Dextramer$^+$ CTLs (99.9%) which did not react with HIV Dextramer (Fig. 2c). These results indicate that $GPC3_{144–152}$ peptide-specific CTL clones were successfully established from PBMCs of patients injected with $GPC3_{144–152}$ peptide vaccine by three different methods. Moreover, the result that patient A2-8 CTL clone that reacted to HepG2 had $GPC3_{144–152}$ peptide specificity verified that $GPC3_{144–152}$ peptide was present naturally on HepG2.

**Analysis of $GPC3_{144–152}$ peptide-specific avidity of three CTL clones.** To further characterize the $GPC3_{144–152}$ peptide-specific avidity of the three CTL clones, we tested for the lysis of T2 cells pulsed with decreasing concentrations of $GPC3_{144–152}$ or $HIV_{19–27}$ peptide ranging from $10^{-6}$ to $10^{-14}$ M. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the recognition efficiency of that clone. The recognition efficiencies of patient A2-9, A2-14, and A2-8 clones were $10^{-10}$, $10^{-10}$, and $10^{-11}$ M, respectively (Fig. 3). These CTL clones did not react against T2 cells pulsed with $HIV_{19–27}$ peptide. These results indicate that the established clones were $GPC3_{144–152}$ peptide-specific and high avidity CTLs.

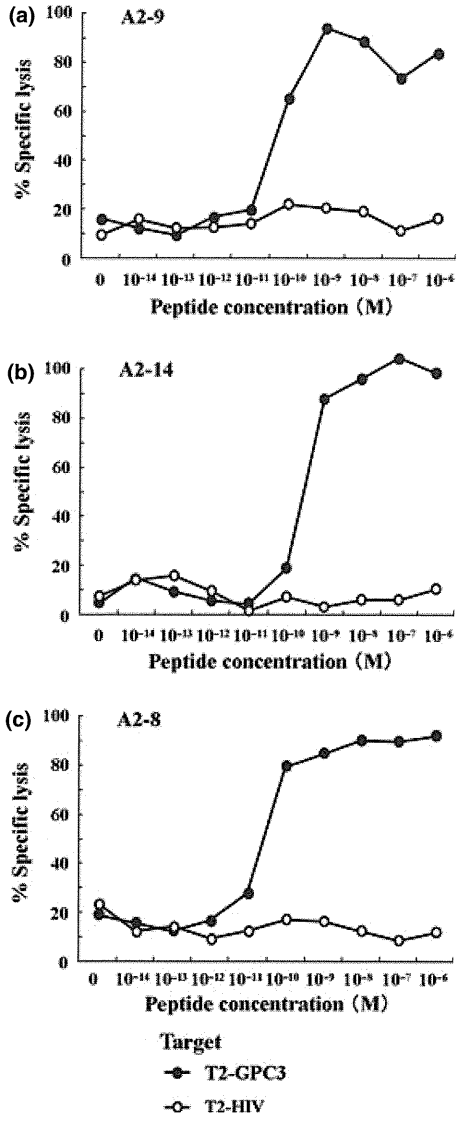


**Fig. 3.** Analysis of the $GPC3_{144–152}$ peptide specific avidity of the three CTL clones. The established CTL clones were tested for their avidities using various concentrations of $GPC3_{144–152}$ (●) or $HIV_{19–27}$ (○) peptide-loaded T2 targets. The peptide concentration at which the curve crossed 50% cytotoxicity was defined as the recognition efficiency of that clone. Effector/target ratio = 10. The recognition efficiencies of patient A2-9 (a), A2-14 (b), and A2-8 (c) CTL clones were $10^{-10}$, $10^{-10}$, and $10^{-11}$ M, respectively.

**Reactivity of three CTL clones against cancer cell lines.** We analyzed the IFN-γ production and cytotoxicity of the established CTL clones against cancer cell lines expressing HLA-A*02:01 and GPC3. We used SK-Hep-1 (GPC3$^-$, HLA-A*02:01$^+$) and a human GPC3 gene transfectant, SK-Hep-1/hGPC3 (GPC3$^+$, HLA-A*02:01$^+$), as target cells. Production of IFN-γ in the three CTL clones was detected against SK-Hep-1/hGPC3, but not against SK-Hep-1 (Fig. 4a). Furthermore, these CTL clones showed specific cytotoxicity against SK-Hep-1/hGPC3 and HepG2 (GPC3$^+$, HLA-A*02:01$^+$), but not against SK-Hep-1 and SW620 (GPC3$^-$, HLA-A*02:01$^+$) (Fig. 4b). These results indicate that all three CTL clones show cytotoxicity and the ability to produce IFN-γ against HLA-A*02:01$^+$ GPC3$^+$ HCC cell lines. Next, we examined whether these CTL clones respond to cancer cells weakly expressing GPC3. We used human melanoma cell line 526mel (GPC3$^+$, HLA-A*02:01$^+$) as a target cell that expresses GPC3 mRNA and protein at a lower level than the HCC cell lines (data not shown). Production of IFN-γ in patient A2-8 CTL clone (recognition efficiency: $10^{-11}$ M) were clearly detected against 526mel, whereas patient A2-9 CTL clone (recognition efficiency: $10^{-10}$ M) showed weak response to 526mel (Fig. 4c). Similarly, patient A2-8 CTL clone showed specific cytotoxicity against 526mel, whereas patient A2-9 CTL clone failed to lyse 526mel (Fig. 4d). These results suggest that higher avidity is essential to react to cancer cells weakly expressing GPC3.

**Analysis of HLA-A2 and GPC3 restriction.** In a cold target inhibition assay, cytotoxicity against SK-Hep-1/hGPC3 of patient A2-9 clone 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 (Fig. 5a). In an HLA blocking experiment, the IFN-γ production of patient A2-9 CTL clone was markedly inhibited by anti-HLA class I mAb and anti-HLA-A2 mAb as compared with that by IgG2a or IgG2b isotype control ($P < 0.05$) (Fig. 5b). Similarly, the cytotoxicity against SK-Hep-1/hGPC3 of patient A2-9 clone was markedly inhibited by anti-HLA class I mAb and anti-HLA-A2 mAb compared with that by IgG2a and IgG2b isotype control ($P < 0.05$) (Fig. 5c). These results clearly indicate that the CTL clone recognized SK-Hep-1/hGPC3 in an HLA-A2-restricted manner.

Next, to ascertain the GPC3 antigen-specific response of a CTL clone, we examined GPC3 knockdown using siRNA on the GPC3$^+$ HepG2 cell line. Representative data are shown in Figure 5(d–f). The GPC3 expression of HepG2 was clearly decreased by GPC3 siRNA on RT-PCR (Fig. 5d). Specifically, the GPC3 expression of HepG2 was decreased from 24 to 72 h following treatment with GPC3 siRNA on Western blot (Fig. 5e). We examined the IFN-γ production of patient A2-9 CTL clone against HepG2 treated with GPC3 siRNA. The IFN-γ production of the CTL clone was significantly decreased by GPC3 siRNA ($P < 0.05$) (Fig. 5f). These results indicate that HLA-A2-restricted $GPC3_{144–152}$ peptide can be processed naturally by cancer cells, and the peptides in the context of HLA-A2 can be expressed on the cell surface of cancer cells in order to be recognized by a $GPC3_{144–152}$ peptide-specific CTL clone.

## Discussion

Salgaller *et al.*[17] failed to detect dose dependency between 1 and 10 mg in terms of the capacity of gp100 peptide to enhance immunogenicity in humans. Previously, we reported that the peptide emulsified with incomplete Freund's adjuvant is stable, although the peptide is easily degraded in serum.[16] In this study, as with our previous report using a mouse model,[16] we


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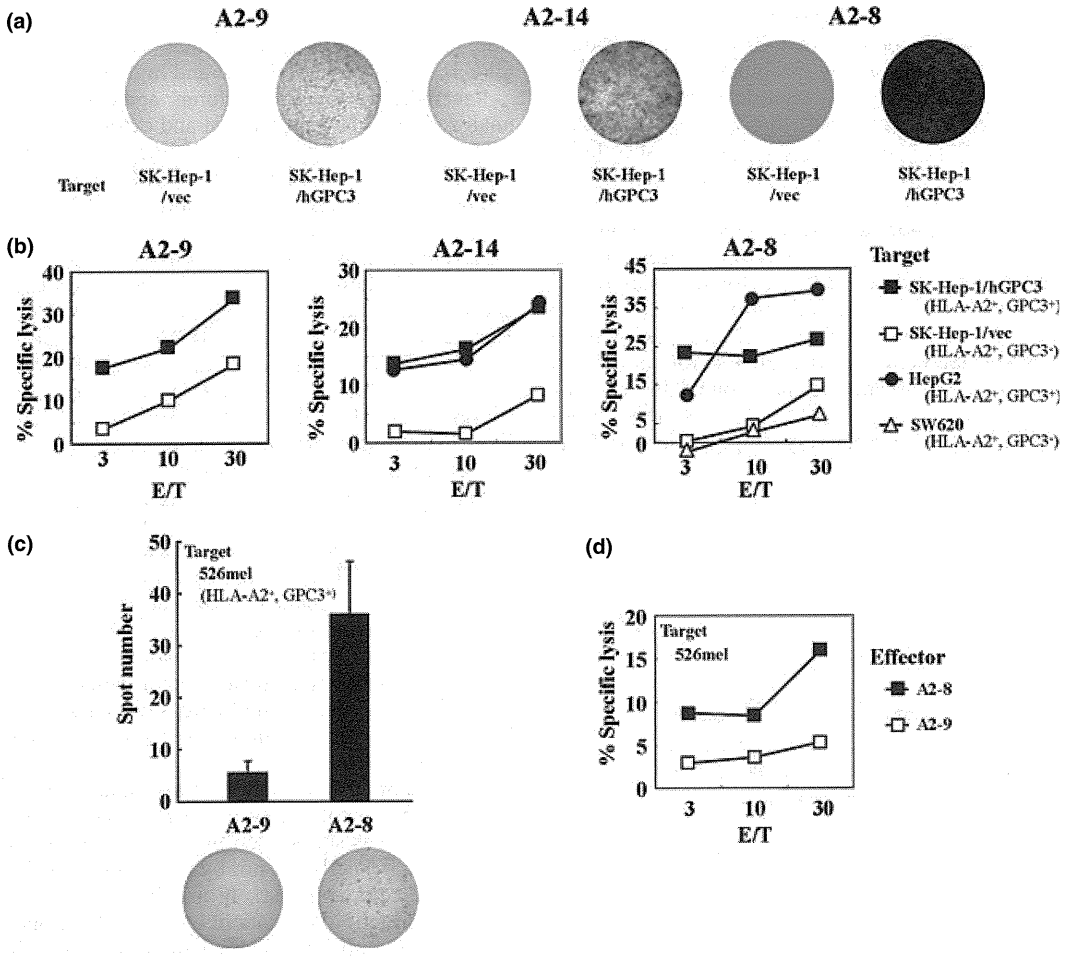


**Fig. 4.** Reactivity of three CTL clones against cancer cell lines. (a) γ-Interferon enzyme-linked immunospot assay of established CTL clones against SK-Hep-1/hGPC3 and SK-Hep-1/vec. Effector/target (E/T) ratio = 0.2. (b) Cytotoxic activities of the three CTL clones against SK-Hep-1/hGPC3 (■), SK-Hep-1/vec (□), HepG2 (●), or SW620 (Δ) analyzed by cytotoxicity assay. (c) γ-Interferon enzyme-linked immunospot assay of established CTL clones against 526mel. E/T ratio = 0.2. (d) Cytotoxic activities of patient A2-8 (■) and A2-9 (□) CTL clone against 526mel analyzed by cytotoxicity assay.

found that the effect of $GPC3_{144–152}$ peptide emulsified with incomplete Freund's adjuvant between 0.3 and 30 mg, to induce specific CTLs, was dose-dependent.

$GPC3_{144–152}$ (FVGEFFTDV) peptide was previously identified as an HLA-A*02:01-restricted peptide.[15] Moreover, we confirmed by binding assay that the peptide could also bind HLA-A*02:06 and HLA-A*02:07 molecules (data not shown). Therefore, we carried out a clinical trial for three types of HLA-A2 patient. Indeed, similar to HLA-A*02:01 patients, $GPC3_{144–152}$ peptide-specific CTLs increased after vaccination in both HLA-A*02:06 and HLA-A*02:07 patients (Fig. 1b). These findings suggest that $GPC3_{144–152}$ peptide is useful for not only HLA-A*02:01 patients but also HLA-A*02:06 and HLA-A*02:07 patients.

Notably, previous reports have shown that vaccination with synthetic peptides occassionally induced ineffective CTL responses due to various underlying mechanisms.[4–9] A possible mechanism is that responding T cells may have a very low affinity such that they recognize only target cells pulsed with high concentrations of the peptide and not tumor cells expressing the relevant epitopes at lower copy numbers. Alternatively, some antigen epitopes were not expressed on the surface of tumor cells.[18,19] When evaluating T-cell response to peptide vaccines, it is important to confirm that responding CTLs lyse human cancer cells. In the present study, although CTL clones established by Dextramer assay could react to HLA-A*02:01$^+$ GPC3$^+$ HCC cell lines, these clones failed to react to the HLA-A*02:01$^+$ GPC3$^+$ melanoma cell line 526mel expressing GPC3 mRNA and protein at a lower level than the HCC cell lines. Therefore, we attempted to establish CTL clones that are more tumor-reactive and with higher avidity than CTL clones established by Dextramer assay. Rubio *et al.*[20] showed that the surface mobilization of CD107a was useful for identifying and isolating functional tumor-reactive T cells with high recognition efficiency directly from PBMCs of cancer patients after vaccination. In the present study, the CTL clone showing the highest avidity ($10^{-11}$ M) and tumor reactivity was established by CD107a mobilization assay. Moreover, this clone could also react to 526mel.

For patients with metastatic melanoma, adoptive cell therapy has emerged as the most effective treatment.[21,22] However, tumor-infiltrating lymphocytes with high avidity for tumor antigens can only be generated from some patients with melanoma.[21] Recent studies have shown that genes encoding T-cell receptors (TCRs) can be isolated from high avidity T cells that recognize cancer antigens, and retroviral or lentiviral vectors can be used to redirect lymphocyte specificity to these cancer antigens.[23–26] In the present study, we were able to successfully establish some high avidity CTL clones. We analyzed the TCR β-chain variable region gene families of these clones by

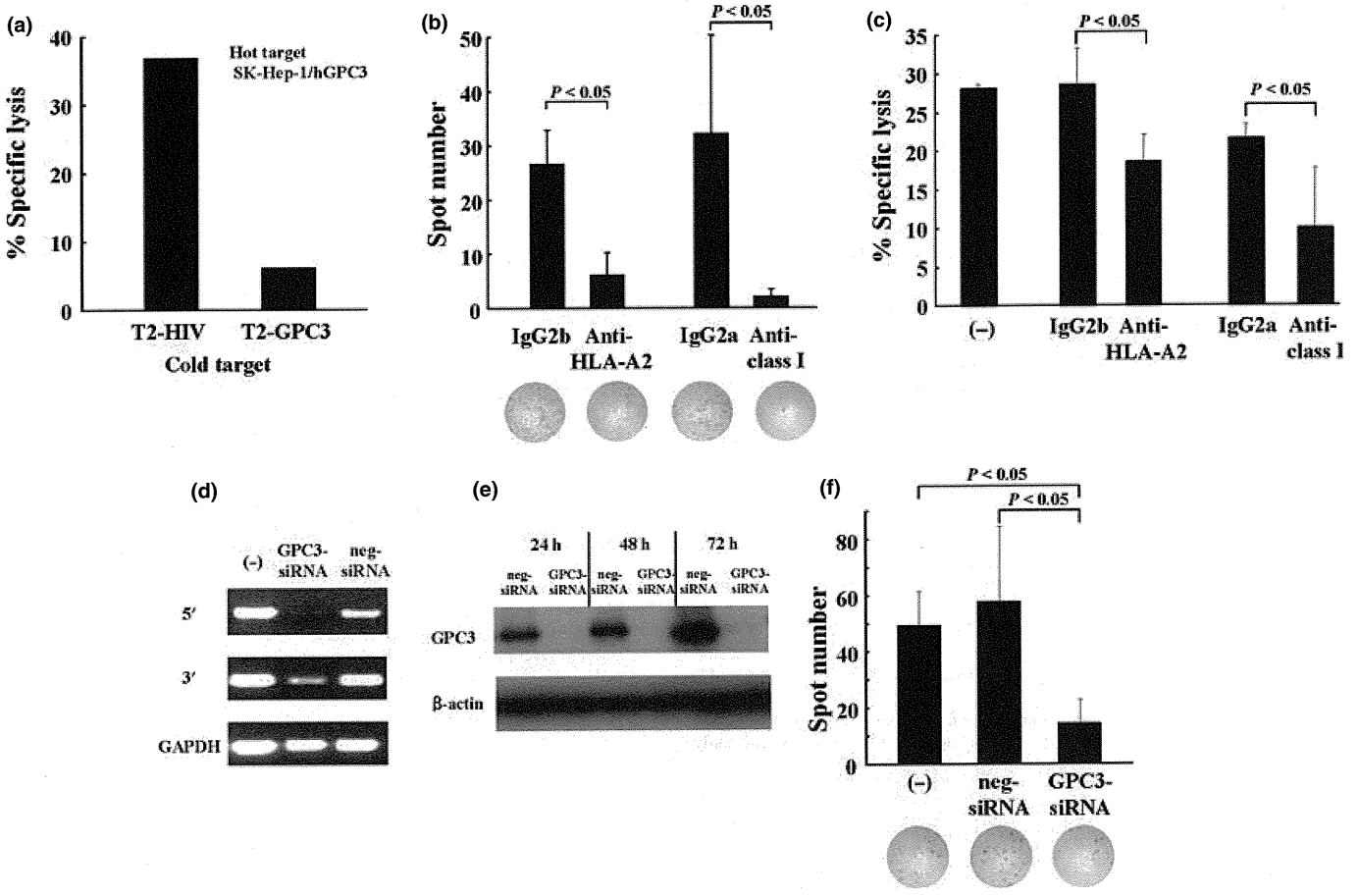


**Fig. 5.** Analysis of HLA-A2 and glypican-3 (GPC3) restriction. (a) Cold target inhibition assay of patient A2-9 CTL clone against SK-Hep-1/hGPC3. Effector/target (E/T) ratio = 30. T2 was prepulsed with either $HIV_{19-27}$ peptide or $GPC3_{144-152}$ peptide, then used as cold target cells. Cold/hot target ratio = 10. The cytotoxicity of the CTL clone was inhibited by T2 pulsed with $GPC3_{144-152}$ peptide but not by T2 pulsed with $HIV_{19-27}$ peptide. (b) Inhibition of interferon (IFN)-γ production by anti-HLA class I mAb and anti-HLA A2 mAb. SK-Hep-1/hGPC3 used as target cells. E/T ratio = 0.02. The IFN-γ production of the CTL clone was markedly inhibited by anti-HLA class I mAb and anti-HLA-A2 mAb as compared with that by IgG2a and IgG2b isotype control ($P < 0.05$). Data are expressed as the mean ± SD. (c) Inhibition of cytotoxicity by anti-HLA class I mAb and anti-HLA A2 mAb. SK-Hep-1/hGPC3 used as target cells. E/T ratio = 30. The cytotoxicity of the CTL clone was markedly inhibited by anti-HLA class I mAb and anti-HLA-A2 mAb compared with that by IgG2a and IgG2b isotype control ($P < 0.05$). (d) The GPC3 expression on HepG2 treated with GPC3-siRNA or negative (neg)-siRNA for 24 h as determined by RT-PCR. (e) The GPC3 expression on HepG2 treated with GPC3-siRNA or neg-siRNA from 24 to 72 h as determined by Western blot analysis. The GPC3 expression of HepG2 was decreased from 24 to 72 h after treatment with GPC3 siRNA. (f) The IFN-γ production of the CTL clone against HepG2 treated with GPC3 siRNA. E/T ratio = 0.02. The IFN-γ production of the CTL clone was decreased by GPC3 siRNA ($P < 0.05$). Data are expressed as the mean ± SD.

RT-PCR and carried out gene sequencing (data not shown). These clones had different TCR genes. Our results raise the possibility that these clones might be applicable to adoptive cell therapy for a large number of HCC patients.

In conclusion, we proved in this study the dose-dependent effects of highly immunogenic $GPC3_{144-152}$ peptide. Furthermore, we provided substantial evidence that CTLs showing not only high avidity but also natural antigen-specific killing activity against HCC cells could be induced in HCC patients by peptide vaccine.

## Acknowledgments

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## Disclosure Statement

The authors have no conflict of interest.

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

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**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

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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 $GPC3_{144\text{-}152}$ peptide-specific CTL clone against cancer cell lines.** $GPC3_{144\text{-}152}$ 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 $HIV_{19\text{-}27}$ peptide or $GPC3_{144\text{-}152}$ peptide, were used as cold target cells.

**CD107a degranulation assay.** $GPC3_{144\text{-}152}$ 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 ($5 \times 10^3$) 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 $GPC3_{144\text{-}152}$ peptide-specific CTL clone recognizes ovarian CCC cell lines.** To ascertain whether the HLA-A2-restricted $GPC3_{144\text{-}152}$ 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 $GPC3_{144\text{-}152}$ 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 $GPC3_{144\text{-}152}$ peptide-specific CTL clone reactivity.

**$GPC3_{144\text{-}152}$ 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

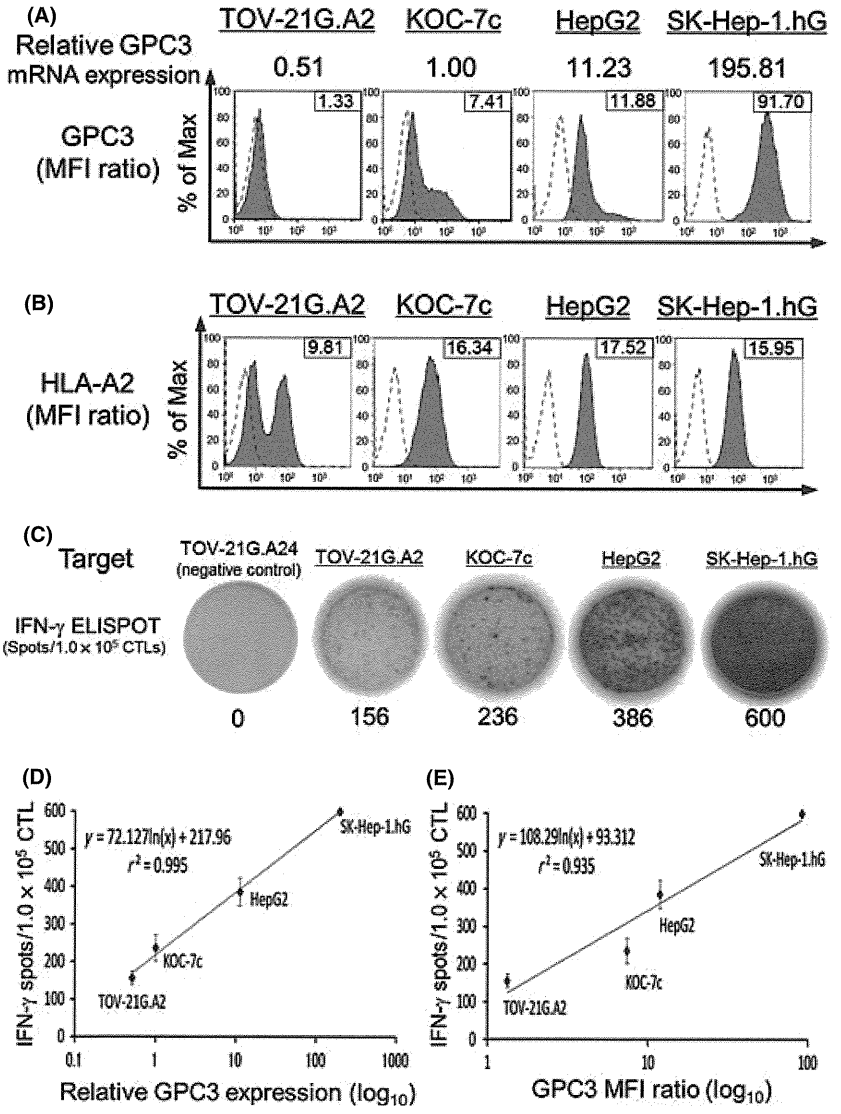


**Fig. 1.** HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone recognizes ovarian clear cell carcinoma (CCC) cell lines. (A) Expression of GPC3 on cancer cell lines. We used two human ovarian CCC cell lines (TOV-21G.A2 and KOC-7c) and two human HCC cell lines. We performed qRT-PCR and flow 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


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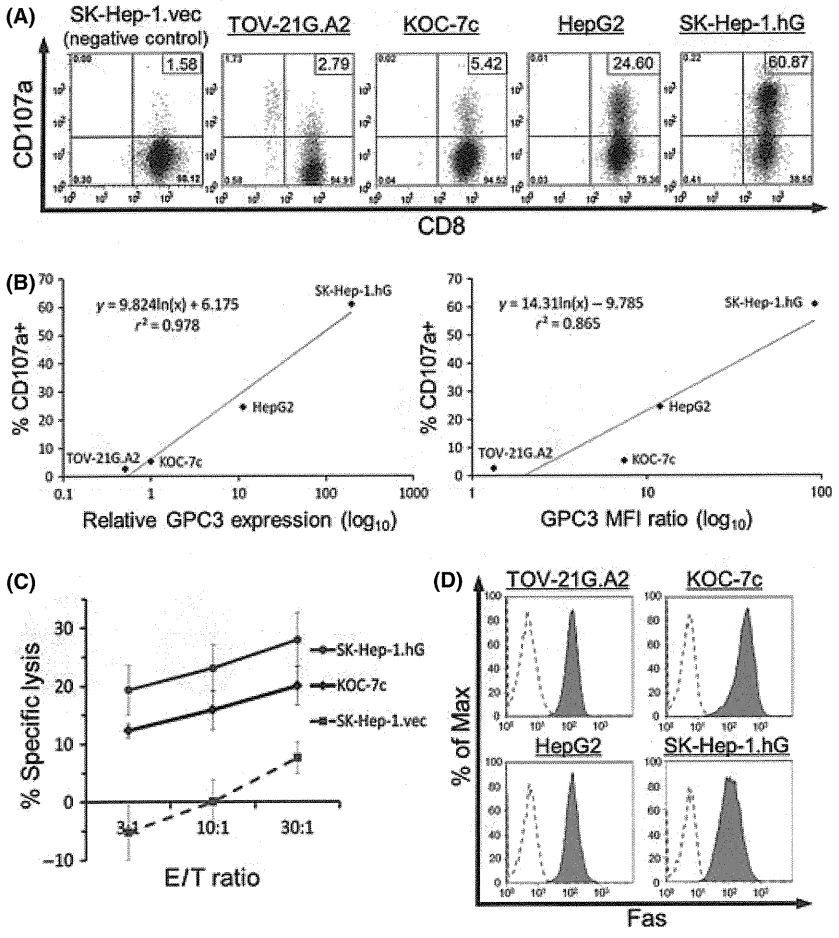


**Fig. 2.** $GPC3_{144-152}$ peptide-specific CTL clone lyses ovarian clear cell carcinoma (CCC) cell lines. (A) CD107a degranulation assay. Representative data are shown. GPC3-specific CTL responses against TOV-21G.A2 and KOC-7c cells exhibited 2.79% and 5.42% CD107a staining, respectively. (B) CD107a 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

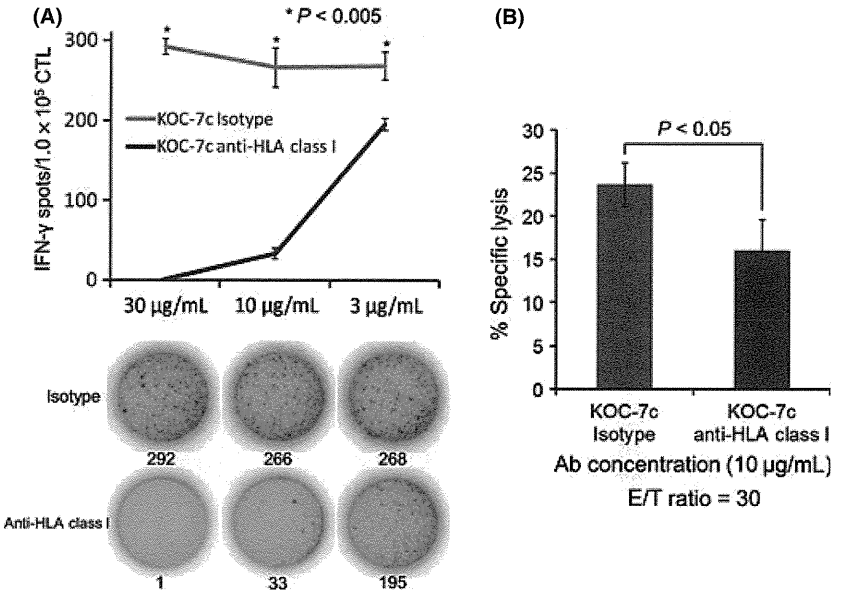


**Fig. 3.** Analysis of HLA class I restriction. (A) Inhibition of IFN-γ production by anti-HLA class I mAb. Effector/target ratio = 2. Data represent the mean ± SD of six wells. IFN-γ production of the CTL clone was markedly inhibited by anti-HLA class I mAb compared with that by isotype control in 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 ($P < 0.05$).

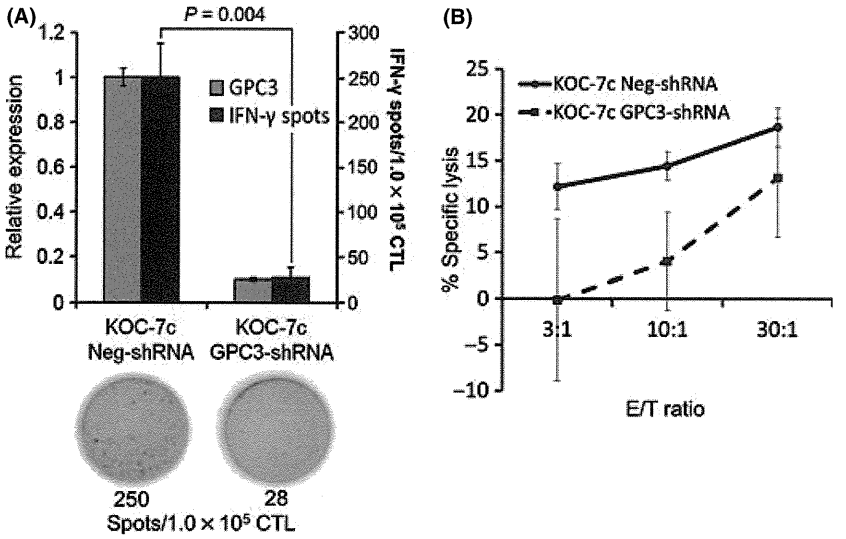


**Fig. 4.** Effect of GPC3 silencing using shRNA on the response of $GPC3_{144-152}$ peptide-specific CTL clone against KOC-7c cells. (A) GPC3 expression of KOC-7c was obviously decreased by GPC3 shRNA on qRT-PCR. IFN-γ production was significantly decreased by GPC3 shRNA ($P = 0.004$). Data represent 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



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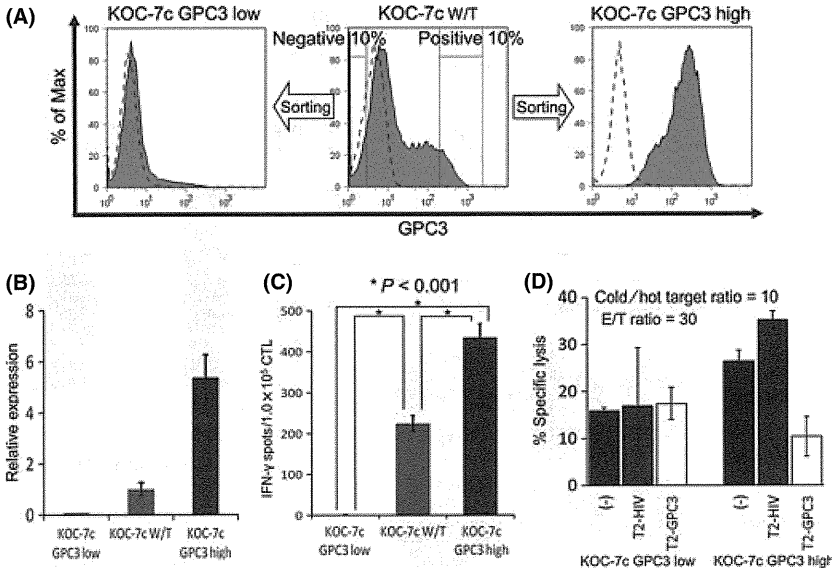


**Fig. 5.** The level of GPC3 expression on the cell surface is responsible for CTL recognition. (A) KOC-7c GPC3 high and GPC3 low cells were sorted as described in the Materials and Methods. (B) Relative GPC3 mRNA expression (ratio to KOC-7c wild type) is shown. Data represent the mean ± SD. (C) IFN-γ 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.

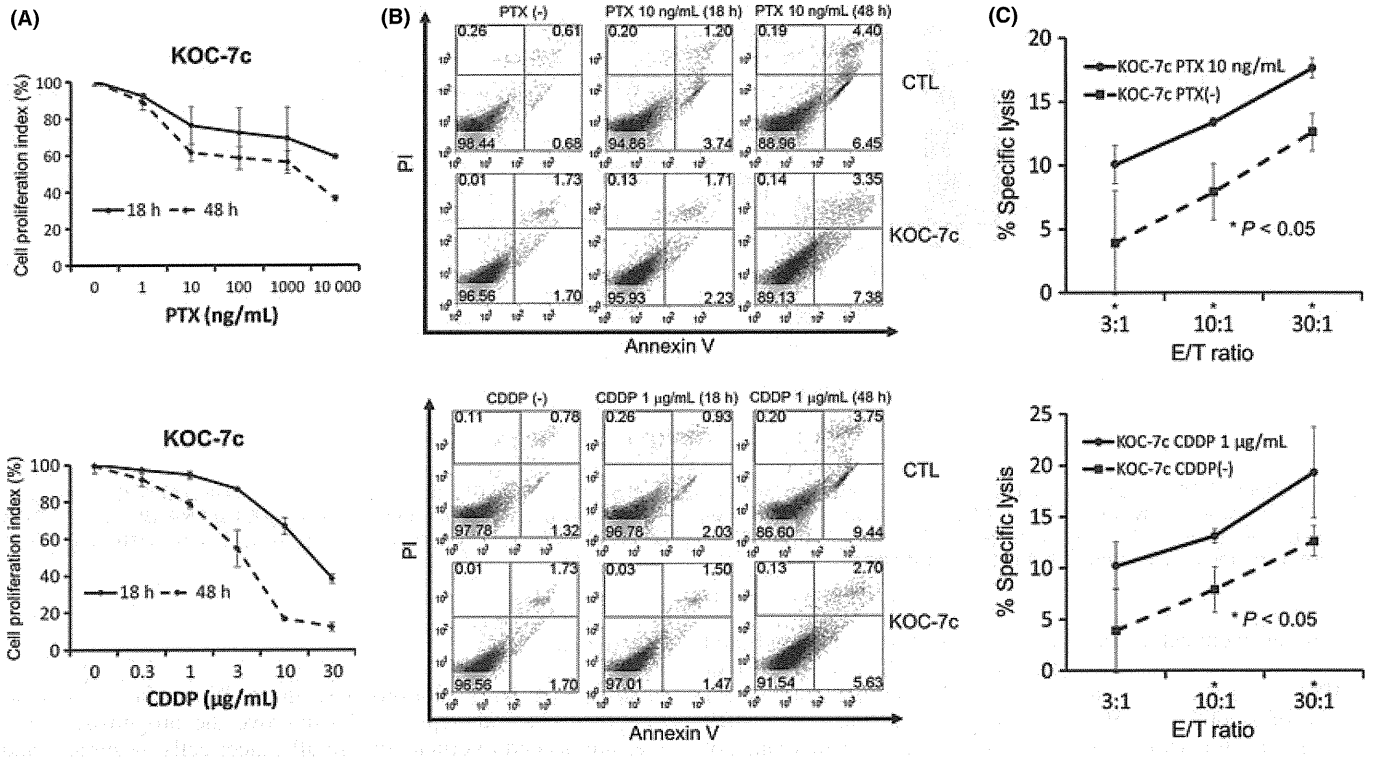


**Fig. 6.** Subtoxic-dose chemotherapy sensitizes KOC-7c cells to the cytotoxic effect of the $GPC3_{144-152}$ peptide-specific CTL clone. We used two agents (paclitaxel [PTX] and cisplatin [CDDP]) to investigate whether they sensitize ovarian clear cell carcinoma (CCC) cells to GPC3-specific lysis. (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.

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## Disclosure Statement

The authors have no conflict of interest.

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# Radiofrequency ablation for hepatocellular carcinoma induces glypican-3 peptide-specific cytotoxic T lymphocytes

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**Abstract.** Glypican-3 (GPC3), a carcinoembryonic antigen, is an ideal target for anticancer immunotherapy against hepatocellular carcinoma (HCC). In this study, we attempted to compare the induction of the GPC3-specific T-cell-mediated immune response after locoregional therapies in HCC patients and tumor-bearing mice. Twenty-seven HCC patients treated with locoregional therapies, including radiofrequency ablation (RFA), surgical resection and transcatheter arterial chemoembolization (TACE), were prospectively enrolled in this study. Additionally, we performed RFA experiments using a mouse model. GPC3-specific T-cell response was investigated pre-treatment and post-treatment by an interferon-γ enzyme-linked immunospot assay using peripheral blood mononuclear cells from HCC patients and lymph node cells from tumor-bearing mice. Circulating GPC3-specific cytotoxic T lymphocytes (CTLs) were increased in 5 of 9 patients after RFA and in 4 of 9 patients after TACE, but in only 1 of 9 patients after surgical resection. All 7 patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas none of the 7 patients did after surgical resection. The number of increased GPC3-specific CTLs after RFA was significantly larger than that after surgical resection (P=0.023). Similarly, the frequency of GPC3-specific CTLs after RFA was significantly greater than that after surgical resection in the mouse model (P=0.049). We validated for the first time the stronger effect on the immune system brought by RFA compared with surgical resection for HCC patients and tumor-bearing mice. Combined treatment of RFA and immunotherapy is a reasonable strategy against HCC.

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*Abbreviations:* GPC3, glypican-3; HCC, hepatocellular carcinoma; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; CTL, cytotoxic T lymphocyte; CT, computed tomography; TNM, tumor-node-metastasis; UICC, the Union for International Cancer Control; PBMC, peripheral blood mononuclear cell; IFN, interferon; ELISPOT, enzyme-linked immunospot; HSP105, heat shock protein 105; CMV, cytomegalovirus; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist II; hTERT, human telomerase reverse transcriptase; MRP3, multidrug resistance-associated protein 3

*Key words:* hepatocellular carcinoma, radiofrequency ablation, glypican-3, cytotoxic T lymphocyte, immunotherapy

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common and most serious cancers worldwide (1). Locoregional therapies, including radiofrequency ablation (RFA), surgical resection, and transcatheter arterial chemoembolization (TACE), are recognized as the gold-standard therapies for HCC patients whose cancer lesions are limited to the liver (2). However, the recurrence rate remains quite high despite potentially curative treatment (3,4). The reasons for this are as follows: first, a multicentric new tumor frequently occurs from underlying active hepatitis or cirrhosis and, second, a small tumor undetectable by imaging modalities frequently exists before treatment and would be left untreated (5). Therefore, the establishment of effective adjuvant therapy to prevent recurrence is urgently required, and

clinical trials are ongoing throughout the world (6). However, at the present time, there is no universal consensus (2,7,8).

Previous studies have reported that local tumor ablation treatments, such as RFA and cryoablation, not only destroy tumor tissue but also induce a marked inflammatory response both locally and systemically (9,10). Unlike surgical resection, tumor ablation treatment generates tumor cell necrosis (11), followed by the release of tumor-associated antigens (12). These antigens can be uptaken, processed, and presented by dendritic cells (10,13), and then an antigen-specific T-cell-mediated immune response can be induced (9). If this induction is sufficiently steady and reliable, it may provide the basis for adjuvant immunotherapy, which is an attractive strategy.

Glypican-3 (GPC3) belongs to the glypican family of heparan sulfate proteoglycans that are linked to the outer surface of the cell membrane through a glycosylphosphatidylinositol anchor (14). GPC3 is one of the carcinoembryonic antigens overexpressed in HCC (15-17). We have shown that GPC3 is an ideal target for anticancer immunotherapy because its expression is specifically detected in ~80% of HCCs even in the early stages and is correlated with a poor prognosis (18-21). Moreover, GPC3-specific cytotoxic T lymphocytes (CTLs) have a high level of killing activity against HCC tumor cells (22). We have finished the phase I clinical trial of a GPC3-derived peptide vaccine for patients with advanced HCC (unpublished data), and just started the phase II clinical trial for adjuvant therapy after curative resection or RFA.

In this study, our aim was to determine if the GPC3-specific T-cell-mediated immune response is strengthened after locoregional therapies in HCC patients and tumor-bearing mice. Moreover, we evaluated the hypothesis that the post-treatment immune response may provide the basis for adjuvant immunotherapy.

## Materials and methods

*Patient population and treatment of HCC.* Twenty-seven patients with primary HCC were prospectively enrolled in this study from January to November 2007 at the National Cancer Center Hospital East, in Japan. The eligibility criteria included primary HCC, which would undergo locoregional therapies with curative intent. Three treatment groups of nine patients each would undergo RFA, surgical resection, or TACE, respectively. Treatment selection in each patient was in accordance with the Japanese HCC treatment guidelines (2). Other inclusion criteria included HLA-A24 or HLA-A2 gene-positive status, as determined by commercially-available genomic DNA typing tests (Mitsubishi Chemical Medience, Tokyo, Japan), and no other active malignancy. HCC was diagnosed using dynamic computed tomography (CT). Tumor stage was assigned according to the tumor-node-metastasis (TNM) classification of the Union for International Cancer Control (UICC) (23). All RFA procedures were performed percutaneously under ultrasound guidance. Curative treatment was defined as complete necrosis of the tumor lesion confirmed by dynamic CT after RFA, a negative surgical margin confirmed histopathologically after resection, and complete lipiodol deposition after TACE.

All patients gave written informed consent before entering the study and this study was approved by the Ethics Committee of the National Cancer Center, conforming to the ethical guidelines of the 1975 Declaration of Helsinki.

*Collection of blood samples and preparation of peripheral blood mononuclear cells.* Venous blood (20-30 ml) from each patient was collected both before treatment and one month after treatment. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using LeucoSep® tubes (Greiner Bio-One, Frickenhausen, Germany) by means of density gradient centrifugation.

*Identification of GPC3-specific CTLs in HCC patients.* In order to identify GPC3-specific CTLs, the proportion of cells producing interferon (IFN)-γ upon stimulation with GPC3 peptide was assessed by an *ex vivo* IFN-γ enzyme-linked immunospot (ELISPOT) assay using pooled PBMCs from HCC patients. Defrosted PBMCs ($1x10^6$ cells/well) were cultured in duplicate using 96-well flat-bottomed plates (BD Biosciences, San Jose, CA) with HLA-A24-restricted $GPC3_{298-306}$ peptide (EYILSLEEL) or HLA-A2-restricted $GPC3_{144-152}$ peptide (FVGEFFTDV) (10 μmol/l) with 100 U/ml recombinant human interleukin-2 (IL-2) for 20 h. The negative control consisted of medium alone or HLA-A24- or HLA-A2-restricted heat shock protein 105 (HSP105) peptide, and the positive control included the HLA-A24- or HLA-A2-restricted cytomegalovirus (CMV) peptide. The number of spots, which indicated the presence of IFN-γ secreting cells, was automatically counted using the Eliphoto system (Minerva Tech, Tokyo, Japan). For an exact comparison of the frequency of GPC3-specific CTLs existing at pre- and post-treatment, the obtained mean values of the number of spots with non-peptide-pulsed samples ($1x10^6$ PBMCs) at pre- and post-treatment were equalized and set to zero, and then the actual number of GPC3-, CMV-, or HSP105-specific spots was calculated. The Δspot was defined as the difference in the number of spots with each antigen between pre- and post-treatment.

*Mice.* Female BALB/c mice ($H\text{-}2^d$), 6-8 weeks of age, were obtained from Charles River Laboratories Japan (Yokohama, Japan). The mice were maintained under specific-pathogen-free conditions. All animal procedures were performed in compliance with the guidelines by the Animal Research Committee of the National Cancer Center, Japan.

*Tumor cell lines.* A subline of the BALB/c-derived GPC3-negative colorectal adenocarcinoma cell line, Colon 26 (24), was provided by Dr Kyoichi Shimomura (Astellas Pharma, Tokyo, Japan). Colon 26/GPC3 is an established stable GPC3-expressing cell line (18). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in humidified 5% $CO_2$ at 37˚C.

*RFA experiment using a mouse model.* The mice were shaved at the tumor area and the contralateral flank. After attachment of the electricity-conducting pad (ground pad) onto the contralateral side, an RFA needle with 5-mm active tip (Cool-tip™, Valleylab, Boulder, CO) was inserted into the middle of the tumor. Impedance could be evaluated on the RFA lesion generator system (RFG-3B model, Radionics, Burlington, MA).

Treatment was started by delivering RFA energy. During two treatment cycles of 10 sec, the temperature could be monitored using the thermistor and thermocouple in the tip of the probe. Treatment was considered successful if a tip temperature of 60-70˚C was reached.

*Identification of GPC3-specific CTLs in mice.* BALB/c mice were immunized beforehand by peptide vaccination with $K^d$-restricted $GPC3_{298-306}$ peptide (50 μg/mouse) emulsified with incomplete Freund's adjuvant twice at a 7-day interval as described previously (20). The day after the second vaccination, the mice were challenged subcutaneously with Colon 26/GPC3 tumor cells ($1x10^5$ cells/100 μl) on their shaved back and, 5 days later, the mice underwent therapeutic RFA or surgical resection for the established tumor. After the next 5 days, the mice were sacrificed and bilateral inguinal lymph nodes were obtained. $CD8^+$ T cells were isolated from lymph node cells using anti-mouse CD8α (Ly-2) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and an IFN-γ ELISPOT assay was performed without prior *in vitro* stimulation. For the IFN-γ ELISPOT assay, $CD8^+$ lymph node cells ($3x10^5$ cells/well) were used as effector cells, and Colon 26 and Colon 26/GPC3 cells ($3x10^4$ cells/well) as target cells. These cells were cultured in duplicate using 96-well flat-bottomed plates (BD Biosciences) with 100 U/ml recombinant murine IL-2 for 20 h. The number of spots after RFA or surgical resection was compared with that without treatment.

*Immunohistochemical analysis.* To investigate GPC3 expression in HCC tissues, we performed immunohistochemical staining of GPC3 in biopsy specimens or resected specimens from HCC patients. The paraffin-embedded blocks were analyzed using monoclonal anti-GPC3 antibody (dilution 1:300, BioMosaics, Burlington, VT) as described previously (17,21). The results were classified into two groups according to the area of GPC3-positive staining cells as follows: -, negative (<10%) and +, positive (≥10%).

To investigate tumor-infiltrating lymphocytes, we performed immunohistochemical staining of CD4 and CD8 in resected specimens from an HCC patient using monoclonal anti-CD4 or CD8 antibody (dilution 1:20, Novocastra, Newcastle upon Tyne, UK).

*Statistical analysis.* Statistical analyses were performed using $\chi^2$ test, Mann-Whitney U test, or Kruskal-Wallis rank test. Differences were considered significant at $P<0.05$. Data were analyzed with the StatView 5.0 software package (Abacus Concepts, Calabasas, CA).

## Results

*Demographics and clinical characteristics.* The characteristics of all 27 patients are represented in Table I. The three groups of 9 patients received RFA (RFA1-9), surgical resection (RES1-9), or TACE (TAE1-9), respectively. Among them, 21 patients had the HLA-A24 gene and 7 had the HLA-A2 gene. One patient had both HLA-A24 and -A2, and the HLA-A2-restricted $GPC3_{144-152}$ peptide was used for the IFN-γ ELISPOT assay in this patient. Among the three treatment groups, tumor size in the RFA group (mean: 16.4 mm) was significantly smaller than that in the resection group (mean: 43.2 mm) (P=0.001) and the TACE group (mean: 44.1 mm) (P=0.001). Similarly, tumor stage in the RFA group was less advanced than that in the resection group (P=0.018) and TACE group (P=0.005). There was no statistically significant difference in Child-Pugh classification grade among the three groups (P=0.128). In this study, all treatments were considered to be curative according to the definitions described in Materials and methods. Moreover, all groups reduced the levels of α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II) in most of HCC patients after treatment (data not shown). The diagnosis of HCC was histopathologically confirmed by biopsy specimens or resected specimens from 21 patients. GPC3 expression was detected by immunohistochemical staining in 14 of 21 patients.

*Analysis of GPC3-specific CTLs in HCC patients.* As shown in Table I, GPC3-specific CTLs were detected in 11 and 15 of 27 patients at pre- and post-treatment, respectively. In total, 19 patients had GPC3-specific CTLs at either pre- or post-treatment. There was no statistically significant correlation between the presence of GPC3-specific CTLs and clinical features, including HLA-A type (P=0.126), age (P=0.750), gender (P=0.764), HCV infection (P=0.674), HBV infection (P=0.764), Child-Pugh classification grade (P=0.404), tumor multiplicity (P=0.674), tumor size (P=0.650), HCC staging (P=0.155), serum AFP level (P=0.288), and serum PIVKA-II level (P=0.094). Among the 21 patients who had the information about GPC3 expression in their HCC tissue, patients with GPC3-expressing HCCs had GPC3-specific CTLs more frequently than those with GPC3-negative HCCs, but the difference was not statistically significant (P=0.053).

*Changes in GPC3-specific CTLs between before and after treatment.* In order to analyze the effect of anticancer treatment on GPC3-specific T-cell response, we compared the frequency of GPC3-specific CTLs in PBMCs before treatment with that after treatment. As shown in Table I and Fig. 1, an increase in GPC3-specific CTLs was found in 5 of 9 patients after RFA and in 4 of 9 after TACE, but in only 1 of 9 patients after resection. Of note, all of the 7 patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas none of the 7 patients with GPC3-expressing HCCs did after surgical resection. The Δspot of GPC3 in the RFA group (mean: 24.4 spots) was larger than that in the resection group (mean: -7.2 spots) (P=0.023). The Δspot of GPC3 in the TACE group (mean, 36.9 spots) was also larger than that in the resection group, but the difference was not statistically significant (P=0.096). In contrast, the Δspot of CMV showed no difference among the three groups (P=0.498). Neither the existence of GPC3-specific CTLs before or after treatment, nor the changes between before and after treatment had statistically significant correlation with patient survival according to the log-rank test in each treatment group (neither disease-free nor overall), with the 27-month mean follow-up period (data not shown).

The representative data on changes in CT images and serum levels of tumor markers between before and after treatment is shown in Fig. 2. All three patients (RFA3, RES6, and TAE5) had GPC3-expressing HCCs. Both the CT images and

Table I. Patient characteristics and glypican-3-specific cytotoxic T lymphocytes.

| Patient | HLA | Age (yrs.) | Gender | Etiology | Child-Pugh | No. of tumor | Tumor size (mm) | T[1] | N[1] | M[1] | AFP (<9.5 ng/ml) | PIVKA-II (<40 mAU/ml) | GPC3 expression[2] | GPC3-specific CTLs[3] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | Pre | Post | Change | Δspot[4] |
| RFA1 | A24 | 73 | F | HBV | A | 2 | 26 | 2 | 0 | 0 | 4.0 | 228 | - | 4 | 0 | - | -4 |
| RFA2 | A24 | 68 | M | HCV | B | 1 | 20 | 1 | 0 | 0 | 5.0 | 300 | + | 10 | 24 | + | +14 |
| RFA3 | A2 | 50 | M | HCV | A | 1 | 15 | 1 | 0 | 0 | 63.3 | 25 | + | 0 | 88 | + | +88 |
| RFA4 | A24 | 79 | F | HCV | A | 1 | 10 | 1 | 0 | 0 | 484.2 | 30 | + | 0 | 10 | + | +10 |
| RFA5 | A24 | 69 | M | HCV | A | 1 | 15 | 1 | 0 | 0 | 2.3 | 57 | - | 0 | 0 | +/- | 0 |
| RFA6 | A24 | 60 | M | HCV | A | 1 | 17 | 1 | 0 | 0 | 15.1 | 23 | - | 0 | 0 | +/- | 0 |
| RFA7 | A2 | 73 | M | HCV | A | 1 | 20 | 1 | 0 | 0 | 97.3 | 51 | + | 3 | 88 | + | +85 |
| RFA8 | A2/A24 | 64 | M | HBV/HCV | B | 1 | 15 | 1 | 0 | 0 | 39.9 | 17 | + | 0 | 31 | + | +31 |
| RFA9 | A2 | 60 | M | HCV | B | 1 | 10 | 1 | 0 | 0 | 92.0 | 19 | - | 19 | 15 | - | -4 |
| RES1 | A24 | 48 | M | HBV | A | 1 | 20 | 1 | 0 | 0 | 19.7 | 38 | + | 32 | 15 | - | -17 |
| RES2 | A24 | 66 | F | HCV | A | 1 | 26 | 2 | 0 | 0 | 63.4 | 77 | + | 20 | 3 | - | -17 |
| RES3 | A24 | 64 | M | HCV | A | 2 | 30 | 2 | 0 | 0 | 10.1 | 276 | + | 12 | 0 | - | -12 |
| RES4 | A2 | 72 | M | - | A | 1 | 60 | 2 | 0 | 0 | 9.2 | 1500 | + | 3 | 1 | - | -2 |
| RES5 | A24 | 70 | M | HCV | A | 1 | 20 | 1 | 0 | 0 | 4.2 | 25 | + | 0 | 0 | +/- | 0 |
| RES6 | A24 | 42 | M | HBV/HCV | A | 2 | 98 | 3 | 0 | 0 | 15115.0 | 22477 | + | 50 | 30 | - | -20 |
| RES7 | A2 | 75 | M | - | A | 1 | 75 | 2 | 0 | 0 | 22.8 | 10341 | - | 0 | 3 | + | +3 |
| RES8 | A24 | 52 | M | HCV | A | 1 | 30 | 1 | 0 | 0 | 16.0 | 234 | + | 0 | 0 | +/- | 0 |
| RES9 | A24 | 60 | M | HBV | A | 1 | 30 | 1 | 0 | 0 | 15.6 | 23 | - | 0 | 0 | +/- | 0 |
| TAE1 | A2 | 64 | M | - | A | 3 | 30 | 2 | 0 | 0 | 10.7 | 98 | + | 0 | 330 | + | +330 |
| TAE2 | A24 | 78 | F | HCV | B | 1 | 60 | 1 | 0 | 0 | 2483.0 | 3932 | ND | 34 | 0 | - | -34 |
| TAE3 | A24 | 77 | F | - | A | >5 | 35 | 3 | 0 | 0 | 180.2 | 11538 | ND | 0 | 3 | + | +3 |
| TAE4 | A24 | 77 | M | HCV | A | 2 | 80 | 4 | 0 | 0 | 20014.0 | 241 | ND | 0 | 0 | +/- | 0 |
| TAE5 | A24 | 55 | M | HBV | A | 2 | 30 | 2 | 0 | 0 | 3.7 | 24 | + | 0 | 23 | + | +23 |
| TAE6 | A24 | 77 | M | - | A | >5 | 42 | 2 | 0 | 0 | 1407.0 | 1661 | ND | 0 | 20 | + | +20 |
| TAE7 | A24 | 63 | F | HCV | A | >5 | 32 | 2 | 0 | 0 | 640.3 | 270 | ND | 0 | 0 | +/- | 0 |
| TAE8 | A24 | 74 | M | - | A | 1 | 18 | 1 | 0 | 0 | 3.8 | 12 | - | 0 | 0 | +/- | 0 |
| TAE9 | A24 | 62 | M | HCV | A | 3 | 70 | 3 | 0 | 0 | 46.8 | 1907 | ND | 10 | 0 | - | -10 |

[1]Tumor stage was assigned according to the tumor-node-metastasis (TNM) classification of the Union for International Cancer Control (UICC). [2]GPC3 expression was evaluated by immunohistochemical staining; +, positive; -, negative. [3]Peripheral blood was taken from each patient before and after treatment, and GPC3-specific CTLs were measured by *ex vivo* interferon-γ enzyme-linked immunospot assay; +, increase; -, decrease; +/-, no change. [4]The Δspot was defined as the difference in the number of spots with each antigen between pre- and post-treatment. F, female; M, male; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist II; GPC3, glypican-3; ND, not determined.

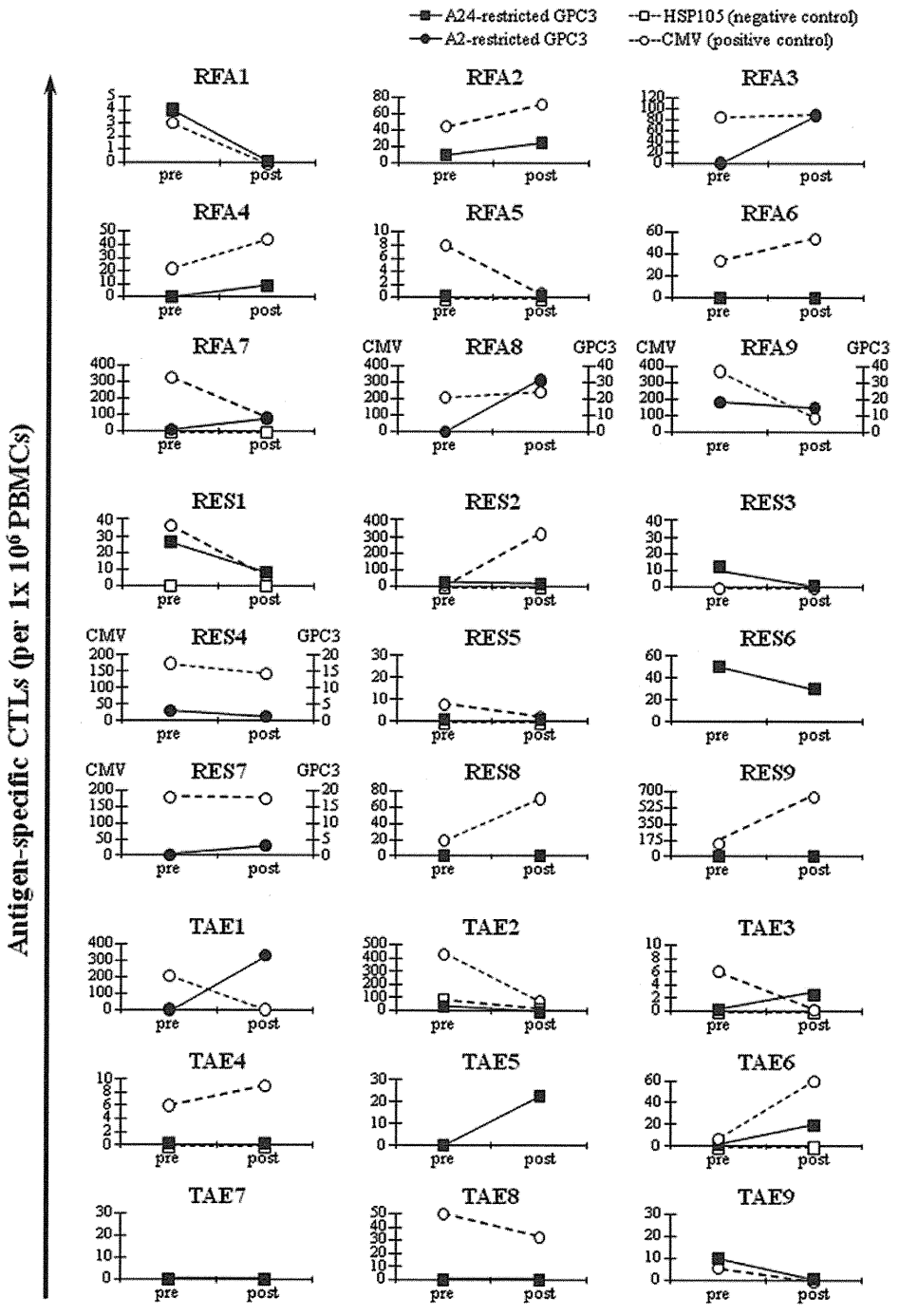


Figure 1. Kinetics of glypican-3 (GPC3)-specific CTLs between before and after treatment in each patient. A direct *ex vivo* interferon-γ enzyme-linked immunospot assay of PBMCs was performed before treatment and one month after treatment. The data are expressed as the number of interferon-γ producing cells, which indicate the CTLs specific with HLA-A24-restricted $GPC3_{298-306}$ peptide (EYILSLEEL) (■) or HLA-A2-restricted $GPC3_{144-152}$ peptide (FVGEFFTDV) (●). Heat shock protein 105 (HSP105) peptide (□) and cytomegalovirus (CMV) peptide (○) were used as the negative and positive control, respectively.

kinetics of tumor markers indicated that their treatment was effective. The frequency of GPC3-specific CTLs increased after RFA (RFA3) and TACE (TAE5), whereas it decreased after surgical resection (RES6).

*RFA has the potential to strongly induce T-cell-mediated immune response: A case report.* A 70-year-old woman was admitted because of recurrent HCCs. Thirteen months earlier, the patient had undergone RFA for primary HCC located in the S5/8 region of the liver. CT detected two recurrent HCCs: one was contiguous to the previously ablated S5/8 region and the other was a distant tumor located in the S6 region. We performed surgical resection for these recurrent HCCs. Immunohistochemical examination of CD8 in the resected tumors revealed that a marked number of $CD8^+$ T cells had infiltrated not only into the surrounding recurrent tumor but also into the distant recurrent tumor after RFA (Fig. 3). On the other hand, few $CD4^+$ T cells were observed in these tumors (data not shown). Immunohistochemical analyses showed the expression of GPC3 and HLA class I in these tumors (data not

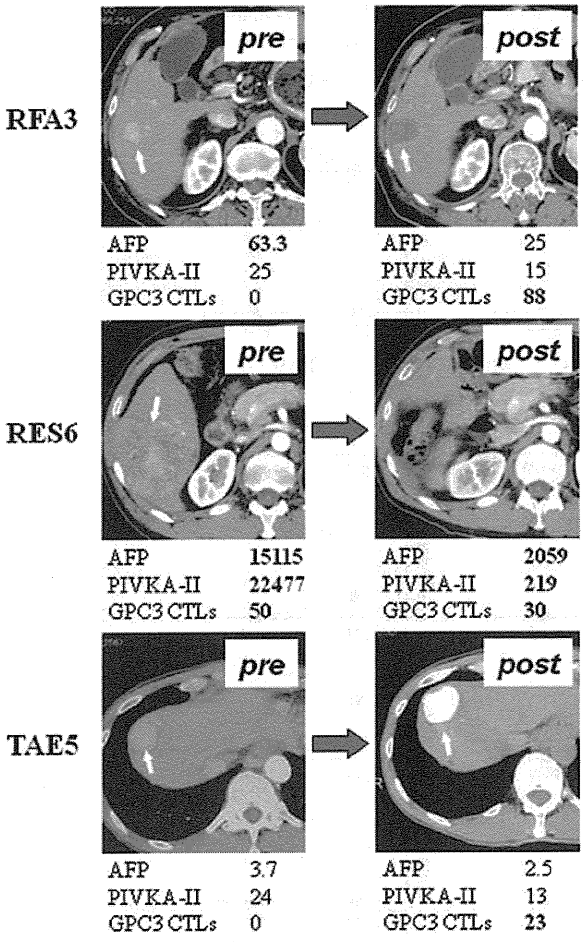


Figure 2. Changes in computed tomography images, serum levels of tumor markers, including α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II), and glypican-3 (GPC3)-specific CTLs in PBMCs between before and after treatment in patients RFA3, RES6, and TAE5. White arrows indicate nodules of hepatocellular carcinoma at pre- and post-treatment. The bold letters show the abnormal levels of tumor markers or the positive response of GPC3 specific CTLs.

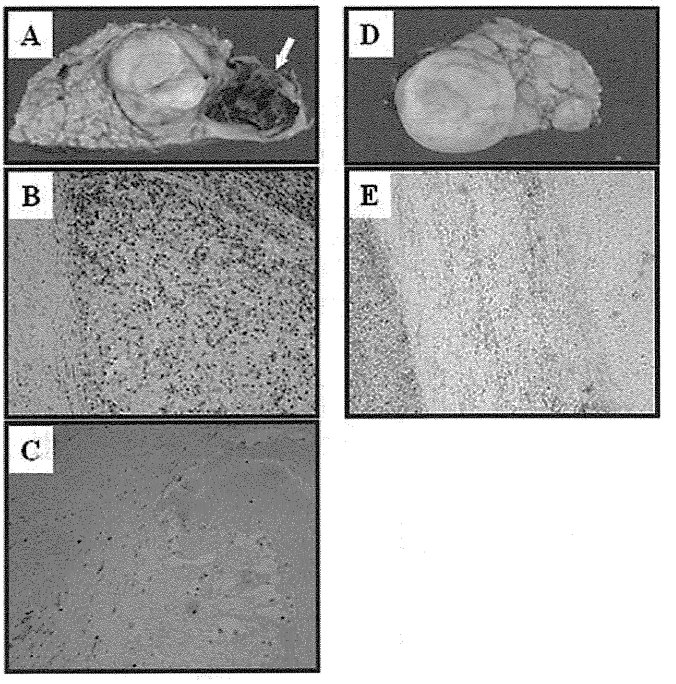


Figure 3. Macroscopic features and immunohistochemical examination of $CD8^+$ T cells in the resected tumors that had recurred after radiofrequency ablation. (A and D) show the cut surface of the resected specimens. (A) The white arrow indicates the post-ablated lesion to which a recurrent tumor was contiguous. The other recurrent tumor was distant from the post-ablated lesion (D). A marked number of $CD8^+$ T cells had infiltrated into the contiguous recurrent tumor (B) and the distant recurrent tumor (E), whereas few $CD8^+$ T cells had infiltrated into the post-ablated necrotic lesion (C). Magnification x100 (B and C) and x40 (E).

shown). These findings suggest that RFA not only activates the immune response systemically but also induces local infiltration of CTLs into the tumors.

*Analysis of immune response induced by RFA in a mouse model.* The experimental schedule is shown in Fig. 4A. The IFN-γ ELISPOT assay with $CD8^+$ T cells from the lymph nodes of mice demonstrated that the number of spots against both Colon 26 (P=0.049) and Colon 26/GPC3 (P=0.049) was larger after RFA compared to without treatment. On the other hand, the number of spots did not increase after surgical resection. These results suggest that RFA induced a significantly larger number of both Colon 26- and Colon 26/GPC3-reactive CTLs compared to no treatment or surgical resection (Fig. 4B).

The difference in number of spots between Colon 26 and Colon 26/GPC3 in each mouse, which represents GPC3-specific CTLs, is shown in Fig. 4C. As an effect of prior peptide vaccination, GPC3-specific CTLs were detected in the no treatment group. The frequency of GPC3-specific CTLs increased after RFA and decreased after surgical resection. As a result, the frequency of GPC3-specific CTLs after RFA was significantly greater than that after surgical resection (P=0.049). These results suggest that RFA induced a significantly larger number of GPC3-specific CTLs compared to surgical resection (Fig. 4C).

## Discussion

We previously reported that 39% of HCC patients had detectable GPC3-specific CTLs by a direct *ex vivo* IFN-γ ELISPOT assay (25). In this study, GPC3-specific CTLs were detectable before treatment in 11 of 27 patients (41%). Additionally, when we analyzed the patients with a prior treatment for HCCs using the same methods, 11 of 21 (52%) patients had detectable GPC3-specific CTLs (data not shown). These results are favorable for anticancer immunotherapy because the antigen-specific T-cell-mediated immune response could be detected without *in vitro* stimulation. As for frequency, GPC3-specific CTLs were detectable in ~40% of HCC patients, whereas AFP-, human telomerase reverse transcriptase (hTERT)-, and multidrug resistance-associated protein 3 (MRP3)-specific CTLs have been detected in 5-20, 6-12, and 14-21% of HCC patients with a single epitope peptide, respectively (26-28). As for tumor stages, a GPC3-specific immune response is frequently detected even in the early stages (24), whereas AFP-specific CTLs are more frequently detected in patients with advanced HCC (26). These results suggest that GPC3 has strong immunogenicity and GPC3-specific T-cell-mediated immunotherapy is suitable for adjuvant therapy against HCC because the induction of tumor-specific immune response in

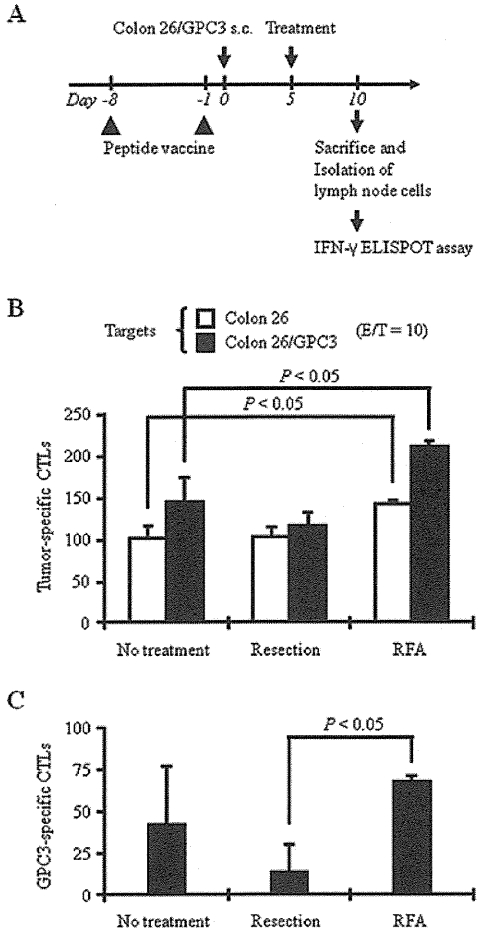


Figure 4. Investigation of the glypican-3 (GPC3)-specific immune response in a mouse model. (A) Experiment schedule. (B) An *ex vivo* interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay of $CD8^+$ lymph node cells (effector, $3x10^5$ cells/well) against Colon 26 and Colon 26/GPC3 (target, $3x10^4$ cells/well). No treatment column indicates the group of mice that received only the peptide vaccination and no therapy for the established tumor. The data are expressed as the mean + SD. Three mice were used for each group. Effector/target ratio=10. (C) The frequency of GPC3-specific CTLs, which is calculated from the difference in the number of spots between Colon 26 and Colon 26/GPC3 in each mouse.

the early stages would be more effective for suppression of tumor growth.

The association between the induction of an antigen-specific immune response and the antigen expression in tumor tissue remains unclear. In this study, we obtained the result that the presence of GPC3-specific CTLs in PBMCs potentially had a positive correlation with GPC3 expression in tumor tissue, but the correlation was not statistically significant. On the other hand, Mizukoshi *et al* showed a negative correlation between the frequency of MRP3-specific CTLs and MRP3 expression level (28). Moreover, Benavides *et al* showed that even antigen-naïve patients had pre-existing immunity (29). First, this may be because of tumor heterogeneity of cancer tissue. In most cases, the whole tumor cannot be evaluated and, in the case of truly antigen-naïve patients, antigen-specific CTLs cannot exist in theory. Second, antigen expression may be negative if antigen-specific CTLs have killed all of the antigen-expressing tumor cells as described by Jäger *et al* (30). As for the changes in an antigen-specific immune response between before and after treatment, in this study, we showed impressive data that all patients with GPC3-expressing HCCs exhibited an increase in GPC3-specific CTLs after RFA or TACE, whereas no patient with GPC3-expressing HCCs did after surgical resection.

This is the first study to compare locoregional therapies, including RFA, surgical resection, and TACE, in terms of antigen-specific T-cell response in HCC patients and tumor-bearing mice. Half the patients after RFA or TACE showed an increase in GPC3-specific CTLs, which might have been induced by the treatment, whereas only 1 of 9 patients after resection showed an increase and more than half the patients after resection showed a decrease. Similarly, the frequency of GPC3-specific CTLs increased after RFA and decreased after resection in a mouse model. These results suggest that RFA induced a stronger GPC3-specific immune response compared to surgical resection. RFA destroys tumor tissue and causes local necrosis followed by the release of tumor-associated antigens (12), whereas all of the tumor-associated antigens must be completely removed after resection. With regard to TACE, whereas the results of an IFN-γ ELISPOT assay after TACE were as encouraging as that after RFA, we have no other favorable data on the immune response after TACE. Although further investigation is required, TACE, which is also a necrosis-inducing treatment, might induce an antigen-specific immune response.

A limitation of this study is the patient selection in the three kinds of locoregional therapy. Current treatment guidelines for HCC including the Japanese ones, which we followed in this study, recommend RFA to earlier HCCs and TACE to more advanced HCCs than those which receive surgical resection (2,31-33). Therefore, selection bias is unavoidable under the circumstances. To overcome this problem, we added a murine study. The advantage of RFA over surgical resection in the induction of GPC3-specific CTLs was demonstrated also in a mouse model.

The correlation between antitumor immune response and clinical response is controversial. In this study, a significant contribution of GPC3-specific CTLs toward an optimal prognosis was not demonstrated. Mizukoshi *et al* reported that enhancement of T-cell response did not last for long and did not contribute to the prevention of HCC recurrence (34). In view of the highly complex nature of the human immune system, patient prognoses might not be determined only by the CTL response. Previous studies have demonstrated that the release of tumor-derived antigens by necrosis-inducing treatment causes sufficient signaling to activate not only antigen-specific CTL response but also antigen-specific helper T-cell response (35,36), antigen-specific antibody response (36), and non-antigen-specific natural killer cell response (37). However, the mechanisms for cancer escape from immunosurveillance would suppress the efficiency of these immune responses (38). In the literature, tumor-infiltrating lymphocytes in HCC are associated with better prognosis (39), but, in our case, tumor-infiltrating CTLs were actually insufficient for suppression of cancer recurrence despite the massive infiltration. For successful anticancer immunotherapy, the development of an innovative strategy to link antitumor immune response with clinical response and to provide a survival benefit for cancer patients is necessary, and so we have just started the clinical trial of a GPC3-derived peptide vaccine for adjuvant therapy after RFA.

In conclusion, our results demonstrate that RFA has a stronger effect on the immune system compared with surgical resection. Although further investigation is necessary, the data on immune response support the rationale for combined immunotherapy for HCC patients.

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