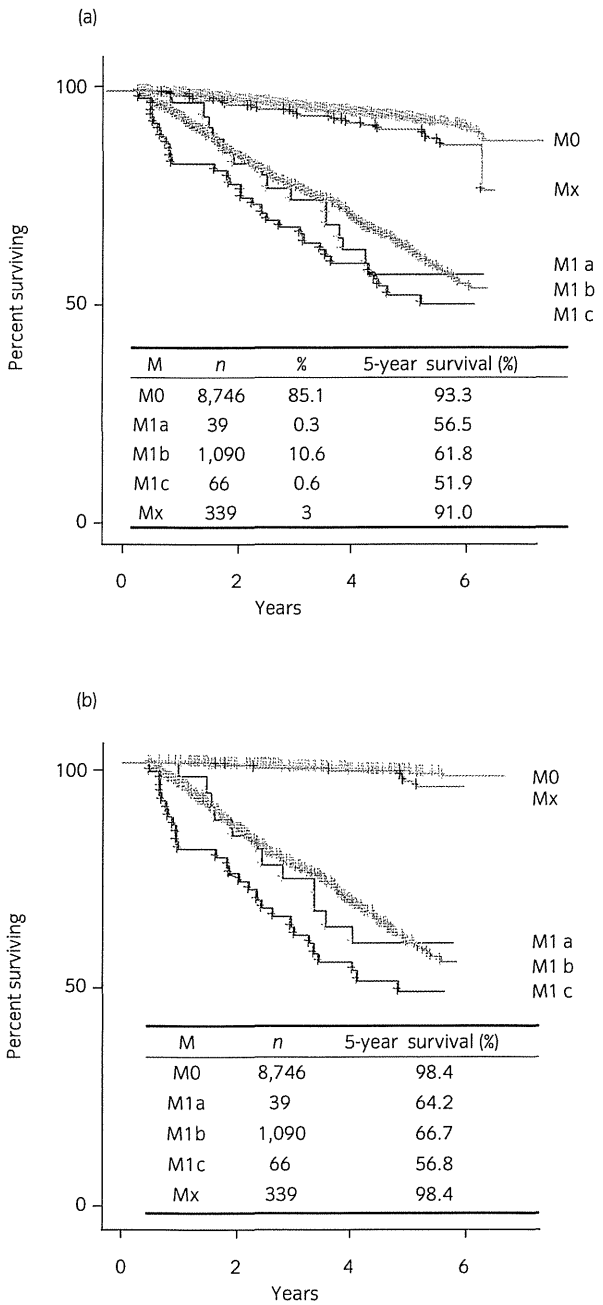

Fig. 1 Kaplan–Meier curves of (a) overall survival and (b) prostate cancer-specific survival according to M stage (n = 10 280).

The outcomes of 3200 RP patients according to NHT duration are summarized (see Table S3, supporting information). Because of uncertain NHT status, 12 patients were excluded. In the RP with NHT group ($n = 1164$), most pathological parameters including node metastasis (pN) and surgical margin status (ew) were better than in those patients without NHT ($n = 2045$; $P < 0.001$), except for seminal vesicle invasion (sv). However, the survival status of RP with NHT group did not differ from the RP without NHT group. The disease-free rate and prostate cancer death rate in the RP group within this observation period of approximately 5 years was approximately 70–75% and less than 1%, respectively.

Hormonal therapy alone

In this registration series, 4934 patients were treated with Hx alone (see Table S4, supporting information). In these patients, 3582 patients (72.6%) had non-metastatic disease (M0) and 1061 patients (21.5%) had bony metastasis (M1b). The combination of luteinizing hormone-releasing hormone (LH-RH) analogs with non-steroidal anti-androgen drugs were used in the majority of the Hx patients (67.4%). In M0 disease, 25% of patients received monotherapy with LH-RH analogs or surgical castration, and 67.4% patients were treated with maximum androgen blockade (MAB). Estrogen or estramustine phosphate therapy as the initial Hx was rare for M0 disease. For M1b disease, 82% of patients received MAB and 14.4% of patients received estrogen or estramustine phosphate as the initial treatment. The 5-year PCSS in patients with M0 disease was 93.3% and in M1b patients, it was 71.2%. In M0 patients, 8.4% of the patients died of other causes, which seemed to be higher when compared with patients treated with other modalities.

Curative radiation for prostate cancer

Rx as a radical treatment was used for 1554 patients. There were 28 patients who received particle radiotherapy and 27 patients were treated by uncertain modality. Excluding these patients, the characteristics of the 1499 patients are summarized (see Table S5, supporting information). Radiation therapy was classified as external beam radiation therapy with Liniac (EBRT; $n = 1241$), brachytherapy (BT; $n = 210$) or a combination (BT + EBRT; $n = 48$). Median age in EBRT was 72.9 years and median PSA was 15.0 ng/mL. In contrast, that in BT was 70.0 years and median PSA was 7.30 ng/mL. When compared with EBRT patients, BT patients were younger and had lower PSA, Gleason scores and earlier stage disease. The median PSA level in patients who received EBRT was 15.0 ng/mL, higher than in RP patients. In 1241 EBRT patients, 88.6% received radiation to the prostate only and the median dose in EBRT was 70 Gy. No cancer deaths were observed in patients who received BT and BT + EBRT. In the EBRT group, 5-year PCSS was 98.3% (see Table S6, supporting information).

Watchful waiting

In this registry, W/W included active surveillance, deferred treatment and palliative observation. At the time of registration, 72.4% of patients were maintained on watchful

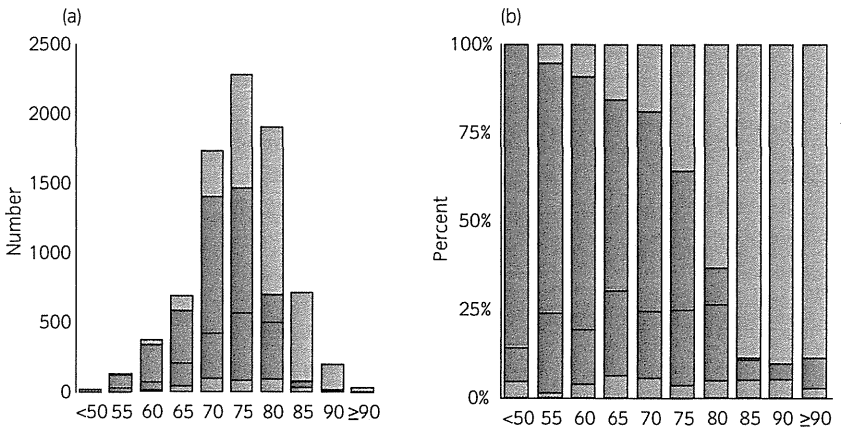

Fig. 2 Age distribution by main treatment modality in patients with T1-4N0M0 prostate cancer ($n = 8424$). (a) Totals and numbers of patients who underwent each treatment modality. (b) Percentages of each treatment by age. Hx, hormone ablation therapy; RP, radical prostatectomy; Rx, radiation therapy; W/W, watchful waiting.

Number of the patients by age and main treatments

	<50	50–55<	55–60<	60–65<	65–70<	70–75<	75–80<	80–85<	85–90<	≥90
Hx	0	7	34	108	329	815	1207	637	184	31
RP	18	94	269	675	982	899	198	4	0	0
Rx	2	30	58	166	326	485	409	41	9	3
W/W	1	2	15	45	100	86	96	37	11	1

waiting. In the W/W group, 0.62% of the patients died of prostate cancer. The incidence was similar to that in the RP patients (see Table S7, supporting information).

Discussion

The present report is the first large-scale study of the characteristics and survival of prostate cancer patients in Japan based on multi-institutional registry data. The estimated number of newly diagnosed prostate cancer patients in Japan in 2005 was 42 997.[3] This registry seems to cover approximately one-quarter of newly diagnosed prostate cancer in Japan. With regard to prostate cancer incidence and mortality, ethnic differences between American or European and Asian men are well known. Understanding the actual situation of Japanese prostate cancer patients is indispensable to addressing many clinical issues regarding prostate cancer treatment.

The incidence of metastatic prostate cancer at the initial registration was 11.6% in the present study. In the USA, 6.5% were distant stage according to the report from the 1990–2000 database of the Surveillance, Epidemiology and End Results (SEER) Program[4], suggesting the incidence of metastatic disease is higher in Japan than in the USA. However, the incidence was 21.3% from the Japanese registration data in 2000.[1] Compared with the data from 2000, the ratio of distant disease in 2004 was reduced by half. However, the number of the distant diseases in 2000 ($n = 964$) was almost the same as that in 2004 ($n = 1195$).

In the report derived from the 1973–2000 database of the SEER Program[4], 5- and 10-year PCSS were approximately 99% and 95%, respectively. Two-thirds of patients were diagnosed with well or moderately differentiated localized or regional prostate cancer. Among these patients, 5- and 10-year PCSS were approximately 100%. In the present study, 5-year PCSS was 94.8%, which resembles the SEER data from 1995. The PCSS of localized or regional prostate cancer was 98.4%, similar to the SEER data. Five-year PCSS of patients with bony metastasis in Japan was 66.7%, which was better than the 27–37% 5-year PCSS in the USA[4]. The reason why Japanese patients with bony metastasis showed a longer survival period than American patients is uncertain.

The main treatment used for non-metastatic prostate cancer patients in Japan was quite different from that in the USA. In the USA, approximately half of prostate cancer patients received surgery and more than one-third underwent Rx.[5] In Japan, Hx comprised of 39.9% of the initial main treatment, even for non-metastatic prostate cancer. One of the reasons for the high rate of Hx might be the relatively advanced age at diagnosis. Another reason might be the high rate of health insurance coverage and indifference about erectile dysfunction. In the present study, the most frequent treatment for non-metastatic prostate cancer in patients less than 70-years-old was RP (62.5%). Essentially, for patients younger than 70-years-old, Japanese urologists might choose treatments in agreement with major guidelines published by the National Comprehensive Cancer Network and the European Association of Urology, among others.

Concerning the administration of Hx medications, MAB therapy was recommended for stage D2 prostate cancer.[6] However, in Japan, 65% of patients with non-metastatic disease received MAB therapy and 25% of them received

LH-RH analogs or surgical castration as monotherapy. The 5-year PCSS of non-metastatic prostate cancer patients in Japan showed excellent results, even in the W/W group. The OS of patients with Hx seemed to be lower than that with other modalities. The patients undergoing Hx are relatively older.

In the present series, detailed data on RP was analyzed. In 2004, open retropubic RP (89.6%) with obturator lymph node dissection (71.6%) was the most common procedure. Interestingly, just 20% of patients received nerve-sparing operations in Japan. In high-volume hospitals in the USA, most radical prostatectomy seems to be carried out using the nerve-sparing technique. For most Japanese men, there might be less concern about sexual function when compared with American men.

The pathological results were sorted by NHT duration, because they might be affected by NHT status. Similar to the data from many randomized controlled studies of NHT[7,8] most pathological findings were improved by longer NHT, except for seminal sv and pN. However, there was no remarkable improvement in prognosis despite longer NHT as previously reported. However, these data came from non-randomized, non-historically controlled patients.

Additionally, the present study might be the largest population study of Rx in Japan. In past years, the trends and patterns of Rx in Japan were reported by the patterns of care study (PCS).[9,10] The age, PSA, Gleason score and radiation dose in the EBRT group of the present study were similar to PCS data. The median PSA of 15.0 ng/mL in the EBRT patients was higher than that of the patients treated with RP. Japanese urologists seemed to select EBRT for treating localized advanced disease. The EBRT group in the registry had a disease-free rate of 58% and a stable disease rate of 22.7%. Recently, higher dose radiation has been recognized to contribute to better cancer control. In 2004, 11.0% of the patients received 72 Gy and 11.4% patients received 76 Gy EBRT. Nearly 50% of patients underwent 68 Gy EBRT. Recently, relatively high dose EBRT in combination with NHT was attempted using the intensity modulated radiotherapy technique.

In conclusion, this is the first report of survival data involving one-quarter of newly diagnosed prostate cancer patients in Japan. In Japan, the patient population, survival period with metastatic disease and the ratio of patients receiving Hx differ from Western countries. Also noteworthy is the reduction in the ratio of metastatic prostate cancer at diagnosis, which was 11.6% in 2004, approximately half the rate in 2000. However, the total number of newly diagnosed patients with metastatic prostate cancer in 2004 was almost same as that in 2000. In terms of localized (cT2 or earlier stage) prostate cancer, Hx was used as the main treatment in 36.7% of Japanese patients. The 5-year survival of patients with localized prostate cancer was excellent irrespective of the main treatment used. Five-year OS and PCSS of patients with M1b disease were superior to that in the USA.

Acknowledgments

These clinicopathological statistics are the results from a number of institutions in Japan (see Appendix I, supporting information). We are grateful for the cooperation of many Japanese urologists. This document was created by the Cancer Registration Committee of the Japanese Urological Association.

Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Distribution of age (A) and PSA (B) in patients with T1-4N0M0 prostate cancer ($n = 8424$) according to treatment. RP, radical prostatectomy; Rx, radiation therapy; Hx, hormone ablation therapy; W/W, watchful waiting.

Fig. S2 cT distribution and the main treatment adopted in patients with T1-4N0M0 prostate cancer ($n = 8424$). The graph A shows totals and numbers of patients who underwent each treatment modality. The graph B shows percentages of each treatment by clinical stage. RP, radical prostatectomy; Rx, radiation therapy; Hx, hormone ablation therapy; W/W, watchful waiting.

Fig. S3 Kaplan–Meier curves of overall survival (A) and prostate cancer-specific survival (B) by main treatment adopted in patients with T1-4N0M0 prostate cancer ($n = 8224$). RP, radical prostatectomy; Rx, radiation therapy; Hx, hormone ablation therapy; W/W, watchful waiting.

Table S1 Characteristics of the registered patients.

Table S2 Characteristics of 3212 radical prostatectomy patients.

Table S3 Outcome of 3200 radical prostatectomy cases with or without neoadjuvant hormonal therapy.

Table S4 Outcome of 4934 patients treated with hormone ablation therapy alone.

Table S5 Characteristics of patients treated with radiation therapy as the main treatment.

Table S6 Outcome of patients treated with radiation therapy as the main treatment.

Table S7 Outcome of 485 patients treated with watchful waiting.

Appendix I Statistics from various institutions in Japan.

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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 ≥50000/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.

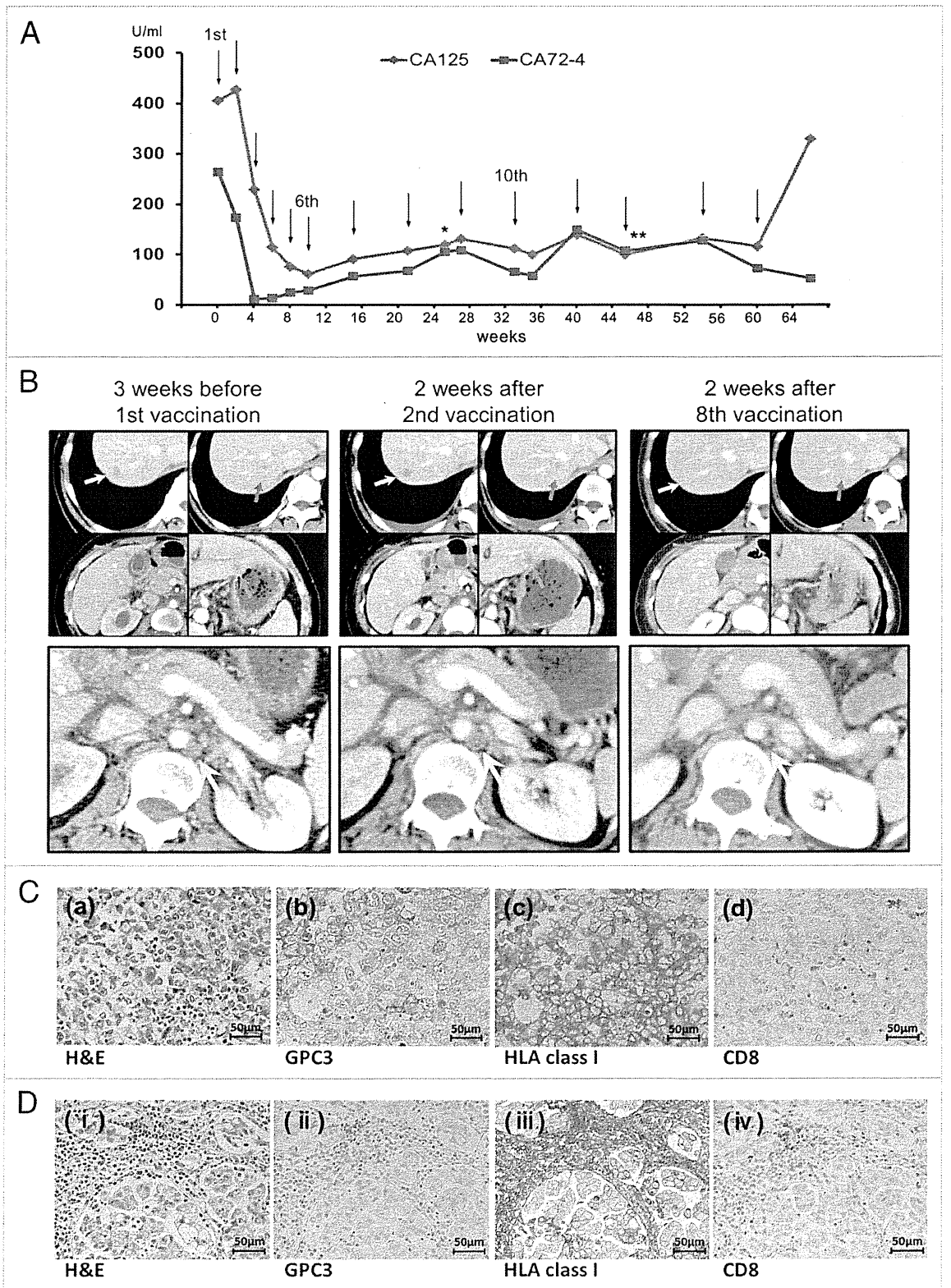

Figure 1. See page 2 for legend.

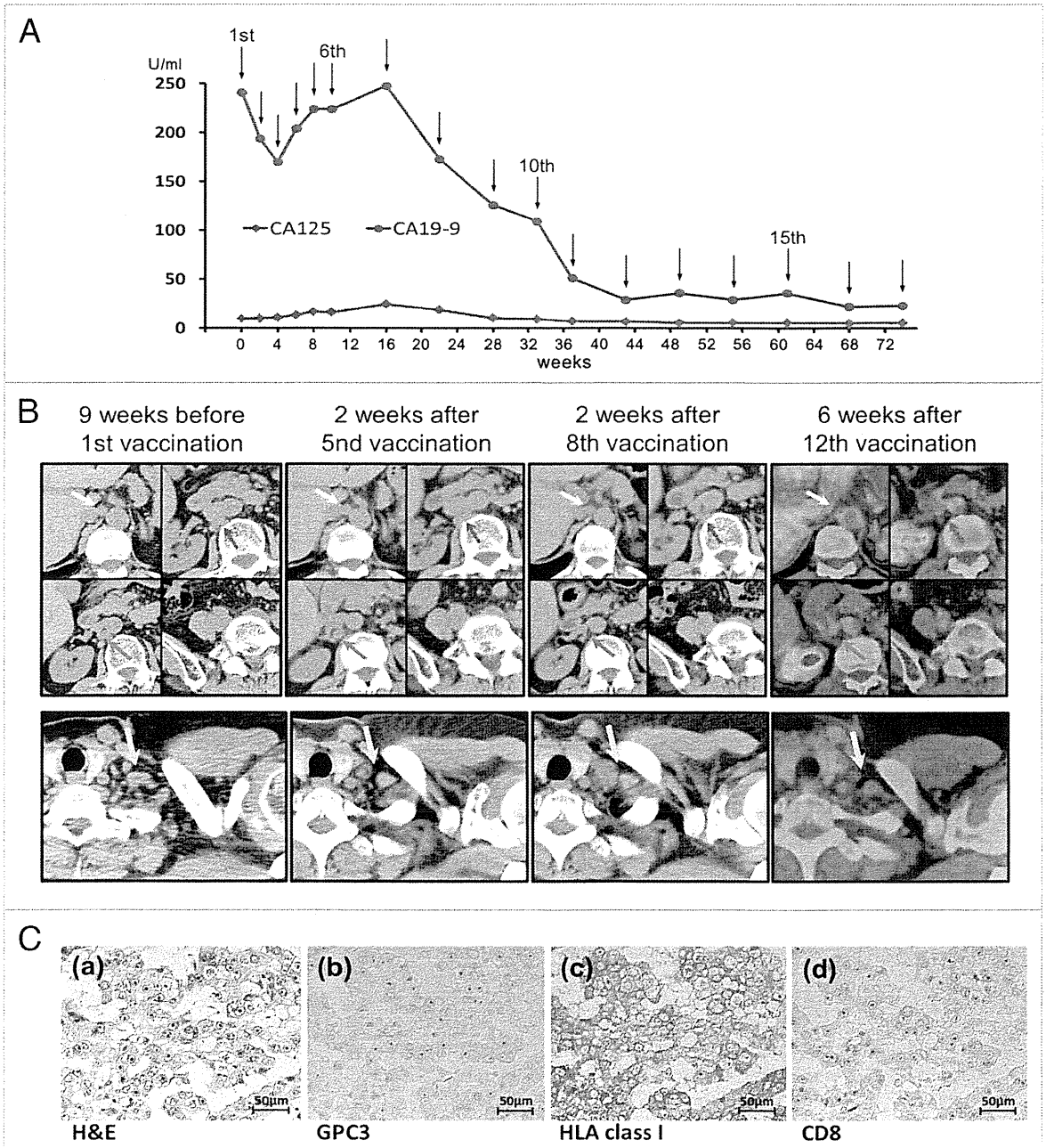

Figure 2. (**A**) Clinical course from the beginning of the GPC3 peptide vaccination. Serum levels of CA19–9 and CA125 decreased after the 7th vaccination. The CA19–9 level decreased to within the normal range. Black arrows indicate vaccinations. (**B**) Plain CT and ^{18}F-FDG PET/CT scans showing retroperitoneal 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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Remarkable tumor lysis in a hepatocellular carcinoma patient immediately following glypican-3-derived peptide vaccination

An autopsy case

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Keywords: peptide vaccine, glypican-3, CTL, HCC, tumor necrosis

Abbreviations: GPC3, glypican-3; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; CTL, cytotoxic T-lymphocyte; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cells; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin

We recently reported the safety, immunological and clinical responses to a GPC3-derived peptide vaccine in a phase I clinical trial of patients with advanced hepatocellular carcinoma (HCC). We conducted a subsequent trial in advanced HCC to assess the histopathological findings before and after vaccination with the GPC3 peptide. Here, we present the clinical course and the pathological study including the autopsy of a patient with advanced HCC in the ongoing clinical trial. A 62-year old patient suffering from HCC refractory to sorafenib therapy received the GPC3 peptide vaccine. The patient had fever and remarkably impaired liver function twice after vaccination. Contrast-enhanced CT after the second vaccination showed multiple low-density areas in the liver tumor, indicating tumor necrosis. In contrast, the tumor thrombus in the right atrium increased. The patient discontinued protocol treatment due to disease progression and died 30 days after the second vaccination. An autopsy was performed to determine the main cause of death and to evaluate the antitumor effect of the vaccination. A histological examination showed central necrosis in most of the intrahepatic tumor. The main cause of death was circulatory failure due to tumor thrombus, which occupied most of the right atrium. An immunohistochemical analysis revealed infiltration of CD8-positive T cells in the residual carcinoma, but not within the cirrhotic area. Ex vivo IFN-γ enzyme-linked immunospot analysis revealed vaccine-induced immune-reactivity against the GPC3 peptide. A histopathological examination at the estimated time of a strong immunological response demonstrated a GPC3 peptide vaccination-induced cytotoxic T-lymphocyte response with an anti-tumor effect.

Introduction

Cancer vaccine targeting hepatocellular carcinoma (HCC) tumor antigens have been tested in clinical trials.[1,2] However, cancer vaccines using tumor-antigen-derived peptides have not demonstrated adequate antitumor efficacy in clinical trials for advanced HCC.[1-3] Glypican-3 (GPC3), a carcinoembryonic antigen, is an ideal target for immunotherapy against HCC because it is overexpressed specifically in HCC (72–81%) and correlates with a poor prognosis.[4-10] GPC3 forms a complex with Wnt molecules and promotes the growth of HCC by stimulating canonical Wnt signaling.[10] We identified HLA-A*24:02-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptides, both of which induce GPC3-reactive cytotoxic T-lymphocytes (CTLs) without inducing autoimmunity.[8,9] We recently reported the safety, immunological, and clinical responses of a GPC3-derived peptide vaccine in a phase I clinical trial of patients with advanced HCC.[11] The results of that trial showed that GPC3 peptide-specific CTLs increased in peripheral blood, and that many CD8-positive T cells infiltrated the tumors in some patients, demonstrating a correlation between the CTL response and overall survival following GPC3 peptide vaccination. Based on these results, we conducted a trial in patients with advanced HCC to assess the clinical outcome and whether tumor-infiltrating lymphocytes with an antitumor effect increased. In all cases, liver biopsies were performed before and after GPC3 peptide vaccination according to the protocol. This trial was approved by the Ethics Committee of the National

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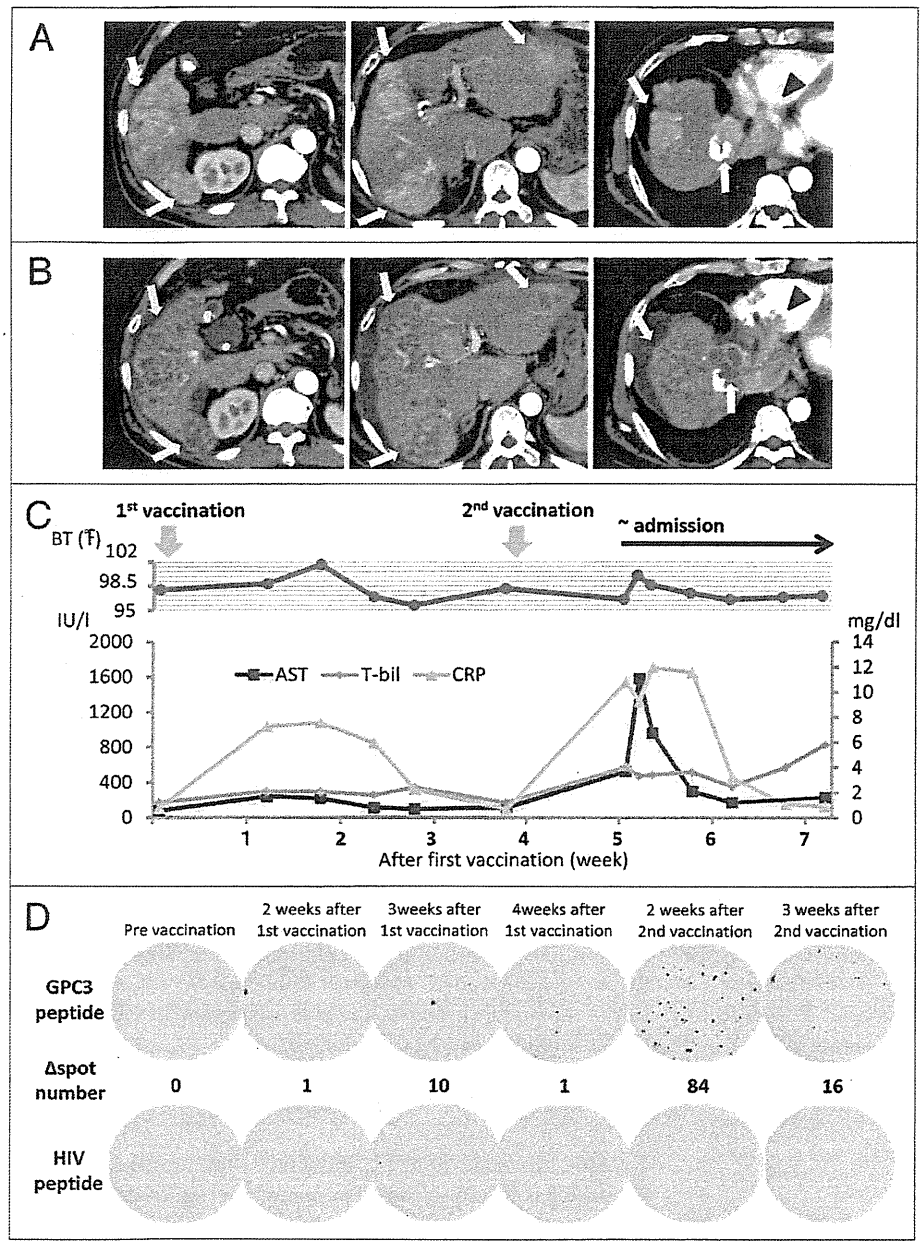

Figure 1. Findings of an early-phase contrast-enhanced CT (CT) scan. (**A**) Contrast-enhanced CT scan before vaccination shows a 68 × 51-mm tumor with multiple intrahepatic tumors (arrow) and a 44 × 30-mm tumor invading the right atrium (arrowhead). (**B**) Contrast-enhanced CT after the second vaccination showing multiple low-density areas in the liver, indicating extensive tumor necrosis (arrow). By contrast, a tumor thrombus in the right atrium increased to a 83 × 50-mm tumor (arrowhead). (**C**) Clinical course from the beginning of GPC3 peptide vaccination. Approximately 1 week after the first vaccination, the patient began reporting general fatigue and showed intermittent fever. Inflammatory and hepatic parameters were elevated (CRP: pink line, AST: red line, T-bil: green line). The abnormal laboratory parameters improved after observation. On day 9 after the second vaccination, the patient was admitted to our hospital as an emergency due to fever and general fatigue, which were similar to his previous symptoms. One day after hospitalization, the inflammatory and hepatic parameters were remarkable. Inflammatory and hepatic parameters improved 1 week after hospitalization. However, his status gradually worsened, and he died on day 30 after the second vaccination. (**D**) Immunological monitoring of the GPC3 peptide-specific T cell responses. Ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assays against GPC3 in 5 × 10^5 peripheral blood mononuclear cells (PBMCs) were performed before and after vaccination. The Δ spot number indicates the number of GPC3 peptide-specific cytotoxic T-lymphocytes (CTLs). The number of interferon (IFN)-γ positive spots increased from 0 to 84 after the second vaccination.

Cancer Center and registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number 000005093). The patient described herein was the first case examined pathologically using autopsy specimens. Here, we present the clinical course and pathological study, including an autopsy, of a patient with advanced HCC who revealed remarkable tumor lysis immediately after the second vaccination in an ongoing clinical trial of a GPC3 peptide vaccine.

Patient presentation. A 62-year-old male had a history of asymptomatic chronic hepatitis C. In September 2009, he was diagnosed with HCC. Laboratory data disclosed no abnormalities. Abdominal CT (CT) scans showed four lesions in the liver, and the patient was treated four times with hepatic artery chemoembolization. In December 2010, CT scans revealed a new lesion indicative of a tumor thrombus extending into the inferior vena cava. The patient was treated with sorafenib. However, the sorafenib treatment was discontinued in January 2011 due to progressive multiple intrahepatic tumors.

As no established therapeutic regimens exist for this condition, he was offered participation in a clinical trial of a GPC3 peptide vaccine for advanced HCC. HLA-typing revealed an HLA-A2 phenotype. The patient had a performance status of 0, and Child-Pugh class B disease. The patient did not have active HBV infection or rapidly progressive tumor thrombus before enrollment, met the eligibility criteria, and was enrolled after providing informed consent. Early-phase contrast-enhanced CT before treatment showed a maximum 68 × 51-mm tumor with multiple intrahepatic tumors and a 44 × 30-mm tumor invading the right atrium (**Fig. 1A**). Pretreatment tumor markers were as follows: α fetoprotein (AFP), 852 ng/mL and des-gamma-carboxy prothrombin (DCP), 1346 mAU/mL. A liver biopsy was performed 1 week prior to GPC3

peptide vaccination according to the protocol. In April 2011, 3 mg of HLA-A2-restricted $GPC3_{144-152}$ peptide (FVGEFFTDV) (American Peptide Co.) emulsified with incomplete Freund's adjuvant (Montanide ISA-51VG; SEPPIC) was injected intradermally as the vaccine following Good Manufacturing Practice guidelines. The patient had a low-grade fever on day 6 following the first vaccination, and inflammatory and hepatic parameters were elevated on day 12 (**Fig. 1C**). The abnormal laboratory findings improved later. Therefore, he received the second vaccination on day 26 after the first vaccination. On day 9 after the second vaccination, the patient was admitted to our hospital with a high fever and general fatigue. On admission, the patient's C-reactive protein (CRP) level (10.76 mg/dL) and laboratory hepatic parameters were elevated. One day after hospitalization, aspartate aminotransferase and alanine aminotransferase and levels were elevated to 1,580 IU/L and 1,112 IU/L, respectively. The prothrombin time-international normalized ratio increased from 1.18 to 1.51. But the patient did not have ammonemia or asterixis. As seen by early-phase contrast-enhanced CT scan, most tumors in the liver were not contrast enhanced. Findings of the CT scan indicated tumor necrosis and regression. In contrast, the size of the tumor thrombus in the right atrium increased to a maximum of 83 × 50 mm (**Fig. 1B**). Levels of the tumor markers AFP and DCP decreased temporarily to 634 ng/mL and 777 mAU/mL, respectively. He was infused with a liver-supporting agent (monoammonium glycyrrhizinate, glycine, and L-cysteine hydrochloride hydrate). The inflammatory and hepatic parameters improved 1 week after hospitalization (**Fig. 1C**). We did not perform a liver biopsy when the hepatic parameters were elevated because they improved promptly. Nevertheless, his status worsened gradually. Protocol treatment was discontinued due to progressive disease and he died 30 days after the second vaccination. Based on the clinical course, we could not rule out the possibility that his condition had worsened as a result of the vaccine. Therefore, an autopsy was performed to determine the main cause of death and the elevated hepatic parameters, and to evaluate the anti-tumor effect of vaccination.

Results

Immunological analysis and autopsy. Generally, CTLs specific for tumor antigens cannot be detected directly ex vivo; they can be detected only after expansion by repeated in vitro stimulation with the antigenic peptide in conjunction with appropriate antigen-presenting cells. This is attributed to the sensitivity of the assay and the low frequency of tumor-antigen-specific CTLs.[12] GPC3 peptide-specific CTLs in PBMCs, which can be detected directly ex vivo without in vitro stimulation, can provide strong immunological evidence. An ex vivo IFN-γ ELISPOT assay was performed, as described previously.[13] The number of GPC3 peptide-specific CTLs increased from 0 to 84 in 5×10^5 PBMCs after the second vaccination (**Fig. 1D**). This result led us to anticipate a good clinical response because the increased number of CTLs and the specific CTL number correlated with the clinical response in a previous trial of the GPC3 peptide vaccine.[11]

A liver biopsy was performed before vaccination according to protocol. Histological examination of the specimen revealed well-differentiated HCC. Immunohistochemical staining showed expression of GPC3 and HLA class I in the cytoplasm and membranes of the carcinoma cells and a few CD8-positive T cells in the carcinoma tissue before vaccination (**Fig. 2A**).

A general autopsy (with the exception of the brain) was performed 2 h following death. Macroscopic findings of the liver revealed multiple macro-nodular lesions with central necrosis mainly in the right lobe (**Fig. 2B**, left). As the tumor occupied most of the right atrium, the main cause of death was circulatory failure due to progressive tumor thrombus (**Fig. 2B**, right). We judged that his condition had worsened as a result of the tumor thrombus. A histological examination showed central necrosis in most of the tumor in the right lobe, and viable carcinoma cells remained around the necrotic tissue, whereas a cirrhotic nodule adjacent to the carcinoma tissue was not necrotic (**Figs. 2C and 3A**). Immunohistochemical staining revealed GPC3-positive carcinoma cells (**Fig. 3A**). There was infiltration of CD8-positive T cells (brown) in the residual carcinoma, but not within the cirrhotic area (**Figs. 2C and 3A**). We did not detect degeneration or necrosis of the hepatocytes in the non-tumor liver parenchyma of the left lobe. These findings suggest that the elevated hepatic parameters in our patient were due to an antitumor effect. We diagnosed that the cause of death was unlikely to be related to vaccine-induced liver injury. We focused on the necrotic area around the cirrhotic nodules, in which CD68-positive macrophages (brown) aggregated (**Fig. 3B**). CD8-positive T cells also infiltrated the marginal zone between the necrotic area and noncancerous cirrhotic nodule, suggesting that carcinoma cells were attacked by CD8-positive T cells, which may have resulted in necrosis (**Fig. 3B**). The histology of the tumor thrombus in the right atrium was similar to that of the intrahepatic tumor. However, viable tumor cells remained in half of the tumor thrombus and little infiltration of CD8-positive T cells was detected (data not shown).

Discussion

To date, the time to CTL induction and subsequent tumor response has been prolonged in cancer vaccine trials.[14] By contrast, no discrepancy regarding the time between CTL induction and tumor response was observed in our phase I trial of a GPC3 peptide vaccine.[11] In this case, central necrosis of each intra-hepatic tumor was observed at the time of a strong immunological response against the GPC3 peptide, immediately after the second vaccination.

We did not perform a liver biopsy when the hepatic parameters were elevated. A biopsy may be necessary to rule out vaccine-induced liver injury when the hepatic parameters are elevated. However, the clinical course and autopsy results suggested that the elevated hepatic parameters in our patient were due to an antitumor effect.

Treatment-induced necrosis is included in the modified RECIST assessment for HCC.[15] Therefore, a positive radiographic response following vaccination, suggesting tumor necrosis, could be evaluated as a treatment response.

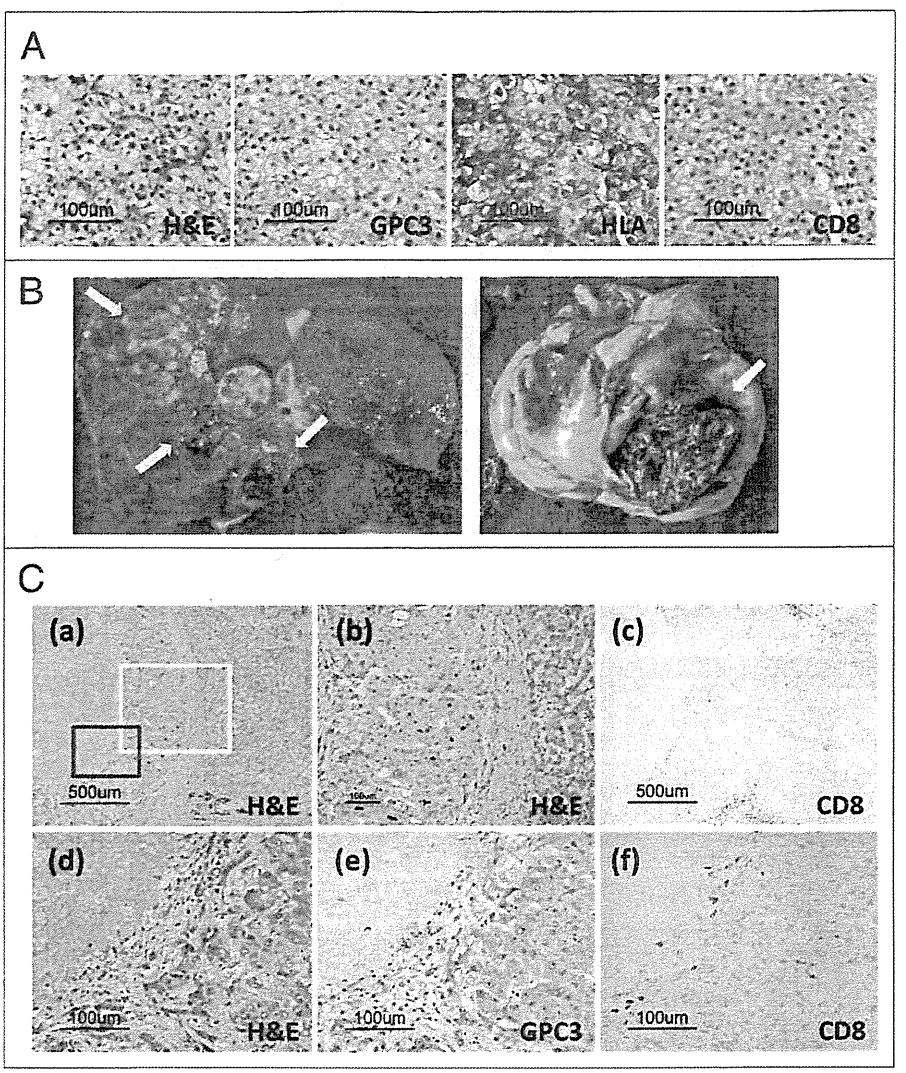

Figure 2. (**A**) Pathological findings of liver biopsy specimens before vaccination. A microscopy image of a hematoxylin and eosin (H&E)-stained section shows well-differentiated hepatocellular carcinoma (HCC). Immunohistochemical staining for GPC3 and HLA class I showed positivity in the cytoplasm and membranes of carcinoma cells, respectively. No CD8-positive T cells were observed in carcinoma tissue before vaccination. (**B**) Macroscopic findings of the liver and heart before formalin fixation at the time of autopsy. Most liver tumors had a necrotic area (arrow). A tumor thrombus occupied most of the right atrium (arrow). (**C**) Pathological findings of the autopsy specimen. (**a**) Microscopic images of H&E-stained sections showing central necrosis of carcinoma tissue, whereas a cirrhotic nodule adjacent to the carcinoma tissue was not necrotic. (**b**) Magnified image of the area enclosed within the white box in (**a**) showing that cancer cells exhibited a morphology (left) different from that of cirrhotic cells (right). (**c**) CD8-positive T cells (brown) infiltrated the carcinoma cells accompanied by necrosis. In contrast, no infiltration of CD8-positive T cells was detected within the cirrhotic nodule. (**d**) Magnified image of the area enclosed within the red box in (**a**) showing necrosis and viable carcinoma cells. (**e**) Positive immunohistochemical GPC3 staining was observed in only the cytoplasm of carcinoma cells. (**f**) CD8-positive T cells infiltrated the necrotic area and carcinoma tissue.

Necrosis was found in the center of each tumor; therefore, the central necrosis caused by ischemia, in addition to CD8-positive T cells attacking tumor cells, may have led to tumor necrosis. Three findings support the hypothesis that tumor necrosis was caused by CD8-positive T cells, as follows: (1) the necrotic changes determined by CT after vaccination, accompanied by clinical laboratory data; this was consistent with an immune response, although no tumor necrosis was evident on the CT before vaccination; (2) no necrosis was evident in the left lobe (no tumors) of the autopsy liver specimen, but it was present in the right liver lobe (tumors present); and (3) CD8-positive T cells infiltrated residual viable tumor cells. The analyses used in this study may contribute to identifying the pathological state after vaccination.

We detected infiltration of CD8-positive T cells into the hepatic tumors, but little infiltration of CD8-positive T cells into

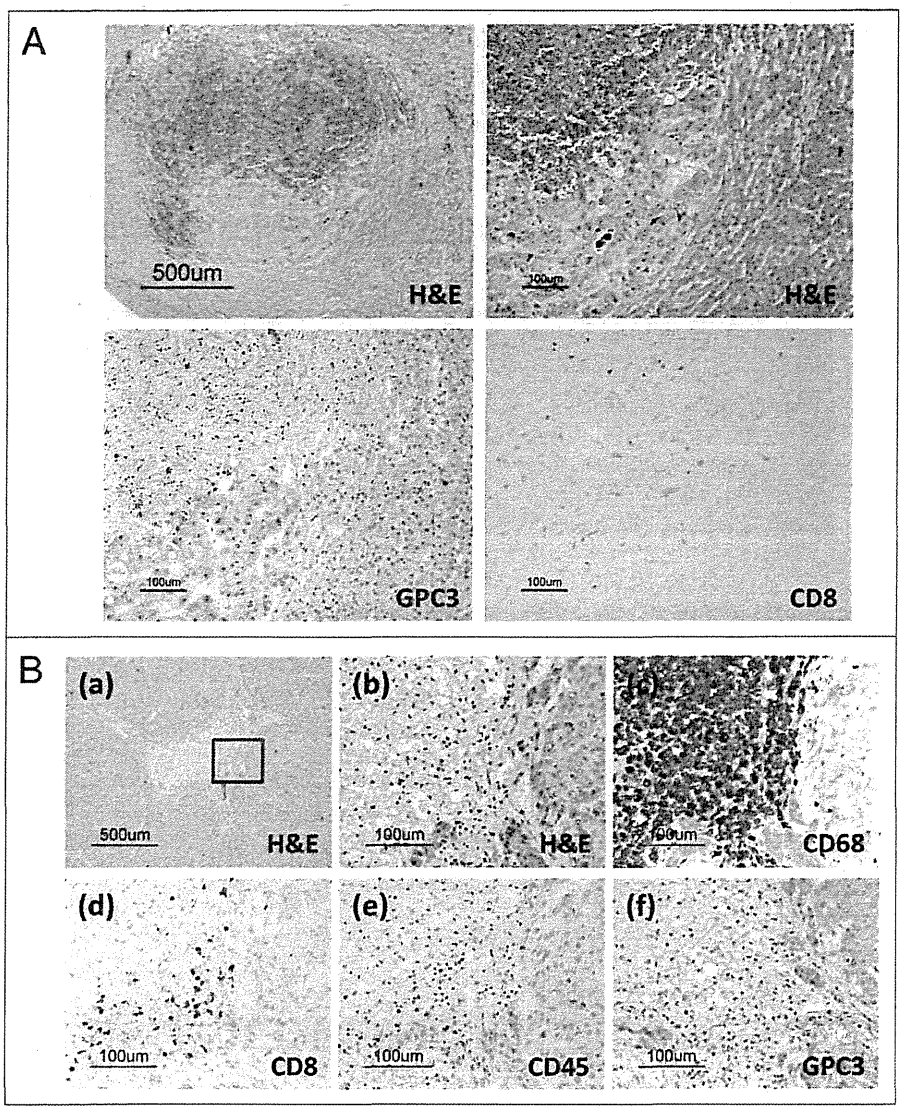

Figure 3. Pathological findings in the autopsy specimen. (**A**) Carcinoma in a cirrhotic nodule. CD8-positive T cells (brown) infiltrated only the carcinoma area, accompanied by necrosis. No infiltration of CD8-positive T cells was detected in the cirrhotic nodule. Only carcinoma cells were GPC3-positive by immunohistochemical staining. (**B**) Necrotic area surrounded by cirrhotic nodules. (**a**) Necrosis was surrounded by viable cirrhotic cells. (**b**) The margin between the necrosis and the cirrhotic nodule. This portion is enclosed by the red box in (**a**). (**c**) CD68-positive macrophages (brown) aggregated in the necrotic area around the cirrhotic nodule. (**d**) CD8-positive T cells (brown) infiltrated the necrotic area but not the cirrhotic nodule. (**e**) CD45-positive lymphocytes infiltrated the necrotic area. Based on the image in (**d**), most of the lymphocytes were CD8-positive T cells. (**f**) Cirrhotic cells did not express GPC3.

the tumor thrombus. This discrepancy may have been caused by the heterogeneity associated with immune-escape mechanisms in tumor cells.

This case report of central necrosis in a patient with HCC might be regarded as spontaneous regression correlated with circulatory failure due to a massive tumor embolism. It was not known whether the tumor necrosis was induced by CTLs, ischemia, or other factors. However, the infiltration of CD8-positive T cells into tumor cells supports immune-related necrosis.

The rate of spontaneous partial regression among patients with HCC is 0.406% compared with the control arm of a randomized clinical trial.[16] In contrast, three of 33 patients who received GPC3 peptide vaccination in the phase I trial had suspicious tumor necrosis on CT scans. In one report, massive infiltration of CD8-positive T cells in the remaining liver tumor and tumor necrosis were identified by histological examination of a biopsy specimen after vaccination.[11] Indeed, on-going clinical trials of the GPC3 peptide vaccine will provide additional information and further demonstrate the antitumor effect.[17,18] Histological results at the estimated time of a strong GPC3-specific CTL response suggest that GPC3 peptide vaccination may be a promising approach to treat HCC.

Materials and Methods

Ex vivo interferon-γ (IFN-γ) enzyme-linked immunospot assay. An ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed to evaluate the antigen-specific CTL response, as described previously.[13] Briefly, peripheral blood (30 mL) was obtained from the patient before the first vaccination and 2 weeks after each vaccination and centrifuged on a Ficoll-Paque gradient. Non-cultured peripheral blood mononuclear cells (PBMCs) (5×10^5/well) were added to plates in the presence of 10 μg/mL peptide antigens and incubated for 20 h. The GPC3 antigen used was the HLA-A2-restricted $GPC3_{144-152}$(FVGEFFTDV) peptide. PBMCs with the HLA-A2-restricted HIV19–27 (TLNAWVKVV) peptide (ProImmune) were used as negative controls. Assays were performed in duplicate.

Immunohistochemical analysis. Immunohistochemical staining with monoclonal antibodies against GPC3 (clone, 1G12; Biomosaics), HLA class I (clone, EMR8/5; Hokudo), CD8 (clone, 1A5; Novocastra), CD45 (cloned 2B11 and PD7/26; Ventana), and CD68 (clone, KP-1; Ventana) was performed according to the manufacturer's protocol.

Disclosure of Potential Conflicts of Interest

The authors have no potential conflicts of interest to declare with regard to this study.

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ORIGINAL ARTICLE

Intratumoral peptide injection enhances tumor cell antigenicity recognized by cytotoxic T lymphocytes: a potential option for improvement in antigen-specific cancer immunotherapy

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Abstract Antigen-specific cancer immunotherapy is a promising strategy for improving cancer treatment. Recently, many tumor-associated antigens and their epitopes recognized by cytotoxic T lymphocytes (CTLs) have been identified. However, the density of endogenously presented antigen-derived peptides on tumor cells is generally sparse, resulting in the inability of antigen-specific CTLs to work effectively. We hypothesize that increasing the density of an antigen-derived peptide would enhance antigen-specific cancer immunotherapy. Here, we demonstrated that intratumoral peptide injection leads to additional peptide loading onto major histocompatibility complex class I molecules of tumor cells, enhancing tumor cell recognition by antigen-specific CTLs. In in vitro studies, human leukocyte antigen (HLA)-A*02:01-restricted glypican-$3_{144–152}$ (FVGEFFTDV) and cytomegalovirus$_{495–503}$ (NLVPMVATV) peptide-specific CTLs showed strong activity against all peptide-pulsed cell lines, regardless of whether the tumor cells expressed the antigen. In in vivo studies using immunodeficient mice, glypican-$3_{144–152}$ and cytomegalovirus$_{495–503}$ peptides injected into a solid mass were loaded onto HLA class I molecules of tumor cells. In a peptide vaccine model and an adoptive cell transfer model using C57BL/6 mice, intratumoral injection of ovalbumin$_{257–264}$ peptide (SIINFEKL) was effective for tumor growth inhibition and survival against ovalbumin-negative tumors without adverse reactions. Moreover, we demonstrated an antigen-spreading effect that occurred after intratumoral peptide injection. Intratumoral peptide injection enhances tumor cell antigenicity and may be a useful option for improvement in antigen-specific cancer immunotherapy against solid tumors.

Keywords Intratumoral peptide injection · Antigen · Immunotherapy · Cytotoxic T lymphocyte

Abbreviations

CTL	Cytotoxic T lymphocyte
HLA	Human leukocyte antigen
GPC3	Glypican-3
HCC	Hepatocellular carcinoma
MHC	Major histocompatibility complex
CMV	Cytomegalovirus
HIV	Human immunodeficiency virus
OVA	Ovalbumin
TAP	Transporter associated with antigen processing
FBS	Fetal bovine serum
IFN	Interferon
ELISPOT	Enzyme-linked immunospot
IFA	Incomplete Freund's adjuvant
PBMC	Peripheral blood mononuclear cell

Introduction

Conventional modalities of cancer treatment, including surgery, radiotherapy, and chemotherapy, have made advancements in recent years, and the survival rate of cancer

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patients has gradually improved; however, these therapies remain far from being satisfactory in most cancers [1, 2]. Therefore, the development of novel treatment modalities, including antigen-specific cancer immunotherapies with peptide vaccines, dendritic cell vaccines, and adoptive cell transfer therapies, is critical for advancing effective cancer treatments [3–5]. While many tumor-associated antigens and epitopes recognized by cytotoxic T lymphocytes (CTLs) have been explored as possible antigen-specific cancer immunotherapies, the results of several anticancer immunotherapy clinical trials have been disappointing [6, 7]. We conducted a clinical trial using the glypican-3 (GPC3) peptide vaccine in advanced hepatocellular carcinoma (HCC) patients. While this carcinoembryonic antigen overexpressed in HCC seemed to be an ideal target for anticancer immunotherapy [8–15], only immunological efficacy was apparent [16], whereas the clinical benefit was limited in patients [17]. Therefore, the establishment of an innovative strategy to link the antitumor immune response with the clinical response and to enhance the power of antigen-specific cancer immunotherapy is urgently required.

In the antigen-specific cancer immunotherapy concept, antigen-specific CTLs recognize and destroy tumor cells that present antigen-derived peptides using cell surface major histocompatibility complex (MHC) class I molecules. However, the density of the antigen-derived peptide endogenously presented on tumor cells is generally low, resulting in the ineffectiveness of antigen-specific CTLs [18]. This low density of presented antigen is one reason why antigen-specific cancer immunotherapy has been ineffective in clinical settings. One solution for overcoming this critical problem is to induce high-avidity CTLs. Such CTLs can recognize a smaller number of peptide–MHC class I complexes and would contribute to a better outcome [19]. Another solution is to enhance tumor cell antigenicity by means of additional peptide loading onto MHC class I molecules. Increasing the density of antigen-derived peptide would facilitate CTL recognition and destruction of the tumor cells.

In this study, we investigated whether intratumoral peptide injection would induce additional peptide loading onto tumor cells, and, if so, whether increased presentation would enhance antigen-specific CTL tumor cell recognition. Moreover, we evaluated whether intratumoral peptide injection could be a useful option for improvement in antigen-specific cancer immunotherapy against solid tumors.

Materials and methods

Synthetic peptides

The peptides used in this study were as follows: human leukocyte antigen (HLA)-A*02:01-restricted $GPC3_{144–152}$ (FVGEFFTDV) peptide (American Peptide Company, Sunnyvale, CA), HLA-A*24:02-restricted $GPC3_{298–306}$ (EYILSLEEL) peptide (American Peptide Company), HLA-A*02:01-restricted cytomegalovirus $(CMV)_{495–503}$ (NLVPMVATV) peptide (ProImmune, Rhinebeck, NY, USA), HLA-A*24:02-restricted $CMV_{341–349}$ (QYDPVAALF) peptide (ProImmune), HLA-A*02:01-restricted human immunodeficiency virus $(HIV)_{77–85}$ (SLYNTYATL) peptide (ProImmune), and H-2 K^b-restricted ovalbumin $(OVA)_{257–264}$ (SIINFEKL) peptide (AnaSpec, Fremont, CA, USA). The peptides were dissolved and diluted in 7 % $NaHCO_3$.

Cell lines

T2 cells (HLA-A*02:01), which lack the transporter associated with antigen processing (TAP), were purchased from Riken Cell Bank (Tsukuba, Japan). The human liver cancer cell line HepG2 ($GPC3^+$, HLA-A*02:01/A*24:02) was purchased from American Type Culture Collection (Manassas, VA, USA). The human liver cancer cell line SK-Hep-1 ($GPC3^-$, HLA-A*02:01/A*24:02), human colon cancer cell line SW620 ($GPC3^-$, HLA-A*02:01/A*24:02), murine lymphoma cell line RMA (OVA^-, H-2 K^b), EL4 (OVA^-, H-2 K^b), and EG7 (OVA^+, H-2 K^b) were kindly provided by Dr. Yasuharu Nishimura (Kumamoto University, Kumamoto, Japan). SK-Hep-1/GPC3 is an established stable GPC3-expressing cell line transfected with a human GPC3 gene, and SK-Hep-1/vec is an established counterpart cell line, in which an empty vector was transfected. EG7 cells are OVA-transfected EL4 cells. Cells were cultured at 37 °C in RPMI 1640 or DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5 % CO_2.

Mice

Female BALB/c nude, NOD/SCID, and C57BL/6 mice (6–8 weeks old) were purchased from Japan Charles River Laboratories (Yokohama, Japan). OT-I mice [20], which are $CD8^+$ T-cell TCR transgenic mice expressing the TCR α-chain recognizing $OVA_{257–264}$ peptide in H-2 K^b, were kindly provided by Dr. Takashi Nishimura (Hokkaido University, Sapporo, Japan). All animal procedures were performed according to the guidelines for the Animal Research Committee of the National Cancer Center, Japan.

Preparation of OT-I mouse-derived $CD8^+$ CTLs (activated OT-I CTLs)

Naïve $CD8^+$ T-cells were purified from the spleens of OT-I mice using MACS anti-CD8a (Ly-2) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). For in vitro

activation, naïve OT-I $CD8^+$ T-cells were incubated with irradiated EG7 cells at a 3:2 ratio in 24-well plates for 3 days. Each well contained 2.4×10^6 OT-I $CD8^+$ T-cells plus 1.6×10^6 EG7 cells in 2 ml of RPMI 1640 medium supplemented with 10 % FBS, penicillin, streptomycin, and 50 μmol/l 2-mercaptoethanol. Activated OT-I $CD8^+$ T-cells were separated from EG7 cells using anti-CD8a magnetic beads before adoptive transfer.

IFN-γ ELISPOT assay

The BD™ ELISPOT set (BD Biosciences, San Jose, CA, USA) was used for an interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay. CTLs were used as effector cells, and tumor cell lines with or without a peptide pulse (10 μg/ml for 1 h) were used as target cells. Effector cells (1×10^3/well) were incubated with target cells (1×10^4/well) in 200 μl of RPMI 1640 medium supplemented with 10 % FBS, penicillin, and streptomycin for 20 h at 37 °C in 5 % CO_2. The number of spots, indicating an antigen-specific CTL response, was automatically counted using the Eliphoto system (Minerva Tech, Tokyo, Japan).

Cytotoxicity assay

The Terascan VPC system (Minerva Tech) was used for cytotoxicity assays. Target cells were labeled with Calcein-AM (Dojindo Laboratories, Kumamoto, Japan) solution for 30 min at 37 °C, washed three times, distributed to 96-well culture plates in duplicate, and incubated with effector cells for 4 h. Fluorescence intensity was measured before and after the 4-h culture, and antigen-specific cytotoxic activity was calculated as described previously [16].

Intratumoral peptide injection

In in vivo studies, tumors implanted on the backs of mice were injected with 50 μg peptide mixed with an equal volume of incomplete Freund's adjuvant (IFA, Montanide ISA-51VG; SEPPIC, Paris, France). The total volume of solution injected was 100 μl in all experiments.

Tumor excision and isolation of tumor cells

To investigate whether the injected peptide was loaded onto HLA class I molecules of tumor cells in a solid mass, an IFN-γ ELISPOT assay was performed using these isolated tumor cells as target cells. Mice were killed and their dorsal tumors were dissected, cut into small pieces, and digested with collagenase (1.5 mg/ml) for 20 min at 37 °C.

In vivo tumor growth inhibition assay

In a peptide vaccine model, H-2 K^b-restricted $OVA_{257–264}$ peptide emulsified with IFA (50 μg/100 μl) was intradermally injected at the base of the tail of C57BL/6 mice, five times at 7-day intervals as described previously [13]. After vaccination, the induction of H-2 K^b-restricted $OVA_{257–264}$ peptide-specific CTLs was detected by IFN-γ ELISPOT assay (data not shown). In an adoptive transfer model, activated OT-I CTL (1×10^7 cells/500 μl) was intravenously injected.

SW620 cells (5×10^6 cells/100 μl) were subcutaneously implanted into the backs of BALB/c nude mice; SK-Hep-1/vec, SK-Hep-1/GPC3, or HepG2 cells (5×10^6 cells/100 μl) were implanted into NOD/SCID mice, and RMA cells (5×10^4 or 5×10^5 cells/100 μl) were implanted into C57BL/6 mice. Tumor volume was monitored twice a week and calculated using the following formula: tumor volume (mm^3) $= a \times b^2 \times 0.5$, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. Mortality and morbidity were checked daily, and the mice were maintained until each mouse showed signs of morbidity or the length or width of the tumors exceeded 30 mm, at which point they were killed for reasons of animal welfare.

Tetramer staining and flow cytometry analysis

For the analysis of local accumulation of antigen-specific CTLs, isolated tumor cells, including tumor-infiltrating lymphocytes, were stained with H-2 K^b OVA Tetramer-PE ($OVA_{257–264}$ [SIINFEKL]; MBL, Nagoya, Japan) for 20 min at room temperature and anti-mouse CD8-FITC (rat monoclonal, clone KT15; MBL) for 20 min at 4 °C. Flow cytometry analysis was carried out using a FACSCanto II flow cytometer (BD Biosciences).

Immunohistochemistry

To investigate whether $CD8^+$ T-cells infiltrated normal tissues due to intratumoral peptide injection in a murine adoptive cell transfer model, we performed immunohistochemical staining of CD8 in tissue specimens from C57BL/6 mice using monoclonal anti-CD8 antibody (dilution 1:20, BioLegend, San Diego, CA, USA).

Statistical analysis

Comparisons of spot numbers and tumor volume at the last time point were performed using the Mann–Whitney U test. Survival was analyzed according to the Kaplan–Meier estimate, and differences between groups were compared using the log-rank test. Differences were considered

significant at $P < 0.05$. Data were analyzed with the statistical package, Dr. SPSS II (SPSS Japan, Tokyo, Japan).

Results

In vitro CTL activity against peptide-pulsed targets

To evaluate the antigen-specific CTL response in vitro, IFN-γ ELISPOT and cytotoxicity assays were performed. In both assays, the two types of effector cells were the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTL clone, which was established from peripheral blood mononuclear cells (PBMCs) of an HCC patient who had received the $GPC3_{144–152}$ peptide vaccine [16], and the HLA-A*02:01-restricted $CMV_{495–503}$ peptide-specific CTL clone, which was established from PBMCs of a healthy volunteer. The target cells were tumor cell lines with or without antigenic peptide pulses.

As shown in Fig. 1a, in an IFN-γ ELISPOT assay, the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs produced IFN-γ in the presence of GPC3-expressing tumor cells, HepG2 and SK-Hep-1/GPC3, without peptide pulse. These effector cells recognized $GPC3_{144–152}$ antigen peptide, which is endogenously presented on the cell surface of the non-peptide-pulsed target cells. The number of IFN-γ-producing cells increased dramatically after the pulse of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide. In contrast, $GPC3_{144–152}$ peptide-specific CTLs did not produce IFN-γ against GPC3-negative tumor cells, SW620 and SK-Hep-1/vec, without peptide pulse. However, a marked increase in IFN-γ-producing cells was detected against these cell lines after the pulse of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide. The IFN-γ-producing cells did not increase after the pulse of HLA-A*24:02-restricted $GPC3_{298–306}$ or HLA-A*02:01-restricted $HIV_{77–85}$ peptide (Fig. 1a). Similarly, HLA-A*02:01-restricted $CMV_{495–503}$ peptide-specific CTLs produced IFN-γ only in the presence of HLA-A*02:01-restricted $CMV_{495–503}$ peptide-pulsed target cells (Fig. 1b).

In a cytotoxicity assay, HLA-A*02:01-restricted $GPC3_{144–152}$ and $CMV_{495–503}$ peptide-specific CTLs showed antigen-specific killing activity according to the peptide density on tumor cells. HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs showed specific cytotoxicity against HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-pulsed SW620 and T2 targets, whereas they did not show cytotoxicity against HLA-A*02:01-restricted $HIV_{77–85}$ peptide-pulsed targets (Fig. 1c). In addition, HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs showed apparent but weak cytotoxicity (13–44 %) against non-peptide-pulsed HepG2 and SK-Hep-1/GPC3 cells, but the cytotoxicity was markedly strengthened (55–99 %) against all examined cell lines after the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide pulse (Fig. 1d). Similarly, HLA-A*02:01-restricted $CMV_{495–503}$ peptide-specific CTLs showed $CMV_{495–503}$ peptide-specific cytotoxicity against all examined cell lines pulsed with $CMV_{495–503}$ peptide (Fig. 1e).

The peptide-specific CTLs showed strong activity against all peptide-pulsed cell lines, regardless of whether the tumor cells expressed the antigen. The density of the HLA-A*02:01-restricted $GPC3_{144–152}$ peptide endogenously presented on tumor cells was not enough to induce strong CTL activity.

Loading of injected peptide onto HLA class I molecules of tumor cells in vivo

As shown in Fig. 2a, BALB/c nude mice were inoculated subcutaneously on their backs with SW620 ($GPC3^-$) tumor cells. When tumor diameters reached 5–7 mm, 50 μg HLA-A*02:01-restricted $GPC3_{144–152}$ peptide was injected into the tumor. After 2–96 h, the tumors were dissected, cut into small pieces, and digested with collagenase (1.5 mg/ml) for 20 min at 37 °C. To investigate whether the injected HLA-A*02:01-restricted $GPC3_{144–152}$ peptide was loaded onto HLA class I molecules of tumor cells in a solid mass, an IFN-γ ELISPOT assay was performed in duplicate using these isolated tumor cells as target cells and HLA-A*02:01-restricted $GPC3_{144–152}$ peptide-specific CTLs as effector cells.

Loading of HLA-A*02:01-restricted $GPC3_{144–152}$ peptide onto HLA class I of tumor cells was detected (Fig. 2b). Without IFA, the density of loaded peptide gradually decreased after intratumoral peptide injection, whereas the loaded peptide density remained for 96 h after injection with IFA, suggesting that IFA is required for long-term stability of the injected peptide (Fig. 2c). Similar data were obtained with a combination of the HLA-A*02:01-restricted $CMV_{495–503}$ peptide and its specific CTLs (data not shown).

Antitumor effect of intratumoral peptide injection in an immunodeficient mouse model

We planned and executed the experimental schedule shown in Fig. 3a. Four tumors were implanted per mouse, and each tumor received a different combination of injections, as shown in Fig. 3b. From 5–7 days after tumor inoculation, mice were treated two or three times in 5-day intervals. The treatment regime was as follows: HLA-A*02:01-restricted $GPC3_{144–152}$ or $CMV_{495–503}$ peptide emulsified with IFA (50 μg/100 μl) was injected into a tumor, and, 2 h later, HLA-A*02:01-restricted $GPC3_{144–152}$ or $CMV_{495–503}$ peptide-specific human CTLs (1×10^7 cells/100 μl) were injected into the tumor.