptide *in vitro*. After 14 days, the population of $CD8^+$ GPC3 $Dextramer^+$ cells was 1.4% of all stimulated cells. We incubated $CD8^+$ T cells with T2 pulsed with $GPC3_{144–152}$, $HIV_{19–27}$ peptide, or HepG2. Approximately 2% and 18.7% of $CD8^+$ T cells mobilized CD107a in response to HepG2 and T2 pulsed with $GPC3_{144–152}$ peptide, respectively, but not in response to T2 pulsed with $HIV_{19–27}$ peptide. $CD107a^+$ $CD8^+$ cells that reacted against HepG2 were sorted to a single cell. The established clone was $CD8^+$ GPC3