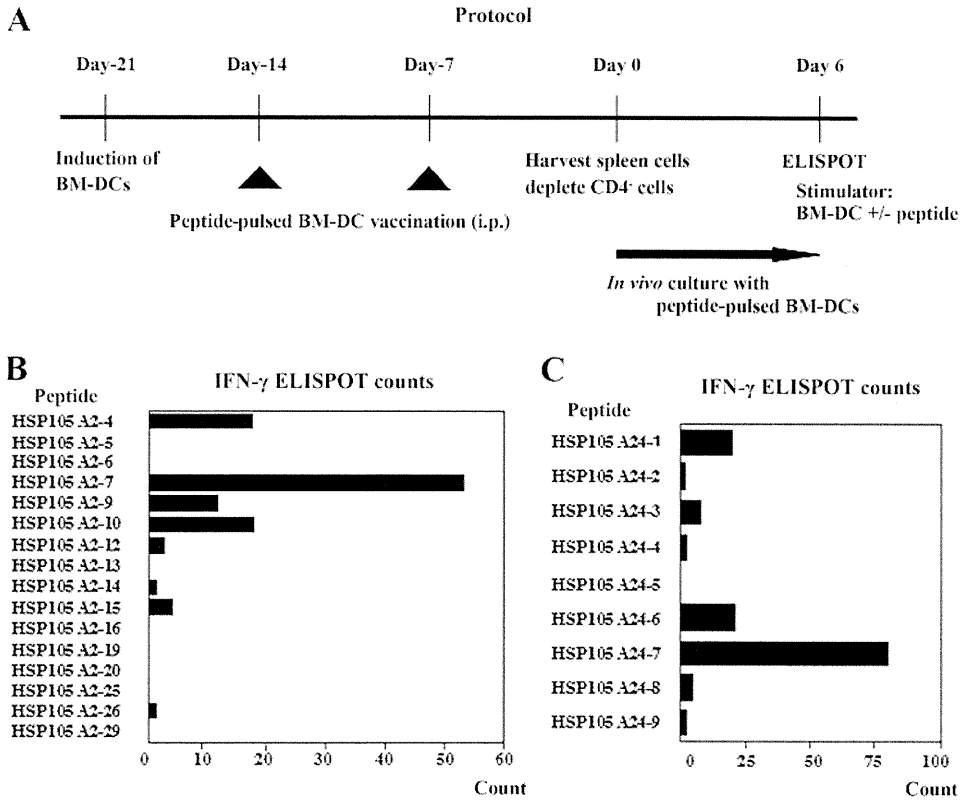

Figure 1. Identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 using HLA-A2.1 Tgm and HLA-A24 Tgm. (A) The protocol used for identification of HLA-A2 or HLA-A24-restricted CTL epitopes of HSP105 is shown. We primed the HLA Tgm with BM-DCs ($5x10^5$) pulsed with the mixture of HSP105-derived peptides carrying the HLA-A2 or HLA-A24 binding motif into the peritoneal cavity once a week for 2 weeks. Seven days after the last DC vaccination, spleens were collected and $CD4^-$ spleen cells ($2x10^6$/well) were stimulated with syngeneic BM-DCs ($2x10^5$/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured $CD4^-$ spleen cells as responder cells in the IFN-γ ELISPOT assay. (B) The bar graphs show the IFN-γ ELISPOT counts per $2x10^4$ $CD4^-$ spleen cells co-cultured with HLA-A2-restricted peptide-pulsed BM-DCs after normalization to counts from cells co-cultured with BM-DCs without peptide loading. (C) The bar graphs show the IFN-γ ELISPOT counts in the HLA-A24-restricted peptides. The columns represent the means from duplicate assays.

Ex vivo IFN-γ ELISPOT assay in peripheral blood in pre-surgical colorectal cancer patients. Ex vivo IFN-γ ELISPOT assays were performed to determine tumor-specific interferon-γ (IFN-γ)-secreting T cells. The 96-well plates were coated with anti-human IFN-γ (BD Biosciences Co., Ltd., USA). After an overnight incubation at 4˚C, the wells were washed and blocked with complete medium for 2 h at room temperature. A total of $1x10^6$ unfractionated PBMCs were added in duplicate wells and incubated at 37˚C for 18-20 h with or without peptides at 0.2 μl/well (1-10 μM). The plate was washed and then incubated with 5 μg/ml biotinylated anti-human IFN-γ antibody for 2 h at room temperature. After washing away the antibodies, streptavidin-HRP was added for 1 h. Finally, the plate was washed and replaced with fresh substrate solution and the reaction was terminated by washing with distilled water. The HLA-A2-restricted CMV peptide (NLVPMVATV) and HLA-A24 restricted CMV peptide (QYDPVAALF), which includes an epitope derived from the CMV pp65 protein, were used as positive controls.

Histological and immunohistochemical analysis. To investigate whether $CD8^+$ T cells infiltrated normal tissues triggered by the HSP105-derived peptide vaccine, we performed immunohistochemical staining with a monoclonal antibody against CD8 (1:100; LifeSpan BioSciences, Inc., Seattle, WA, USA) in tissue specimens from HLA-A2 Tgm immunized with the HSP105 peptides, as previously described (7). Immunohistochemical staining with rabbit polyclonal antibodies against HSP105 (1:200; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA) was performed according to the manufacturer's instructions.

Results

Identification of HLA-A2-or HLA-A24-restricted CTL epitopes in HLA Tgm. We designed pools of HSP105 peptides possessing amino acid sequences conserved between humans and mice that have a highly predicted binding score to HLA-A2 (pool of 16 different peptides) or HLA-A24 (A*24:02) (pool of 9 different peptides) (Table I). $CD4^-$ spleen cells were obtained from Tgm immunized twice i.p. with BM-DCs that had been pulsed with each peptide mixture; the spleen cells were then stimulated *in vitro*, again with the BM-DCs pulsed with each peptide mixture (Fig. 1A).

The IFN-γ ELISPOT counts, normalized to those of spleen cells co-cultured with BM-DCs without peptide loading, clearly indicated a HSP105 A2-7 peptide-specific response in the $CD4^-$ spleen cells (Fig. 1B). These $CD4^-$ spleen cells ($2x10^4$/well) showed 55±29.7 spot counts/well in response to the BM-DCs pulsed with the HSP105 A2-7 peptide, whereas they showed 23±31.1 spot counts/well in the presence of

Table I. HSP105-derived peptides conserved between human and mouse HSP105 predicted to bind to HLA-A2 or HLA-A24.

Peptides	Position	Subsequent residue listing	HLA-A2 binding score
HSP105 A2-4	120-128	MLLTKLKET	107
HSP105 A2-5	141-149	VISVPSFFT	55
HSP105 A2-6	155-163	SVLDAAQIV	37
HSP105 A2-7	169-177	RLMNDMTAV	591
HSP105 A2-9	202-210	DMGHSAFQV	21
HSP105 A2-10	222-230	VLGTAFDPFL	759
HSP105 A2-12	275-284	KLMSSNSTDL	276
HSP105 A2-13	276-284	LMSSNSTDL	26
HSP105 A2-14	300-309	KMNRSQFEEL	50
HSP105 A2-15	304-313	SQFEELCAEL	32
HSP105 A2-16	313-321	LLQKIEVPL	36
HSP105 A2-19	434-442	FLRRGPFEL	43
HSP105 A2-20	458-467	KIGRFVVQNT	76
HSP105 A2-25	668-676	LLTETEDWL	401
HSP105 A2-26	675-684	WLYEEGEDQA	146
HSP105 A2-29	757-765	EVMEWMNNV	15

Peptides	Position	Subsequent residue listing	HLA-A24 binding score
HSP105 A24-1	180-188	NYGIYKQDL	240
HSP105 A24-2	214-223	AFNKGKLKVL	30
HSP105 A24-3	251-260	KYKLDAKSKI	110
HSP105 A24-4	305-313	QFEELCAEL	47
HSP105 A24-5	433-442	TFLRRGPFEL	33
HSP105 A24-6	613-622	MYIETEGKMI	90
HSP105 A24-7	640-649	EYVYEFRDKL	330
HSP105 A24-8	725-733	HYAKIAADF	140
HSP105 A24-9	739-748	KYNHIDESEM	82

The binding scores were estimated by using BIMAS software: http//bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform.

BM-DCs pulsed with the HSP105 A2-4 peptide. A similarly strong response was observed for the HSP105 A24-7 peptide (Fig. 1C). $CD4^-$ spleen cells ($2x10^4$/well) showed 79.5±27.6 spot counts/well in response to the BM-DCs pulsed with the HSP105 A24-7 peptide, whereas they showed 20.5±14.8 spot counts/well in the presence of BM-DCs with the HSP A24-6 peptide. These assays were performed twice with similar results and they suggest that the HSP105 A2-7 and A24-7 peptides are potential CTL epitope peptides in both HLA Tgm and humans.

Identification of a CTL epitope in BALB/c mice and CTLs that are cytotoxic against C26 tumors in mice. There were similar structural motifs within the peptides that bound to human HLA-A24 and mice K^d. We selected those peptides with binding motifs for both HLA-A24 and K^d molecules and prepared 9 different synthetic peptides (HSP105-1-9). When we tested these peptides for their potential to induce *in vitro* tumor reactive CTLs in spleen cells derived from BALB/c mice immunized with the HSP105 peptides, only the HSP105 24-1 peptide-induced CTLs showed specific cytotoxicity against C26 tumors ($HSP105^+$, H-$2K^d$) (Fig. 2). The cytotoxicity against C26 was attenuated by HSP105 siRNA. These findings indicate that the HSP105 A24-1 peptide has the capacity to induce tumor reactive CTLs and that peptide vaccination-primed CTLs are reactive to this peptide *in vivo*. We would expect this HSP105 A24-1 (NYGIYKQDL) peptide to also be an epitope for human CTLs.

HSP105-reactive CTLs from PBMCs of HLA-A2-positive colorectal cancer patients and CTLs induce cytotoxicity against HSP105-expressing cancer cells. We generated a CTL line from PBMCs of colorectal patients by stimulation with the HSP105 A2-12 peptide. As shown by ^{51}Cr release assays, the resulting CTLs showed HSP105-specific cytotoxicity against SW620 cells ($HSP105^{+++}$, HLA-A2) and against T2 cells pulsed with the HSP105 A2-12 peptide ($HSP105^-$, HLA-A2), but not against HepG2 cells ($HSP105^{\pm}$, HLA-A2) or T2 cells pulsed with an irrelevant peptide (Fig. 3A). HSP105 siRNA decreased the cytotoxicity against SW620 cells. We investigated the effects of the HSP105 A2-12 peptide-reactive CTL lines on the mice implanted with the SW620 cells. Fourteen days after inoculation of HSP105 A2-12 peptide-reactive CTLs, there was an apparent reduction in tumor size in the SW620 compared to that in untreated mice (Fig. 3B). These results clearly indicate the efficacy of HSP105 A2-12 (KLMSSNSTDL) peptide-reactive CTL injection therapy for $HSP105^+$ tumors in mice.

Detection of HSP105-specific CTLs in peripheral blood of pre-surgical patients with colorectal cancer. Our results suggest that the four peptides, HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGIYK QDL) and HSP105 A24-7 (EYVYEFRDKL), are HSP105-derived, HLA-A2, or HLA-A24-restricted CTL epitopes. To determine the frequencies of the HSP105-derived T cells specific for these peptide in pre-surgical colorectal cancer patients, we analyzed the PBMC responses for each peptide using the ELISPOT assay. HSP105 expression was detected in 20 of 21 (95%) patients, consistent with previous studies (4). HSP105-specific T cells secreting IFN-γ were detected in patients stimulated with the HSP105 A2-7 (4 patients), HSP105 A2-12 (6 patients), HSP105 A24-1 (2 patients) and HSP105 A24-7 (6 patients) peptides (Table II). ELISPOT assay detected positive IFN-γ responses to at least one of the HSP105-derived peptides in PBMCs in 14 of the 21 patients. In contrast to the results for colorectal cancer patients, the 4 peptides were not recognized by PBMCs from healthy donors. Both the ratio of normal donors who showed positive T-cell responses to CMV-derived peptides and the frequencies of the specific T cells were identical to those of the colorectal cancer patients (data not shown).

HSP105-derived peptide immunization does not induce autoimmunity in HLA-A2 Tgm. HSP105 in normal adult mice is expressed in only certain tissues, and expression in these tissues is less than that in C26 tumor cells, suggesting a low

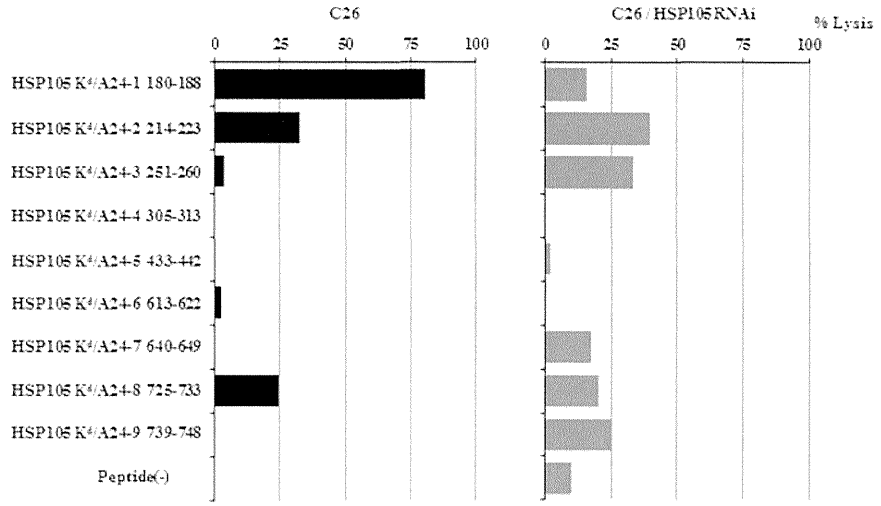

Figure 2. Identification of an HSP105-derived HLA-A24 and K^d-restricted CTL epitope. BALB/c mice were immunized with 9 HSP105 peptides. Using the ^{51}Cr release assay, sensitized spleen cells that had been stimulated *in vitro* with each HSP105 peptide (10 μmol/l) and cultured for 5 days with 100 U/ml interleukin-2 were examined for CTL activity against C26 cells and C26 cells transfected with HSP105 siRNA (C26/HSP105 RNAi). Values represent the percentage of specific cell lysis, based on the mean values from triplicate assays.

Table II. Expression of HSP105 in colorectal cancer tissue and quantification of HSP-specific CTLs in colorectal cancer patients.

						[c]Spot number of peptide-specific CTLs				
HLA-A2-positive patients	Age (yrs.)	Gender	HLA	Stage[a] of tumor	HSP105 expression[b]	HSP105 A2-7		HSP105 A2-12		CMV
1	62	M	0201/2601	IIIB	++	27	+	126	+	160
5	79	M	0207/1101	IIIB	++	0	-	2	-	10
6	51	M	0201/0206	I	+	0	-	49	+	136
8	55	M	0206/2402	I	±	0	-	0	-	66
11	69	M	0206/2402	IIIC	+	143	+	0	-	0
12	61	M	0201/3303	I	±	2	-	45	+	367
13	64	F	0201/2601	IIIC	±	0	-	2	-	254
14	66	M	0206/2402	IIIC	-	13	+	0	-	58
15	78	M	0201/1101	IIA	+	0	-	5	+	57
16	51	F	0206/2601	IV	±	31	+	7	+	15
17	63	F	0206/1101	IIA	++	0	-	25	+	96
HLA-A2402-positive patients						HSP105 A24-1		HSP105 A24-7		CMV
2	64	F	2402	IV	++	2	-	44	+	6
3	60	M	2402/3101	IIIC	++	0	-	0	-	11
4	71	F	2402/3101	IIA	++	25	+	51	+	12
7	47	M	2402/3101	IIIA	++	4	-	6	+	3
9	66	M	2402	IV	++	8	+	6	+	7
10	60	M	2402/3101	I	++	1	-	19	+	26
18	64	M	1101/2402	IV	+	0	-	2	-	40
20	46	F	1101/2402	IIIB	++	4	-	7	+	5
21	66	F	2402	I	++	3	-	0	-	38

F, female; M, male. [a]Stage, staging was performed according to the TNM classification (Union for International Cancer Control; UICC). [b]HSP105 expression, staining intensity of tumor cells was scored on a scale according to the following four grades: -, absent; ±, weak; +, moderate; ++, strong. [c]Spot number indicates the number of peptide-specific CTLs calculated by subtracting the spot number in a well of no peptide. -, Spot number <5; +, Spot number ≥5.

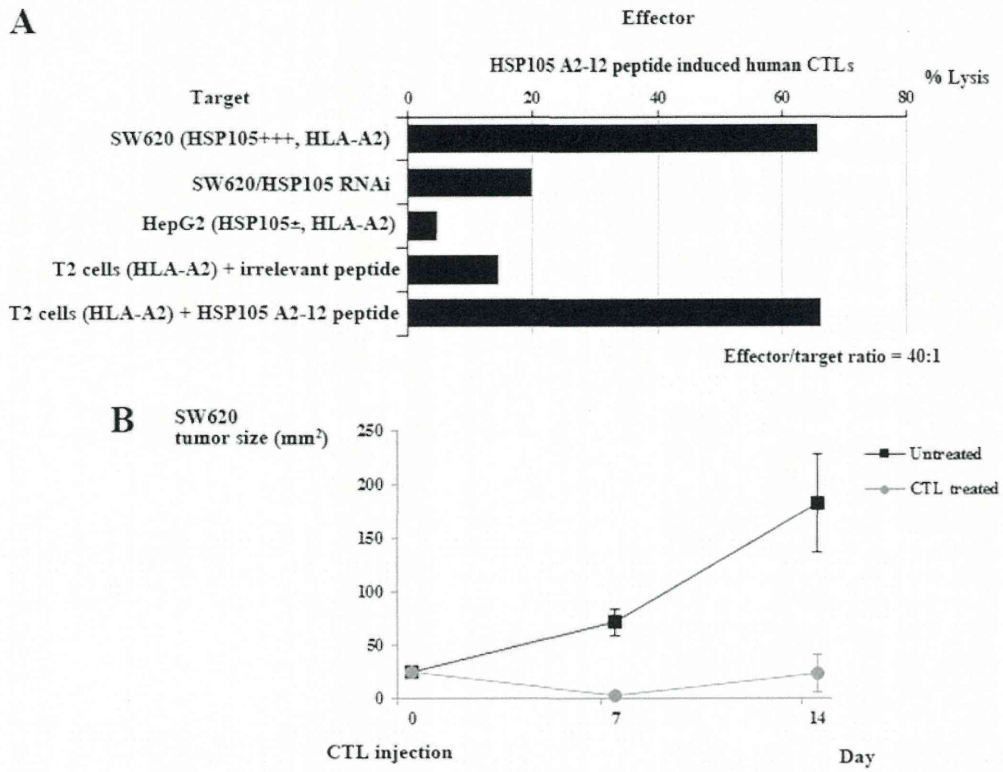

Figure 3. CTL induction from PBMCs of HLA-A2-positive cancer patients. (A) HSP105 peptide-reactive CTLs were generated from $CD8^+$ T cells of HLA-$A2^+$ colorectal cancer patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the HSP105 A2-12 peptides, the CTLs were subjected to a standard ^{51}Cr release assay at the indicated effector/target ratio (40/1). Their cytotoxicity against SW620 cells ($HSP105^{+++}$, HLA-A2), SW620 cells transfected with HSP105 siRNA ($HSP105^-$), HepG2 cells ($HSP105^{\pm}$, HLA-A2), T2 cells pulsed with an irrelevant peptide ($HSP105^-$, HLA-A2) and T2 cells pulsed with the HSP105 A2-12 epitope peptide were all examined by ^{51}Cr release assay. Values represent the percentage of specific cell lysis, based on the mean values from triplicate assays. (B) There was marked growth inhibition of SW620 cells ($HSP105^+$) engrafted into nude mice after intratumoral injection of human CTLs induced by the HSP105 peptides. When tumor size reached 25 mm^2 on day 9 after s.c. tumor implantation, human CTLs ($3x10^6$) reactive to the HLA-A2-restricted HSP105 peptide, generated from an HLA-$A2^+$ donor, were i.t. inoculated. Tumor sizes in nude mice administered the HSP105 epitope peptide-induced CTL lines (n=3), or no treatment (n=3), are shown. The mean tumor size (mm^2) for each group of mice was expressed, and bars represent SD.

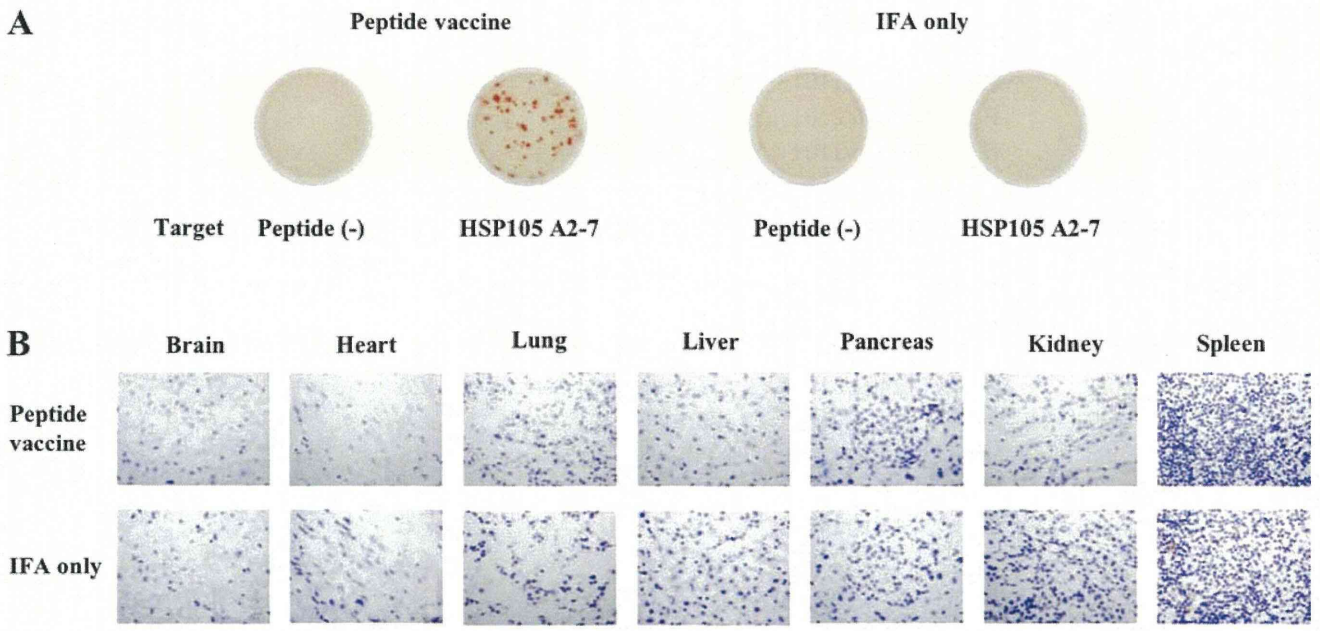

Figure 4. The HSP105 peptide vaccine induces HSP105 peptide-specific CTLs, while CD8 T cells do not infiltrate into normal tissues. (A) HSP105 peptide-specific CTLs were induced in the spleen cells of immunized mice with the HSP105 peptide vaccine. IFN-γ ELISPOT assays were performed using BM-DCs pulsed with HSP105 A2-7 and non-pulsed BM-DCs as target cells. The representative data are shown (n=6). (B) Immunohistochemical staining with anti-CD8 mAb was performed in tissue specimens of HLA-A2 Tgm immunized with the HSP105 A2-7 and A2-12 peptides. The tissue specimens were removed and analyzed 7 days after the second vaccination (original magnification, x400). The representative data are shown (n=3).

risk of damage to normal tissues posed by HSP105 antigen-induced immune responses (6). To investigate whether immunization of the mice with HSP105-derived peptides causes autoimmunity, HLA-A2 Tgm were immunized with the HSP105 A2-7 and A2-12 peptides emulsified in incomplete Freund's adjuvant at 7-day intervals and then sacrificed 7 days after the second vaccination. Using the IFN-γ ELISPOT assay, we confirmed the induction of HSP105 peptide-specific CTLs in the spleen cells of immunized mice (Fig. 4A). We did not detect any pathological changes, such as $CD8^+$ lymphocyte infiltration or tissue destruction/repair, in the brain, heart, lung, liver, pancreas, or kidney of HLA-A2 Tgm (Fig. 4B). These results indicate that the HSP105 peptide-reactive $CD8^+$ CTLs did not attack the healthy tissue specimens that we evaluated.

Discussion

Heat shock proteins (HSPs) have essential functions in the regulation of protein folding, conformation, assembly and sorting. They function as molecular chaperones to maintain the native conformational states of proteins, preventing protein aggregation (18). HSPs are classified into several families based on their molecular weight, including HSP105/110, HSP90, HSP70, HSP60, HSP40 and HSP27 (19). HSP105 is a stress protein within the HSP105/110 family that we previously reported to be overexpressed in a variety of human cancers but with little to no expression in normal tissues, aside from the testis. Thus, HSP105 presents a promising candidate for a target antigen in cancer immunotherapy (3-7). In particular, HSP105 is specifically overexpressed in colorectal cancer (83%) (4). Furthermore, HSP105 is expressed in highly metastatic colon cancer cell lines and its expression is correlated with advanced clinical cancer stages and positive lymph node involvement (20). When considering immunogenic target molecules for cancer immunotherapy, it is important to select a tumor antigen that does not run the risk of becoming lost during immunoediting (21). We reported previously that siRNA-mediated suppression of HSP105 protein expression induced apoptosis in various types of cancer cells, but not in fibroblasts (12). Therefore, it is possible that tumor cells do not lose HSP105 expression, allowing for continued growth.

Advances in molecular biology and tumor immunology have paved the way for identification of a large number of tumor-associated antigens (TAAs) and antigenic peptides recognized by tumor reactive CTLs; hence, peptide-based cancer immunotherapy has become an intensely studied field (22,23). Several HSPs, including HSP70, HSP90 and gp96, bind and deliver (through receptor-mediated endocytosis of HSP) antigenic peptides to the antigen-processing pathway of antigen-presenting cells (APCs) and these peptides are then presented on major histocompatibility complex (MHC) class I molecules. This HSP-mediated pathway has been demonstrated to evoke potent antiviral and antitumor immune responses (24). On the other hand, many researchers have identified MHC class I-presenting peptide epitopes derived from HSP (25). Furthermore, HSP105 itself may induce $CD8^+$ T cells to become reactive towards tumor cells that express HSP105, using HSP105-DNA and HSP105-pulsed DC vaccines in mice (6-8).

We found 4 peptides [HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGI YKQDL) and HSP105 A24-7 (EYVYEFRDKL)] to be potential HSP105-derived, HLA-A2 or A24-restricted CTL epitopes. There was a discrepancy between the expected HSP105 CTL epitopes in Tgm and in PBMCs of colorectal cancer patient. To identify the HSP105-derived CTL epitope peptides, we analyzed the PBMC responses to each of the 4 peptides in colorectal cancer patients using the *ex vivo* IFN-γ ELISPOT assay.

In this study, we used an *ex vivo* assay to detect HSP105-specific IFN-γ-secreting T cells in PBMCs from 14 of 21 pre-surgical patients with colorectal cancer. Generally, CTLs specific for tumor antigens cannot be detected directly *ex vivo*; rather only after expansion by repeated *in vitro* stimulation with the antigenic peptide in the appropriate antigen-presenting cells. This is attributed to assay sensitivity and the low frequency of tumor antigen-specific CTLs (26). HSP105-specific CTLs in PBMCs, which can be detected directly *ex vivo* without *in vitro* stimulation, provide strong immunological evidence of HSP105-derived CTL epitopes, which we were able to identify in this study. However, because the prognosis of the pre-surgical patients was affected by various factors, it was difficult to evaluate the correlation between a positive CTL response before surgery and clinical improvement at the present stage; an increase in the number of patients at each stage and further analyses of this relationship are necessary.

Although the SEREX method facilitated the identification of tumor antigens that could be recognized by antibodies and $CD4^+$ T cells, few of their T-cell epitopes have been determined (27). We previously reported in mice that HSP105-DNA and HSP105-pulsed DC vaccines induced a reaction in $CD4^+$ T cells and $CD8^+$ T cells towards tumor cells expressing HSP105 (6-8). HSP105 was identified by SEREX (3) and thus, HSP105-specific $CD4^+$ T cell reactions may be induced by HSP105 immunization. It was shown that antigen-specific $CD4^+$ T cells are required to activate memory $CD8^+$ T cells into fully functional effector killer cells (28). We are now preparing a clinical trial to investigate HSP105-based immunotherapy for HSP105-expressing tumors, including those from colorectal cancer. We plan to use the HSP105 epitope peptides identified in this study as an initial attempt. We expect that HSP105-based immunotherapy will be a novel treatment strategy for colorectal cancer patients.

Acknowledgements

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CASE REPORT

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Significant clinical response of progressive recurrent ovarian clear cell carcinoma to glypican-3-derived peptide vaccine therapy

Two case reports

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Keywords: Glypican-3, peptide vaccine, refractory disease, ovarian clear cell carcinoma, clinical response

Abbreviations: HLA, human leukocyte antigen; UMIN-CTR, University Hospital Medical Information Network Clinical Trials Registry; CT, computed tomography; GMP, Good Manufacturing Practice; RECIST, Response Evaluation Criteria in Solid Tumors; PR, partial response; ^{18}F-FDG PET, Fluorine-18-fluorodeoxyglucose positron emission tomography; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cell

Carcinoembryonic antigen glypican-3 (GPC3) is expressed by > 40% of ovarian clear cell carcinoma (CCC) and is a promising immunotherapeutic target. We previously reported the safety of and immunological and clinical responses to a GPC3-derived peptide vaccine in a phase I clinical trial of patients with advanced hepatocellular carcinoma (HCC). Although the efficacy of the GPC3-derived peptide vaccine against HCC patients was evaluated, other GPC3-positive cancer patients have not yet been investigated. Therefore, we conducted a phase II trial to evaluate the clinical outcome of ovarian CCC patients treated with a GPC3-derived peptide vaccine. The GPC3 peptide was administered at a dose of 3 mg per body. Patients received an intradermal injection of the GPC3 peptide emulsified with incomplete Freund's adjuvant. Vaccinations were performed biweekly from the first until the 6th injection and were then repeated at 6-week intervals after the 7th injection. Treatment continued until disease progression. We herein present two patients with chemotherapy-refractory ovarian CCC who achieved a significant clinical response in an ongoing trial of a GPC3 peptide vaccine. Case 1, a 42-year-old patient with advanced recurrent ovarian CCC with liver and retroperitoneal lymph node metastases, received the HLA-A24-restricted GPC3 peptide vaccine. Contrast-enhanced CT at week 10 revealed a partial response (PR) using RECIST criteria. Case 2 was a 67-year-old female with multiple lymph node metastases. She was injected with the HLA-A2-restricted GPC3 peptide vaccine. According to RECIST, PR was achieved at week 37. The stabilization of their diseases over one year provided us with the first clinical evidence to demonstrate that GPC3 peptide-based immunotherapy may significantly prolong the overall survival of patients with refractory ovarian CCC.

Introduction

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecological malignancy. Ovarian clear cell carcinoma (CCC) accounts for 5–25% of all EOC, depending on the geographic location. It accounts for <10% of all EOC diagnosed in the USA.[1] In contrast, the incidence of CCC is reportedly >15% of EOC in Japan.[2] Compared with other EOC subtypes, CCC is associated with a poorer prognosis and increased chemoresistance.[2,3] In particular, the response rate of recurrent CCC to salvage chemotherapy was reported to be less than 10%.[4] Progression-free survival was also less than 6 mo, even in patients who achieved a response when treated with conventional anti-cancer cytotoxic agents.[5] The long-term clinical outcome of patients with recurrent CCC is extremely poor.[6] Therefore, new treatment modalities are urgently required for patients with CCC refractory to chemotherapy.

Immunotherapy is a potentially attractive option for EOC. Glypican-3 (GPC3) is useful not only as a novel tumor marker, but also as an oncofetal antigen for immunotherapy. It is specifically overexpressed in hepatocellular carcinoma (HCC).[7] Previous studies demonstrated that GPC3 was also overexpressed in several malignant tumors, including ovarian CCC.[8-12]

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 (CTLs).[13] We recently reported the safety of and immunological and clinical responses to a

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GPC3-derived peptide vaccine in a phase I trial for advanced HCC patients.[14] We are currently conducting a phase II trial with a GPC3-derived peptide vaccine in ovarian CCC patients (UMIN-CTR: 000003696).

This study presents, for the first time, two patients with refractory ovarian CCC who achieved a significant clinical response in an ongoing trial of a GPC3 peptide vaccine.

Materials and Methods

Patient eligibility

This study describes two patients from our GPC3 peptide vaccination trial. This clinical trial was approved and monitored by the Institutional Review Board at Nagoya University School of Medicine. Patients with progressive metastatic ovarian CCC were enrolled after providing written, informed consent. The following eligibility criteria were used: diagnosis of ovarian CCC on the basis of histological examinations; no expectation of a response to other therapies; an Eastern Cooperative Oncology Group performance status of 0–2; age between 20 and 80 y; HLA-A24- or HLA-A2-positive status as determined using commercially available genomic DNA typing tests; and adequate organ function (white blood cell count ≥2000/mm^3, platelets ≥50 000/mm^3, serum creatinine ≤2.1 mg/dl, total bilirubin ≤3.6 mg/dl, aspartate aminotransferase ≤165 IU/L, alkaline phosphatase ≤1795 IU/L). The following exclusion criteria were applied: other active malignancies; clinically serious infection; active gastrointestinal bleeding; severe cardiac insufficiency; severe interstitial pneumonitis; massive ascites and/or hydrothorax; concurrent treatment with steroids or immunosuppressive agents; and unsuitability for the trial based on a clinical judgment.

Immunohistochemical analysis

Surgical specimens were stained with hematoxylin and eosin or monoclonal antibodies against GPC3 (clone 1G12; dilution 1:300; BioMosaics), CD8 (clone 1A5; dilution 1:80; Novocastra), and HLA class I (clone EMR8/5; dilution 1:1000; Hokudo), according to the manufacturers' directions.

Ex vivo IFN-γ enzyme-linked immunospot assay

An ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay was conducted to measure the antigen-specific CTL response, as described previously.[14] Non-cultured PBMCs were added to plates in the presence of peptide antigens (10 μg/mL) and incubated for 20 h at 37 °C in 5% CO_2. The numbers of PBMCs plated per well for case 1 and case 2 were 5×10^5 and 2.5×10^5, respectively.

GPC3 double-determinant ELISA

Double-determinant (sandwich) ELISA of GPC3 was performed as described previously.[14] The serum-soluble protein GPC3 was detected by indirect ELISA using an anti-human GPC3 monoclonal antibody (clone 1G12), anti-human GPC3 sheep polyclonal antibody (R&D Systems), and recombinant human GPC3 (R&D Systems).

Case 1

A 42-y-old nulligravid Japanese female was referred to us in November 2011. She had been diagnosed with an ovarian tumor when she presented with bilateral lower extremity deep vein thrombosis. She had undergone laparotomy including total abdominal hysterectomy, bilateral salpingo-oophorectomy, and sampling of the pelvic lymph nodes at a nearby hospital in August 2010. There were residual tumors of peritoneal dissemination and enlarged retroperitoneal lymph nodes. Histopathology revealed ovarian CCC and the presence of pelvic lymph node metastases. Therefore, her initial clinical stage was IIIC. She was treated postoperatively with six cycles of paclitaxel and carboplatin (TC) chemotherapy. After TC chemotherapy, she once again underwent laparotomy including omentectomy and pelvic and para-aortic systemic lymphadenectomy in March 2011, and a complete response was achieved. The histology of the resected tumor revealed retroperitoneal lymph node metastases, and no pathological chemotherapeutic effects were observed. Thus, she received four cycles of postoperative chemotherapy with irinotecan and cisplatin. CT scans revealed the enlargement of the retroperitoneal lymph nodes and calyx of the right kidney in August 2011. The multiple metastases rapidly progressed, and oral opioids were administered to relieve back pain.

After confirming her HLA type as HLA- A*24:02, she was enrolled in a phase II trial of the GPC3 peptide vaccination. She began receiving intradermal injections of 3 mg of HLA-A24-restricted $GPC3_{298–306}$ GMP grade peptide emulsified with incomplete Freund's adjuvant in November 2011. Vaccinations were performed biweekly from the first until the 6th injection and were repeated at 6-wk intervals after the 7th injection according to the trial schedule.

Pretreatment tumor markers were as follows: CA125, 405.4 U/ml and CA72-4, 264.1 U/ml. The serum levels of these tumor markers decreased after the initiation of treatment (**Fig. 1A**). The pretreatment serum GPC3 protein (17.3 ng/ml) was detectable, but changes in the serum levels of GPC3 unlike other tumor markers had been broadly flat while there was an increase or decrease (data not shown). Liver and para-aortic lymph node metastases grew during the first few weeks before tumor regression. According to RECIST, PR was achieved at week 10. Oral opioids were discontinued. Right hydronephrosis, pleural effusion, and ascites disappeared. Liver and para-aortic lymph node metastases were no longer visible on contrast-enhanced CT after 23 wk of exposure to the vaccine (**Fig. 1B**). However, we

Figure 1 (See opposite page). (**A**) Clinical course from the beginning of the GPC3 peptide vaccination. Serum levels of CA125 and CA72–4 decreased after the initiation of therapy. Black arrows indicate vaccinations. The asterisk indicates right inguinal lymph node resection biopsy. The double asterisk indicates bilateral inguinal lymphadenectomy. (**B**) Contrast-enhanced CT scan showing liver (white, red, blue, and orange arrows) and paraaortic lymph node (yellow arrows) metastases. The size of metastases increased immediately following the initiation of the GPC3 peptide vaccination; however, tumor sizes decreased markedly within three months. (**C, D**) Pathological findings of primary ovarian carcinoma (**C**) and right inguinal lymph node biopsy specimens (**D**). A microscopy image of a hematoxylin and eosin (H&E)-stained section shows CCC (**a, i**). Immunohistochemical staining for GPC3 and HLA class I showed positivity in the primary ovarian carcinoma, respectively (**b, c**). Most CCC cells in the resected right inguinal lymph node metastasis appeared to lack GPC3 expression and a reduction in the expression of HLA class I (**ii, iii**). Immunohistochemical analysis showed a few CD8-positive T cells in the primary ovarian CCC tissue (**d**), whereas there was little infiltration of CD8-positive T cells in the resected right inguinal lymph node metastasis (**iv**). Original magnification, x200.

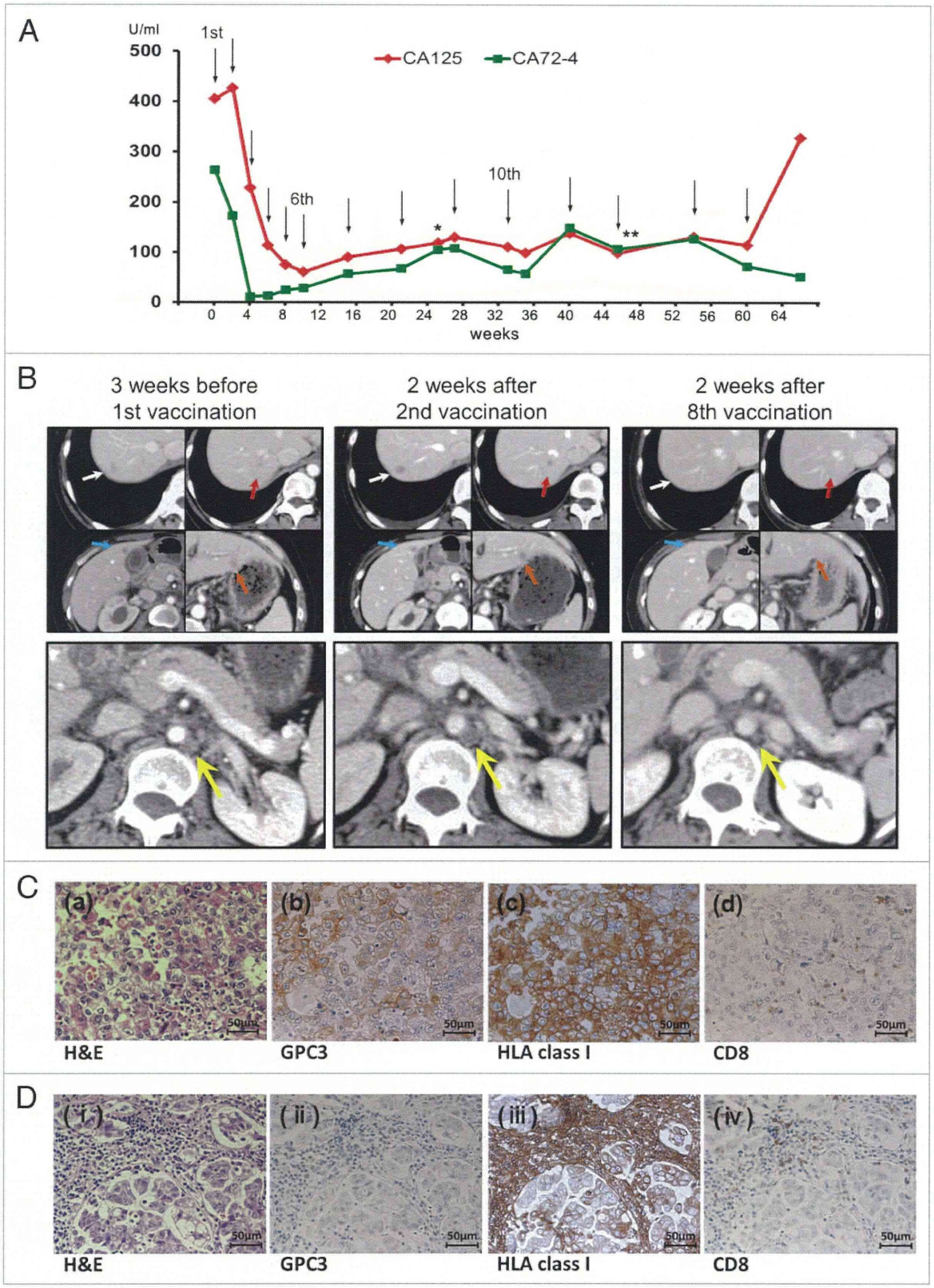

Figure 1. See page 2 for legend.

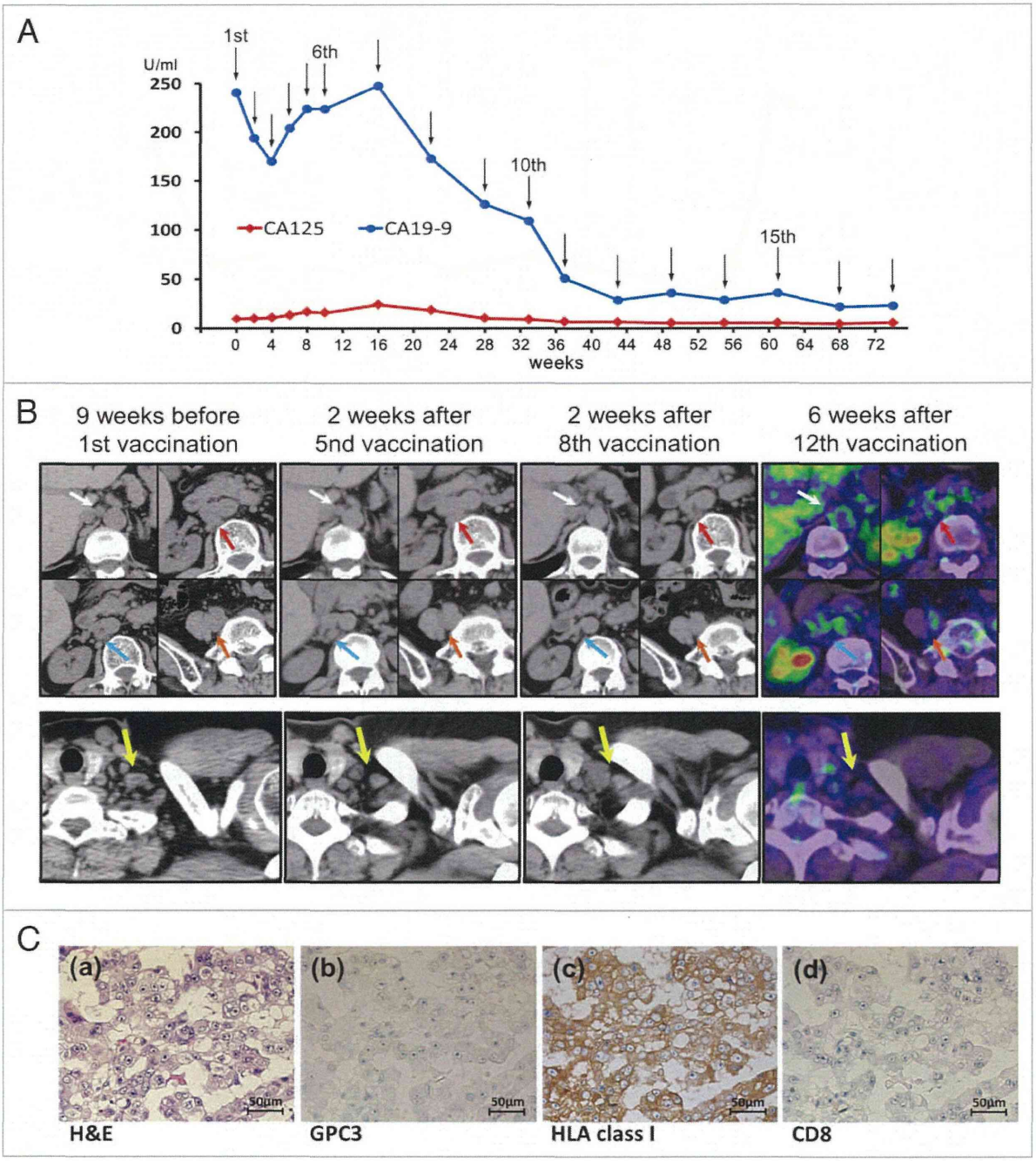

Figure 2. (**A**) Clinical course from the beginning of the GPC3 peptide vaccination. Serum levels of CA19–9 and CA125 decreased after the 7th vaccination. The CA19–9 level decreased to within the normal range. Black arrows indicate vaccinations. (**B**) Plain CT and ^{18}F-FDG PET/CT scans showing retroperitoneal lymph node (white, red, blue and orange arrows) and Virchow's node (yellow arrows) metastases. These metastases were negative on ^{18}F-FDG PET/CT at week 49. (**C**) Pathological findings of primary ovarian carcinoma. A microscopy image of a hematoxylin and eosin (H&E)-stained section shows CCC (**a**). Immunohistochemical staining was performed for GPC3, HLA class I, and CD8. (**b, c, d**). The expression of HLA class I was positive, while that of GPC3 was not, and there was no infiltration of CD8-positive T cells. Original magnification, x200.

observed the slow growth of the bilateral inguinal lymph nodes during the same period. Therefore, right inguinal lymph node resection biopsy was performed at week 25. A histological examination of the biopsied specimen revealed the metastasis of CCC. Immunohistochemical staining was performed with monoclonal antibodies against GPC3, HLA class I, and CD8. Immunohistochemical staining revealed the expression of GPC3 and HLA class I in the cytoplasm and membranes of carcinoma cells and a few CD8-positive T cells in the primary ovarian CCC tissue (**Fig. 1C**), whereas most CCC cells in the resected right inguinal lymph node metastasis appeared to lack GPC3 expression, showed a reduction in the expression of HLA

class I, and there was little infiltration of CD8-positive T cells (**Fig. 1D**).

Because the metastases that had disappeared remained absent, followed by stable disease, we continued to administer the vaccine. The treatment was eventually discontinued due to the development of lower abdominal subcutaneous metastases and progressive disease after the 14th vaccination.

Case 2

A 67-y-old parous Japanese female was referred to our hospital in March 2012. She had undergone laparotomy including total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and sampling of the retroperitoneal lymph nodes in September 2010. Residual enlarged right common iliac and para-aortic lymph nodes were noted. A histological examination showed ovarian CCC. Her initial clinical stage was IIIC due to retroperitoneal lymph node metastases. She was treated postoperatively with nine cycles of weekly TC chemotherapy. Pelvic and para-aortic lymph nodes persisted following weekly TC chemotherapy and increased in size. Two subsequent regimens yielded no response: three cycles of second-line chemotherapy with irinotecan and nedaplatin, and one cycle with gemcitabine and docetaxel as third-line chemotherapy.

She had the HLA-A*02:01 genotype, and began receiving 3 mg of the HLA-A2-restricted $GPC3_{144-152}$ peptide vaccine in April 2012. Pretreatment tumor markers were as follows: CA19-9, 241 U/ml and CA125, 9.8 U/ml. The serum levels of these tumor markers decreased after the 7th vaccination. CA19-9 levels had decreased to within the normal range by week 43 (**Fig. 2A**). The serum GPC3 protein was undetectable.

She achieved stable disease except for Virchow's node by week 24. However, she showed PR at week 37 (**Fig. 2B**). The response resulted in almost the complete resolution of all measurable lesions on plain CT. Unfortunately, she had a past history of allergies to CT contrast media; therefore, we performed ^{18}F-FDG PET/CT to improve the diagnostic accuracy of residual tumors at week 49. ^{18}F-FDG PET/CT showed mild positive FDG uptake in four masses (smaller than 1 cm in size) in the right common iliac lymph node and intraperitoneal dissemination. Although ^{18}F-FDG PET did not always reflect malignancy or detect metastatic lesions, the presence of residual tumors was still suspected.

Because she remained progression free at week 74, she is still receiving the trial treatment. The expression of HLA class I was positive, while that of GPC3 was not positive in the primary ovarian CCC tissue, and there was no infiltration of CD8-positive T cells (**Fig. 2C**).

No adverse effects of the vaccination were observed in either case, except for a local inflammatory response with erythema at the injection site and low-grade fever.

Discussion

Most gynecological oncologists are aware that recurrent or persistent ovarian CCC has a chemoresistant phenotype. Although the different histological types of EOC may represent different diseases with unique clinical and molecular characteristics, ovarian CCC is still currently being treated in the same manner as other EOCs because of its low rate of incidence among EOCs in western countries. Novel treatment approaches should be adopted for ovarian CCC, especially in cases that are recurrent or refractory to previous therapies. Between 5 and 10% of all currently open clinical trials for ovarian cancer patients evaluate approaches using immune-based therapies. Although most immunotherapeutic strategies for ovarian cancer treatment investigated so far are capable of inducing antigen-specific immunity, the unequivocal clinical benefit for these patients has not yet been demonstrated.[15] To date, we have confirmed that a HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone can recognize and kill HLA-A2-positive and GPC3-positive ovarian CCC cell lines.[16]

Based on these conditions, we conducted a trial to assess the clinical outcome of ovarian CCC patients treated with a GPC3-derived peptide vaccine. In the ongoing clinical trial, 20 refractory patients were enrolled until the end of August 2013. Ten of these patients were vaccinated at least six times, and a significant clinical response was achieved in two patients (2/10, 20%) who received the HLA-A24 or A2-restricted GPC3 peptide vaccine. In spite of resistance to multiple chemotherapeutic drugs, the stabilization of their diseases over one year suggests the efficacy of the GPC3 peptide vaccination.

Two patterns of responses, fast and slow, after the initial increase in the total tumor burden were observed in this study. In case 1, a fast response was preceded by an apparent early enlargement in liver and para-aortic lymph node metastases. Metastases were stable for several months in case 2 after beginning the vaccination treatment, except for Virchow's node, which showed radiographical progression; however, all radiographically measurable metastases thereafter almost completely regressed. We hypothesize that the immune response elicited during the first several weeks of vaccination, similarly to ipilimumab,[17] may be mistaken for progressive disease: CTL infiltration and immune-mediated inflammation may not be radiographically distinguishable from a growing tumor. The initial tumor enlargement was suspected to be caused by inflammation. Meanwhile, bilateral inguinal lymph node metastases grew without reductions within the same patient in case 1. Disease progression may occur as part of a "mixed response", i.e., the regression of some lesions and apparent progression of others.

The difference in effectiveness may have been caused by the heterogeneity associated with immune-escape mechanisms, including the downregulation of cancer-specific antigens and/or HLA class I in tumor cells. The intratumor heterogeneity of GPC3 expression was observed at different levels in our preliminary study depending on the locations and timing of biopsies. Although no correlation was observed between the degree of GPC3 expression and $GPC3_{298-306}$ peptide-specific CTL response, GPC3 expression in bilateral inguinal lymph node metastasis in case 1 may be associated with the clinical benefits of GPC3 peptide vaccine therapy. On the other hand, we were unable to perform biopsies of retroperitoneal lymph node metastases in case 2. Therefore, further pathological analysis was limited. Although GPC3 immunohistological expression was

negative in part of the primary ovarian CCC tissue, its expression may be positive in other parts of the tumor. It may be difficult to predict the clinical response against metastatic tumors based on the strength of GPC3 expression in the primary tumor.

In a phase I trial of GPC3-derived peptide vaccination, while GPC3 immunohistological expression was detected in more than 80% of advanced HCC patients, pretreatment serum GPC3 protein was detectable in only approximately half of those patients. Unlike case 1, the serum GPC3 protein in case 2 was undetectable by ELISA using a monoclonal antibody (clone 1G12) and sheep polyclonal antibody. Delayed clinical response may have been due to lower expression of GPC3 than the detection limit of assays using clone 1G12 in metastatic sites.

Ex vivo IFN-γ ELISPOT analysis in these two cases revealed vaccine-induced immune reactivity against the GPC3 peptide (data not shown). Although we were unable to discuss whether there were any differences in the quantity or quality of the responses between the two cases because the number of PBMCs plated per well did not correspond, there were more GPC3 peptide-specific CTL spots and less non-specific background spots in case 2 than in case 1.

It is difficult to confirm whether tumor regression was actually induced by peptide-specific CTLs or other mechanisms. As one of several possibilities, antigen spreading may have occurred following the GPC3 peptide-specific CTL response after the vaccination and contributed decisively to tumor regression.

Although many clinical trials have been conducted with cancer peptide vaccines, none of these have succeeded in Phase 3. The development of a biomarker to select potential responders would contribute significantly to potential success in a Phase 3 trial. In an effort to identify such biomarkers, we consider it possible to predict a response based on HLA class I/peptide complex expression on the cell surface in prevaccine biopsy specimens. Thus, we have attempted to prepare monoclonal antibodies against the HLA-A24/GPC3$_{298\text{-}306}$ peptide complex and HLA-A2/GPC3$_{144\text{-}152}$ peptide complex. Further analyses are needed in the future.

These results provide the first clinical evidence to demonstrate that GPC3 peptide-based immunotherapy is a promising treatment for patients with ovarian CCC. The complexity of the immune response and impact of each individual patient's status on the immune system create challenges for predicting the time course of the response. Ongoing and future trials will yield information on the best clinical use of this vaccine and the most appropriate method for assessing the response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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