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Phase I clinical trial of a five-peptide cancer vaccine combined with cyclophosphamide in advanced solid tumors

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ABSTRACT

We designed a phase I trial to investigate the safety, immune responses and clinical benefits of a five-peptide cancer vaccine in combination with chemotherapy. Study subjects were patients positive for HLA-A2402 with locally advanced, metastatic, and/or recurrent gastrointestinal, lung or cervical cancer. Eighteen patients including nine cases of colorectal cancer were treated with escalating doses of cyclophosphamide 4 days before vaccination. Five HLA-A2402-restricted, tumor-associated antigen (TAA) epitope peptides from KOC1, TTK, URLC10, DEPDC1 and MPHOSPH1 were injected weekly for 4 weeks. Treatment was well tolerated without any adverse events above grade 3. Analysis of peripheral blood lymphocytes showed that the number of regulatory T cells dropped from baseline after administration of cyclophosphamide and confirmed that TAA-specific T cell responses were associated significantly with longer overall survival. This phase I clinical trial demonstrated safety and promising immune responses that correlated with vaccine-induced T-cell responses. Therefore, this approach warrants further clinical studies.

1. Introduction

Although many studies have demonstrated the effectiveness of cancer vaccines, no vaccine has shown survival benefits in randomized phase III clinical trials [1,2]. Cancer vaccines alone appear to be unable to outperform conventional therapies; however, a combination of agents aimed at controlling immune tolerance to cancer vaccines might improve outcomes.

Recently, progress has been made in the development of immunological therapies aimed at inhibiting immune tolerance. For example, anti-PD-1 antibody alone improves clinical outcome in malignant melanoma and non-small lung cell cancer [3,4]. Although immune cell therapies, which represent one class of approach to targeting tumor-associated antigens (TAAs), have shown promise [5,6], the requirement of apheresis is burdensome for end-stage cancer patients. The greatest advantage of cancer vaccines is that they are safe and well tolerated in most cases, and injection-site reaction is the only major adverse event revealed by previous studies [2]. In addition, multiple-peptide vaccines appear more promising than single-peptide vaccines, because such

Abbreviations: CPM, cyclophosphamide; Treg cells, regulatory T cells; CTLs, cytotoxic T lymphocytes; TAA, tumor-associated antigen; PFS, progression free survival; OS, overall survival.

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vaccines are theoretically more likely to prevent escape by cancer cells with many genetic mutations [7,8,9]. The use of cancer vaccines in conjunction with agents intended to control immune tolerance in end-stage cancer patients still represents a reasonable approach, and should be carefully evaluated in clinical trials. However, confirmation of expression of TAAs in target lesions to justify the immunological efficacy of cancer vaccine therapy is not easy in end-stage cancer patients. Moreover, the safety and cost-effectiveness of using a combination of agents to control immune tolerance such as anti-PD-1 antibody have not yet been resolved in practical medicine.

In light of the situation described above, we carefully selected five peptides derived from TAAs, KOC1, DEPDC1, MPHOSPH1, TTK and URLC10, which are highly expressed in solid tumors (esophageal, gastric, colon cancer, cholangiocellular carcinoma, pancreatic cancer, small cell and non-small cell lung cancer, and cervical cancer), and designed a phase I clinical trial using this multiple-peptide cancer vaccine in combination with cyclophosphamide (CPM), without prior pathological confirmation of TAA expression. CPM selectively depletes CD4 + CD25 + regulatory T (Treg) cells and restores T and NK effector function in patients with end-stage cancer [10]. Additionally, in a randomized trial of patients with metastatic renal cell carcinoma, pretreatment with CPM before multiple-peptide cancer vaccination conferred a survival benefit, associated with the immune response, in comparison to patients who did not receive CPM [11]. Because CPM has a long history of use in patients with various cancers, and is available at a much lower price than antibody preparations, CPM is worthy of further study as an agent for controlling immune tolerance. The expression of TAAs in the primary lesion was retrospectively examined as much as possible to pathologically confirm the actual expression of these target antigens in the vaccinated patients. These findings make it possible to discuss the relationships between antigen expression and the induction of immune responses by vaccination. Finally, based on one case of a long-term survivor who received radiation therapy before and after this trial, we discuss the possible usefulness of radiation therapy as a modality that could be combined with cancer vaccines [12].

2. Materials and methods

2.1. Study design

This was a phase I, open-label study of CPM. Eighteen patients were treated in cohorts of six with escalating CPM doses (150, 300 and 600 mg/m^2). CPM was administered over 2 h as an intravenous infusion once per course. Five peptides derived from TAAs of KOC1, DEPDC1, MPHOSPH1, TTK and URLC10, which are highly expressed in esophageal, gastric, or colon cancers, cholangiocellular carcinoma, pancreatic cancer, small cell and non-small cell lung cancer, and cervical cancer, as described below in detail, were administered subcutaneously as vaccines, once per week for 4 weeks (Suppl. Fig. 1). Our study was primarily aimed at determining the feasibility and safety of these vaccinations, and secondarily at determining whether these vaccines could induce antitumor immune responses without prior confirmation of the expression of these TAAs in patient tumor specimens, because patients' tumors were considered to express at least one of these TAAs (Suppl. Table 1).

Additionally, patients without gastrointestinal bleeding, pleural effusion, and ascites received 350,000 IU of Proleukin (IL-2; Chiron, Amsterdam, The Netherlands) subcutaneously for 3 days after each vaccination. After the first course, all patients were observed closely for 1 week. If patients agreed to continue and they were able to tolerate vaccination, a new course was delivered, followed by 3 weeks of observation.

Toxicity and clinical outcomes were evaluated for all patients who received more than four vaccinations. Blood samples for immune response tests were obtained every week during each course and 4 weeks after the final injection. Computed tomography (CT) assessed clinical responses before and after vaccination. Every measurable lesion was evaluated by the Response Evaluation Criteria in Solid Tumor (RECIST) criteria.

2.2. Patient eligibility

The disease inclusion criterion was locally advanced, metastatic, and/or recurrent esophageal, gastric or colon cancer, cholangiocellular carcinoma, pancreatic cancer, small cell or non-small cell lung cancer, or cervical cancer with measurable disease. Other inclusion criteria was as follows: age, 20–80 years; *HLA-A*2402* positivity, as determined by DNA typing of HLA-A genetic variations using a WAKFlowHLAtyping Kit on a Luminex Multi-Analyte Profiling system (Wakunaga, Hiroshima, Japan), as described elsewhere [13]; Eastern Cooperative Oncology Group performance status of 0–1; no active brain metastases; life expectancy ≥ 3 months; and adequate hematological (2000/μL < WBC count < 15,000/μL; platelet count ≥ 75,000/μL), renal (serum creatinine < 2.0 mg/dL), and hepatic (AST, ALT < 3X ~ ULN value) function. Patients must have recovered from toxic effects of any previous therapy at least 4 weeks before entering the trial, and also had to be negative for syphilis sero-diagnosis, hepatitis B antigen, and antibodies against hepatitis C, HIV, and HTLV-1. Exclusion criteria are described elsewhere [14]. The study was approved by the Institutional Ethical Review Board of Kyushu University (#19-40) and is registered with ClinicalTrials.gov (NCT00676949). Written informed consent was obtained from all patients.

2.3. Dose limiting toxicity (DLT) and maximum tolerated dose (MTD)

The DLT of CPM administered with peptide vaccines was determined during the first course and defined by the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 as grade 4 (leukopenia and neutropenia), grade 3–4 (thrombocytopenia), or grade 3–4 (non-hematological). At least six patients were enrolled at each dose level. If DLT was observed after the first course, three additional patients were enrolled at the same dose. If no patients experienced DLT, the dose was escalated. Dose was never escalated for individual patients. MTD was the dose that produced DLT in two of six patients or all three initial patients.

2.4. Peptides and vaccination

Patients positive for HLA-A2402 were vaccinated with five peptides derived from KOC1 (KTVNELQNL), DEPDC1 (EYYELFVNI), MPHOSPH1 (IYNEYIYDL), TTK (SYRNEIAYL) and URLC10 (RYCNLEGPPI), all of which bind the HLA-A24 molecule. Profiles of the five TAAs targeted in this trial are shown in Table I. These novel TAAs were identified from 32,000 human genes using cDNA microarray analysis coupled with laser microdissection [14,15,16]. All of the proteins from which the TAAs were derived are involved in transcription and cell proliferation. These TAAs were expressed at high levels in lung, cervical, and cholangiocellular carcinoma (CCC), and moderately in esophageal, gastric, colon, and pancreatic cancer, as determined by microarray analyses performed by ourselves or reported elsewhere [14,17,18]. According to these results, with lower 95% confidence bound of probabilities, at least one of the five TAAs was considered to be expressed in NSCLC (prob ≥ 0.9929), SCLC (prob ≥ 0.9735), esophageal cancer (prob ≥ 0.9845), stomach cancer (prob ≥ 0.8290), colon cancer (prob ≥ 0.6433), cervical cancer (prob ≥ 0.9960), cholangiocellular carcinoma (prob ≥ 0.9875), and pancreatic cancer (prob ≥ 0.8035) (Suppl. Table 1). The establishment of CTL clones with specific cytotoxic activities against target tumor cells positive for HLA-A24 and expressing these five peptides and were able to induce TAA-specific T cell responses in cancer patients, as reported previously [19,20,21]. The purity (>97%) and identity of the peptides were determined by analytical high-performance liquid chromatography and mass spectrometry, respectively. Endotoxin levels and bioburden of the peptides were

tested and determined to be within acceptable levels for Good Manufacturing Practice grade for vaccines (NeoMPS, San Diego, CA, USA). Peptides were dissolved in dimethylsulfoxide at a concentration of 20 mg/mL, and aliquots of 2 mg of each peptide were stored at −80 °C. Just before use, the stock solutions were diluted with 2 mL sterile saline, mixed with an equal volume (2 mL) of incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France), and emulsified in a 5-mL sterilized syringe. Finally, 1 mg (in 1 mL) of each peptide emulsion (five peptides per vaccination) was injected subcutaneously into the femoral or axillary area. These peptides were confirmed to induce activity restricted to HLA-A24 and tumor-specific CTLs in peripheral blood mononuclear cells (PBMCs) [19,20,22].

2.5. Enzyme-linked immunospot (ELISPOT) assay

TAA-specific T-cell response was estimated by ELISPOT following sensitization in vitro, as described previously [20,21,23] with some modification. Frozen PBMCs from patients were thawed together, and viability was confirmed at more than 90%. PBMCs (5×10^5/mL) were cultured for 2 weeks at 37 °C with 10 mg/mL of each peptide and 100 IU/mL of IL-2 (Novartis, Emeryville, CA, USA). Peptide was added to the culture at days 0 and 7. After $CD4^+$ cells were depleted using a Dynal CD4-positive isolation kit (Invitrogen, Carlsbad, CA, USA), an IFN-γ ELISPOT assay was performed using the Human MabTech PLUS kit (Nacka Strand, Sweden). Briefly, human TISI cells from the B-lymphoblastoid cell line, which are positive for HLA-A*2402 (IHWG Cell and Gene Bank, Seattle, WA, USA), were incubated overnight with 20 mg/mL peptides (DEPDC1, KOC1, MPHOSPH1, TTK, and URLC10), and then residual peptide was washed out to prepare peptide-pulsed TISI cells as stimulator cells. Prepared $CD4^-$ cells were cultured in 96-well plates (Millipore, Bedford, MA, USA) at 37 °C overnight with peptide-pulsed TISI cells (2×10^4 cells/well) at responder:stimulator ratios of 1:1, 1:2, 1:4, and 1:8 . Non-peptide-pulsed TISI cells were used as negative stimulator controls. To confirm productivity of IFN-γ responder cells, as a positive control, cells were stimulated with PMA (phorbol myristate acetate, 66 ng/mL) and ionomycin (3 mg/mL) overnight, and then subjected to the IFN-γ ELISPOT assay (2.5×10^3 cells/well) in the absence stimulator cells. All ELISPOT assays were performed in triplicate wells. The plates were analyzed on an automated ELISPOT reader, ImmunoSPOT S4, using the ImmunoSPOT Professional Software Version 5.0 (Cellular Technology Ltd, Shaker Heights, OH). The number of spots specific for peptide was calculated by subtracting the number in control wells from the number in wells containing peptide-pulsed TISI cells. The sensitivity of our ELISPOT assay was consistent with the average for ELISPOT panels of the Cancer Immunotherapy Consortium [CIC, (http://www.cancerresearch.org/consortium/assay-panels/)]. Positivity of antigen-specific T-cell response was defined quantitatively according to our original evaluation tree algorithm, as reported previously ([21], Suppl. Fig. 2).

2.6. Flow cytometry

We performed flow cytometry on thawed PBMCs in acid–citrate–dextrose isolated by Ficoll gradient. We multiplied lymphocyte frequencies by the absolute number of lymphocytes from a complete blood count obtained on the same day. To phenotype CD8, we directly conjugated monoclonal antibodies specific for PE-Cy7-conjugated CD3/SK-7, PerCP-conjugated CD8/SK1, APC-conjugated Mouse Anti-Human CD45RA/HI100, and FITC-conjugated Mouse Anti-Human CD62L/DREG-56 (Becton Dickinson, San Diego, CA, USA). For Treg analysis, we used a Human Regulatory T-cell Staining Kit (eBioscience, San Diego, CA, USA) including PE-Foxp3 PCH1010, FITC-CD4, and APC-CD25. Intracellular staining was performed according to the supplier's protocol. A total of 100,000 live events were analyzed on a FACSCalibur instrument using the ProCellQuest software (BD Biosciences, San Jose, CA, USA).

Expression of T-cell receptors specific for peptide in Case 17, who exhibited a strong signal in the ELISPOT assay, was analyzed on a FACSCanto II (Becton Dickinson, San Jose, CA, USA) using epitope peptide-MHC tetramer-PE derived from URLC10 (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). HIV-derived epitope peptide (RYLRDQQLL)-MHC tetramer-PE was used as the negative control. Briefly, cells were incubated with peptide-MHC tetramer-PE for 10 min at room temperature, and then treated with FITC-conjugated anti-human CD8 mAb, APC-conjugated anti-human CD3 mAb, PE-Cy7-conjugated anti-human CD4 mAb, and 7-AAD (BD Pharmingen, San Diego, CA, USA) at 4 °C for 20 min.

2.7. Immunohistochemical analysis

To confirm the expression of TAAs in the patients' original tumors, formalin-fixed tumor slides were stained by immunohistochemistry (IHC) and evaluated by light microscopy. IHC staining of DEPDC1, URLC10, KOC1, MPHOSPH1 and TTK antigens of tumors and normal tissues were investigated using 3 μm thick sections of formalin-fixed, paraffin-embedded tissue block or biopsy samples. The primary antibodies used in this study were as follows; 16E9 mouse monoclonal anti-human DEPDC1 antibody (final concentration 1.0 μg/mL), 3B53G11 mouse monoclonal anti-human URLC10 antibody (final concentration 0.01 μg/mL), 1F12E4 mouse monoclonal anti-human KOC1 antibody (final concentration 1.0 μg/mL) and 4-9A-5H rat monoclonal anti-human MPHOSPH1 antibody (final concentration 5.0 μg/mL) were provided by OncoTherapy Science, Inc., Kanagawa, Japan which also provided the five TAA peptides vaccine. A rabbit polyclonal anti-human TTK antibody (NBP1-85392; Novus Biologicals, Littleton, CO, USA) was purchased and used as dilution 1:200. The isotype control antibodies used in this study were as follows; Rabbit Immunoglobulin Fraction (Normal) (DAKO X0903) was purchased and used as dilution 1:4000 (final concentration 0.5 μg/mL). Rat Serum (Normal) (DAKO X0912) was purchased and used as dilution 1:2000 (final concentration 35.7 μg/mL). Mouse IgG1 100 mg/L (DAKO X0931), Mouse IgG2a 200 mg/L (DAKO X0943) and Mouse IgG2b 100 mg/L (DAKO X0944) were purchased and used as final concentration 1 μg/mL). Immunochemical reaction was detected using the secondary antibody system as follows; EnVision™ Detection System/HRP, Rabbit/Mouse (DAB+), RUO (K5007; Dako Inc., Glostrup, Denmark) for 16E9, 3B53G11, 1F12E4, and antihuman TTK antibody and N-Histofine® Simple Stain Mouse MAX-PO (Rat) (Nichirei Bioscience, Tokyo, Japan) for 4-9A-5H according to the manufacturer's instructions. Sections were treated with 3,3′-diaminobenzidine (DAB) tetrahydrochloride as chromogenic development and counterstained with hematoxylin. The IHC signal was scored using the semi-quantitative “Allred score system” [24] as described below. The average estimated intensity of staining-positive cells was evaluated by an intensity score (IS): 0, none; 1, weak; 2, intermediate; 3, strong. The proportion of staining-positive tumor cells was evaluated by a proportion score (PS): 0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to <1/3; 4, 1/3 to 2/3; 5, over 2/3. Total score (TS): sum of IS and PS, yielding a range from 0 to 8. We defined ‘negative’ as TS 0, 1 and 2; ‘positive low’ as TS 3 up to the median (highlighted in light orange); and ‘positive high’ as TS greater than or equal to the median (highlighted in dense orange) (Suppl. Table 2). Each IHC-stained slide was scored by the same pathologist (S.O.) who was not given any information about the patients' clinical profiles, including immunological and clinical outcomes.

2.8. Assessment and statistical considerations

The primary endpoints were safety and tolerability. Secondary endpoints were MTD and immune response. We used the Kaplan–Meier method to estimate the distribution of progression-free (PFS) and overall survival (OS) rates. PFS was calculated from study entry to the date of radiographic disease progression using the RECIST criteria. OS

was expressed in days from study entry to last follow-up or death from any cause. The cutoff date for this analysis was September 18, 2011. Intent-to-treat statistics were analyzed using JMP ver 8.01. All of the data were analyzed by a statistician (J.K.)

3. Results

3.1. Profile of patients and toxicities

Patient baseline characteristics are shown in Table 1 and Table 2. The study included nine cases of colorectal cancer, three cases of CCC, three lung cancers (two non-small cell, one small cell), and one each of esophageal, gastric, and cervical cancer. Vaccinations were tolerated well, with no adverse events above grade 3. Ten of 18 patients experienced grade 1 (nine patients) or grade 2 adverse events (AE) at the vaccination site. Case 11 experienced a grade 2 AE of painful edematous and erythematous change at the injection site after the second course of vaccination. Case 12 had AEs of grade 1 (leukopenia) 4 days after CPM administration and grade 2 (lower gastrointestinal bleeding) after the third course of vaccination (Table 3).

3.2. Clinical responses

Clinical and immune responses by CPM dose are summarized in Table 4. Seven patients received IL-2. PFS and OS in all patients were 3.5 and 6.5 months, respectively. Although survival was not dependent on CPM dose, the 600 mg/m^2 group exhibited significantly longer survival than other groups (OS: 9.2 months, log-rank test $P = 0.032$, Suppl. Fig. 3). OS for groups with 3 or more cases of the same primary disease were 9.4 and 5.9 months for CRC and CCC, respectively. Stable disease (SD, PFS >2 months) was observed in 11 patients. Among those patients, two exhibited significant tumor reduction. In the first case, Patient 8, a 61-year-old male, had a history of rectal cancer followed by local recurrence involving iliac bone metastasis 3 years after surgery. MRI findings of proximal femurs and pelvis revealed reduced size of the left femur (*a*: the upper figures) and a left inguinal lymph node metastasis (*b*: the lower figures) 60 days after the first course of vaccination (Fig. 1a). At the first time, this patient had been suffering from left leg edema and pain, resulting in gait disturbance. After the first vaccination, his leg edema disappeared and he became able to walk by himself, leading to an improved quality of life. He received three courses of vaccination and remained in SD until the third vaccination. In the second case, Patient 17, a 40-year-old, male, had a history of inferior thoracic esophageal cancer followed by a relapse at the descending thoracic aorta. He received proton beam therapy (PBT) (55 Gy/30 fractions) at the relapsed legion followed by the remarkable shrink, but celiac lymph nodes metastases newly appeared (Fig.1b, -1 month). He subsequently received the first course of vaccination. Two months later, the multiply metastasized celiac lymph nodes progressed (Fig. 1b, 2-months), and he then received PBT (59.4 Gy/27 fractions) for celiac lymph nodes metastases followed by the second course of vaccination. (Fig. 1b, 8, 11 months). Since then, he has received 8 courses of vaccination every 6 months, total of 38 vaccinations, and finally achieved complete response (CR). His CR has persisted for 5 years as of the preparation of this manuscript (Fig. 1b, 50 months).

3.3. TAA-specific CD8 T-cell responses and survival

As summarized in Table 4, nine of 13 patients with sufficient PBMCs for ELISPOT assay showed responses specific to TAA for at least one of five antigens after vaccination (69%, 95% Confidence interval: 42.3–87.3). TAA-specific T-cell responses were seen in 67% of patients positive for TTK, 44% for KOC1, 22% for DEPDC1, 22% for MPHOPH1 and 44% for URLC10 (details of immune responses determined by ELISPOT in Suppl. Table 3). Fig. 1b and c shows data from ELISPOT and multimer assays against URLC10 in Case 17. ELISPOT assays revealed that T-cell responses specific to URLC10 in PBMCs 2 months after the first vaccination, whereas no specific T-cell response was observed PBMCs prior to vaccination, indicating that antigen-specific responses were induced by vaccination (Fig. 2a). In addition, multimer analysis revealed that the proportion of URLC10 peptide-specific CD8+ T cells increased at least a thousandfold (pre: 0.03%, post: 43.1%) after

Table 1
Patient baseline characteristics.

Characteristic		Level 1 150 mg/m2 (n = 6)		Level 2 300 mg/m2 (n = 6)		Level 3 600 mg/m2 (n = 6)		P
		No.	%	No.	%	No.	%	
Age, years								0.230 N/S
	Median	61		49		57		
	Range	54–71		40–60		31–80		
Sex								0.507 N/S
	Male	3		4		2		
	Female	3		2		4		
ECOG performance status								1.000 N/S
	0	2	33.3	2	33.3	2	33.3	
	1	4	66.7	4	66.7	4	66.7	
	2	0	0.0	0	0.0	0	0.0	
	3	0	0.0	0	0.0	0	0.0	
	4	0	0.0	0	0.0	0	0.0	
Primary disease								
	Colorectal cancer	4	66.7	2	33.3	3	50.0	
	Lung cancer	0	0.0	3	50.0	0	0.0	
	Cholangiocell carcinoma	2	33.3	0	0.0	1	16.7	
	Gastric cancer	0	0.0	1	16.7	0	0.0	
	Esophageal cancer	0	0.0	0	0.0	1	16.7	
	Cervical cancer	0	0.0	0	0.0	1	16.7	
Target lesions								
	Liver meta	2	20.0	4	36.4	2	22.2	
	Lung tumor or meta	3	30.0	3	27.3	1	11.1	
	Lymph nodes meta	0	0.0	1	9.1	2	22.2	
	Bone meta	1	10.0	2	18.2	0	0.0	
	Adrenal gland meta	0	0.0	1	9.1	1	11.1	
	Ovary meta	1	10.0	0	0.0	1	11.1	
	Skin meta	1	10.0	0	0.0	0	0.0	
	Pelvic tumor (local rec)	2	20.0	0	0.0	2	22.2	

Table 2
Individual patient characteristics.

CPM	Patient	Gender	Age	Disease	PS	Prior therapy		Stage	Metastatic sites
						Surgery	Chemotherapy, irradiation		
150 mg/m^2	1	M	59	CCC	1	+	GEM	rec.	Liver, pancreas, celiac LNs
	2	F	54	CRC	1	+	FOLFOX4 (5FU/LV/L-OHP), FOLFIRI (5FU/LV/CPT-11)	rec.	Ovary, peritoneum, sigmoid colon
	3	F	58	CRC	1	+	5FU/LV, S-1, RT	rec.	Celiac LNs, sacral vertebral bone
	4	F	52	CCC	1	+	5FU/CDDP, GEM/CDDP, PTX	rec.	Lung
	5	M	60	CRC	1	+	S-1, FOLFOX4, mFOLFOX6, FOLFIRI/BV, RT	rec.	Celiac LNs, cervical vertebral bone
	6	M	51	CRC	1	+	FOLFOX4, FOLFIRI, mFOLFOX6/BV, Cetuximab/CPT-11, RT	rec.	Celiac LNs, skin, lung
300 mg/m^2	7	M	70	NSCLC	1	−	CBDCA/PTX, DTX, RT	rec.	Lung, liver, sacral vertebral bone, rib
	8	M	61	CRC	1	+	5FU, UFT/LV, mFOLFOX6 (5FU/LV/L-OHP), FOLFIRI, RT	rec.	Iliac bone, lung
	9	M	65	SCLC	1	+	CDDP/CPT-11, CDDP/VP16, CBDCA/VP16, DXR/VDS/ACNU, AMR, RT	rec.	Pleura, hilar LNs, liver, adrenal grand
	10	F	80	NSCLC	1	-	CBDCA/PTX, S-1	rec.	Spinal bones, humerus bone, rib, hilar LNs
	11	F	64	CRC	0	+	UFT, FOLFOX4, FOLFIRI/BV	rec.	Peritoneum, ovary
	12	M	64	GC	1	+	S-1, CDDP, PTX, CPT-11	rec.	Celiac LNs, peritoneum
600 mg/m^2	13	F	49	CRC	1	+	mFOLFOX6, FOLFIRI, RT	IV	Liver, sacral vertebral bone
	14	M	44	CCC	0	+	GEM, S-1	rec.	Liver
	15	F	40	CC	1	+	CDDP/5FU, CBDCA/PTX, CPT-11/CDDP, RT	rec.	Pelvis, piriformis muscle
	16	F	62	CRC	1	+	S-1, FOLFOX/BV, CPT-11/Capecitabine	rec.	Lung, pleura
	17	M	40	EC	0	+	5FU, CDDP, RT, Proton	rec.	Celiac LNs
	18	M	31	CRC	1	+	UFT, FOLFOX4, FOLFIRI/BV, MTX/5FU, RT	rec.	Celiac LNs

Abbreviation: CPM, cyclophosphamide; PS, performance status; M, male; F, female; rec., recurrent status; LNs, lymph nodes; CCC, cholangiocellular carcinoma; CRC, colorectal cancer; NSCLC, non-small cell lung carcinoma; SCLC, small cell lung carcinoma; GC, gastric cancer; EC, esophageal cancer; CC, uterine cervical cancer
GEM, gemcitabine; 5FU, fluorouracil; LV, leucovorin; L-OHP, oxaliplatin; CPT-11, irinotecan; S-1, tegafur and gimeracil, oteracil potassium; RT, radiation therapy; CDDP, cisplatin; PTX, paclitaxel; BV, bevacizumab;
CBDCA, carboplatin; DTX, docetaxel; UFT, tegafur and uracil; VP-16, etoposide; DXR, doxorubicin; VDS, vindesine sulfate; ACNU, nimustine hydrochloride; AMR, amurubicin; MTX, methotrexate.

vaccination with this protocol (Fig. 2b). No significant correlation of immune responses with the primary diseases was observed (CRC: 4/6 cases [67%], LC: 1/2, CCC: 1/2, GC: 1/1, EC: 1/1 and CC: 1/1). A log-rank test between positive and negative ELISPOT findings revealed that TAA-specific T cell responses induced by vaccine were associated significantly with longer OS ($P = 0.0010$, Fig. 3).

3.4. Expression of DEPDC1, MPHOSPH1, URLC10, KOC1, and TTK

We next examined the expression of five antigens in primary lesions by IHC in 15 patients whose pathological samples were available retrospectively. Pathological analyses confirmed the expression of at least three antigens in all 15 patients: two patients were positive for three antigens; seven patients were positive for four, and six patients were positive for all five. Expression frequencies of each antigen were 100% (15/15) for TTK and MPHOSPH1, 93% (14/15) for KOC1 and URLC10, and 47% (7/15) for DEPDC1 (Table 5; details shown in Suppl. Table 2). Representative IHC data are shown in Fig. 4. The number of samples obtained for both ELISPOT and pathological findings were 40 (n = 11, Suppl. Table 3), of which 15 were ELISPOT-positive. Antigen expression was confirmed in 14 of 15 ELISPOT-positive samples, i.e., the frequency of antigen expression among ELISPOT-positive samples was 93% (14/15). On the other hand, IHC revealed TAA expression in 33 samples, of which 14 were ELISPOT-positive, i.e., the frequency of ELISPOT positivity among samples with TAA expression was 42% (14/33).

3.5. Reduction in the number of Treg cells and survival

Because both absolute numbers of Treg cells and the ratio of Treg cells to CD4 + T cells varied among patients, we evaluated the inhibitory effect of CPM on Treg cells as reduction relative to baseline. Differences in the degree of reduction on the number of Treg cells from baseline to post-CPM (just after CPM and before the first vaccination) are shown in Fig. 5a and Table 4. Reductions in the number of Treg cells after CPM administration depended on dose, and approached maximum reduction at a CPM dose of 300 mg/m^2. On the other hand, % CD4 did not decrease with increasing CPM dose (Fig. 5b). Using a Weibull parametric model, we found that a higher degree of reduction of the number of Treg cells correlated significantly with longer OS ($P = 0.023$, Fig. 5c), and it was also reproducible among the nine CRC cases in our study ($P = 0.030$). In addition, because the target cancers were not of the same type, we analyzed the effect of relative changes in the number of Treg cells on OS using a multivariate Weibull parametric model to adjust for differences among cancer types (likelihood ratio test of the effect), showing significant differences as follows: when the parameter was cancer, likelihood ratio chi-square was 14.3 and *P*-value was 0.0136; when the

Table 3
Overall therapy-associated toxicities.

Toxicity	Level 1 150 mg/m^2 (n = 6)			Level 2 300 mg/m^2 (n = 6)			Level 3 600 mg/m^2 (n = 6)			Total
	Grade			Grade			Grade			
	1	2	3	1	2	3	1	2	3	
Injection site reaction	4	0	0	1	0	0	4	1	0	12
Leukopenia	0	0	0	0	0	0	1	0	0	1
Lower GI hemorrhage	0	0	0	0	0	0	0	1	0	1

parameter was relative changes in the number of Treg cells, likelihood ratio chi-square was 8.9 and *P*-value was 0.0028.

3.6. IL-2 administration effects on Treg and CD4 T lymphocytes

IL-2 is involved in proliferation of Treg cells, so we examined its stimulatory effect by comparing the number of Treg and CD4 cells between the IL-2 (n = 7, CPM150 x3, CPM300 x2, CPM600 x2) and no IL-2 groups (n = 11, CPM150 x3, CPM300 x4, CPM600 x4). In contrast to CPM, IL-2 selectively increased Treg count, but not the overall number of CD4 cells (the mean of the number of Treg cells: IL-2-, 17.0/μl; IL-2 +, 25.4/μL; $P = 0.1657$, Suppl. Fig. 4). In addition, the frequencies of induced, TAA-specific T-cell responses were higher in the no IL-2 group (IL-2 +: 2/4 cases, IL-2-: 7/9).

3.7. Other immune parameters and survival

We used a Weibull parametric model to evaluate the effects of number and phenotypes of CD4 + and CD8 + T cells in order to determine whether other immune parameters, besides Treg count, influenced clinical outcome. Higher naïve CD8 count before vaccination was highly correlated with longer OS ($P < 0.0001$, Fig. 5d, Table 4). This observation was reproducible among the nine CRC cases in our study ($P = 0.001$). In addition, because the target cancers were not of the same type, we analyzed the effect of naïve CD8 count on OS using a multivariate Weibull parametric model to adjust for differences among cancer types (likelihood ratio test of the effect), showing significant differences as follows: when the parameter was % naïve CD8, likelihood ratio chi-square was 20.8 and *P*-value was <0.0001; when the parameter was disease, likelihood ratio chi-square was 8.8 and *P*-value was 0.1156. To assess CD8 memory differentiation, cells were categorized into three subpopulations (naïve, effector memory [EM], and central memory [CM]), with two parameters (CD62L and CD45RA) after gating CD8 + T cells [25,26]. The diverse antigenic repertoire of TAA-specific immune responses was associated with a higher frequency of naïve CD8 T cells, corresponding to survival (Fig. 5e). Although statistically insignificant, CPM at doses of 300 mg/m^2 and 600 mg/m^2 tended to increase the EM fraction after vaccinations. IL-2 had no effect on CD8 subpopulation, although EM fraction increased slightly after vaccination in the population that received IL-2 (Suppl. Table 4).

4. Discussion

In this phase I clinical study, we found that a five TAA-epitope peptide vaccine in combination with CPM was tolerated well and provided some clinical benefits to patients. During the preparation of this manuscript, Walter et al. reported a phase I and II clinical trial in which a combination of CPM, GM-CSF, and a 10-peptide cancer vaccine was tested against metastatic renal cell carcinoma [11]. They randomized their subjects to receive either 300 mg/m^2 of CPM before vaccination or no pretreatment, and demonstrated that immune responders pretreated with CPM survived longer. Thus, the design of their clinical trial was similar to that of ours, and several of their findings were consistent with our results. Differences between the two studies included the dose of CPM and the identity of the cytokine administered (IL-2 in our study, GM-CSF in theirs) to augment immune responses.

Although some clinical trials in the past decade investigated immunotherapies combined with CPM, they were not based on definite evidence of the optimum dose of CPM [27]. In those studies, patients received 200–300 mg/m^2 CPM on days 1–4 before treatment because an early study in mice found that both relative and absolute numbers of Treg cells was lowest 4 days after low-dose CPM treatment [28]. Our results suggested that even a single dose of CPM resulted in the reduction of the number of Treg cells, and demonstrated that this reduction corresponded to survival benefits. Our data also showed that the frequency of CD4 + T cells did not decrease after administration of CPM, and the frequency of CD4 + T cells after vaccination was correlated with PFS (also reproducible among nine CRC patients, $P = 0.034$, data not shown), suggesting that a higher proportion of CD4 cells after vaccination might be associated with clinical benefits. Longer survival among patients who received 600 mg/m^2 of CPM could not be explained by the cytotoxic effects of CPM because 1) survival was not correlated with CPM dose, but 2) survival was associated with several immune parameters including positive ELISPOT results. A previous report also showed that CPM caused no difference in survival among non-immune responders, suggesting that a single dose of CPM does not have

Table 4
Immune responses and clinical responses.

CPM	Patient	Disease	IL-2	RECIST	Survival (days)		Elispot		% relative Treg	% naïve CD8
					PFS	OS	TAA	PMA	(post CPM)	(pre Vx)
150 mg/m^2	1	CCC	+	SD	179	179	NA	NA	68.4	6.8
	2	CRC	−	PD	50	363	NA	NA	70.3	27.2
	3	CRC	+	SD	198	676	NA	NA	72.8	45.4
	4	CCC	−	SD	115	183	+++	+	73.7	31.2
	5	CRC	+	PD	50	107	−	+	211.3	4.7
	6	CRC	−	SD	102	114	−	+	262.3	21.6
	Median				109	181			126.5	22.8
300 mg/m^2	7	LC	−	PD	51	126	−	+	130.4	4.8
	8	CRC	−	OR	163	211	+	+	83.9	20.4
	9	LC	+	PD	29	120	++	+	61.1	7.3
	10	LC	+	PD	53	448	NA	NA	41.1	31.2
	11	CRC	−	SD	162	330	+	+	107.5	4.7
	12	GC	−	PD	31	135	++	+	67.6	21.6
	Median				52	173			81.9	18.6
600 mg/m^2	13	CRC	−	PD	31	112	NA	NA	60.6	11.8
	14	CCC	+	SD	84	173	−	+	132.7	12.9
	15	CC	−	SD	110	280	++	+	30.3	55.4
	16	CRC	−	SD	275	280	++	+	73.5	26.7
	17	EC	+	OR	730*	730*	++	+	51.4	56.0
	18	CRC	−	SD	165	402	+++	+	126.9	56.7
	Median				138	280			79.2	36.5

Abbreviations: CCC, cholangiocell carcinoma; CRC, colorectal cancer; GC, gastric cancer; CC, cervical cancer; EC, esophageal cancer; SD, stable disease; PD, progressive disease; OR, objective response; PFS, progression free survival; OS, overall survival; CPM, cyclophosphamide; TAA, tumor-associated antigen; PMA, phorbol myristate acetate (positive control).
+, indicates one peptide response; NA, not assessed; *, indicates sensor data.

antitumor activity by itself but instead supports the effects of the vaccine as an immunomodulator [11]. Taken together, these data indicate that a single dose 600 mg/m^2 of CPM could provide an optimum balance between reduction of the number of Treg cells and maintenance of CD4 + T cells.

In contrast to CD4 + CD25 + Foxp3 + Treg cells, less is known about CD8 + Treg cells in cancer. Nevertheless, increasing evidence indicates that CD8 + Treg cells also accumulate in the tumor microenvironment cells [29,30]. Although CD4 + CD25 + Treg cells are naturally generated in the thymus and can be detected in the periphery, CD8 + CD25 + Treg cells are not detectable in the PBMCs of cancer patients suggesting that CD8 + Treg cells are induced in the tumor microenvironment or in a cytokine milieu favoring Treg cell induction [31]. Since it is well documented that most CD8 + Treg cells are generated by antigen stimulation, cancer vaccine may induce CD8 + Treg cells [32,33]. CPM inhibits generation and function of CD8 + Treg cells as well as CD4 + CD25 + Treg cells [34]. Thus, pretreatment by CPM as designed in our study may prevent induction of CD8 + Treg cells due to vaccination. In addition, their suppressive function can be regulated by TLR8 ligands in prostate cancer [29]. Although these studies suggest possibilities to manipulate CD8 + Treg cells, further study to develop monitoring CD8 + Treg cells in tumor infiltrating lymphocytes (TILs) is needed.

Our results correspond with those of previous reports showing that low-dose IL-2 therapy preferentially expanded Treg under other clinical conditions [35,36], suggesting that cancer vaccine probably does not need low-dose IL-2. Although IL-2 has been expected to exert an anticancer effect by augmenting immune responses specific to TAA as at beginning of this trial, accumulating evidence subsequently suggested that IL-2 exerts negative effects on anticancer therapies by increasing Treg through the IL-2 receptor. Actually IL-2 may exacerbate gastrointestinal bleeding and effusion into body cavity as adverse events, administration of IL-2 in end-stage cancer patients should be careful. In particular, we had difficulties to administrate in the patients with growing gastrointestinal lesions. These clinical practical issues resulted in two groups, IL-2 administrated and not administrated. The design in this study is not appropriate to conclude, however, the results could not show positive effect on induction of vaccine-specific T cells because frequencies of induced vaccine-specific T-cell responses were higher in the no IL-2 group. On the other hand, the mean of the number of Treg cells was higher in IL-2 receiving group than no IL-2 group after vaccination and administration of IL-2(Suppl. Fig. 4). In addition, the number of Treg among the three groups with different CPM doses in IL-2 receiving group could not prove significant difference (data not shown).

In this study, CRC was the most frequent type of cancer (nine cases). Forty-eight studies of immunotherapy against advanced CRC conducted between 1998 and 2010, including one clinical trial of peptide vaccination, showed an overall response rate of 1.7% [37]. In this study, OS was 9.4 months in the nine CRC patients. This outcome would be promising even compared with 6.1 months by the treatment with cetuximab as the third line therapy for advanced CRC [38]. On the other hand, OS was 5.9 months in the three CCC patients, which was comparable to the treatment with S-1 as the second line therapy in gemcitabine-refractory biliary tract cancer [39]. Although the contribution of immune responses to survival is unknown due to the small number of patients, a significant association between reduction of Treg count before vaccination and survival was reproducible among nine CRC patients. Another phase I trial for advanced CRC patients who received

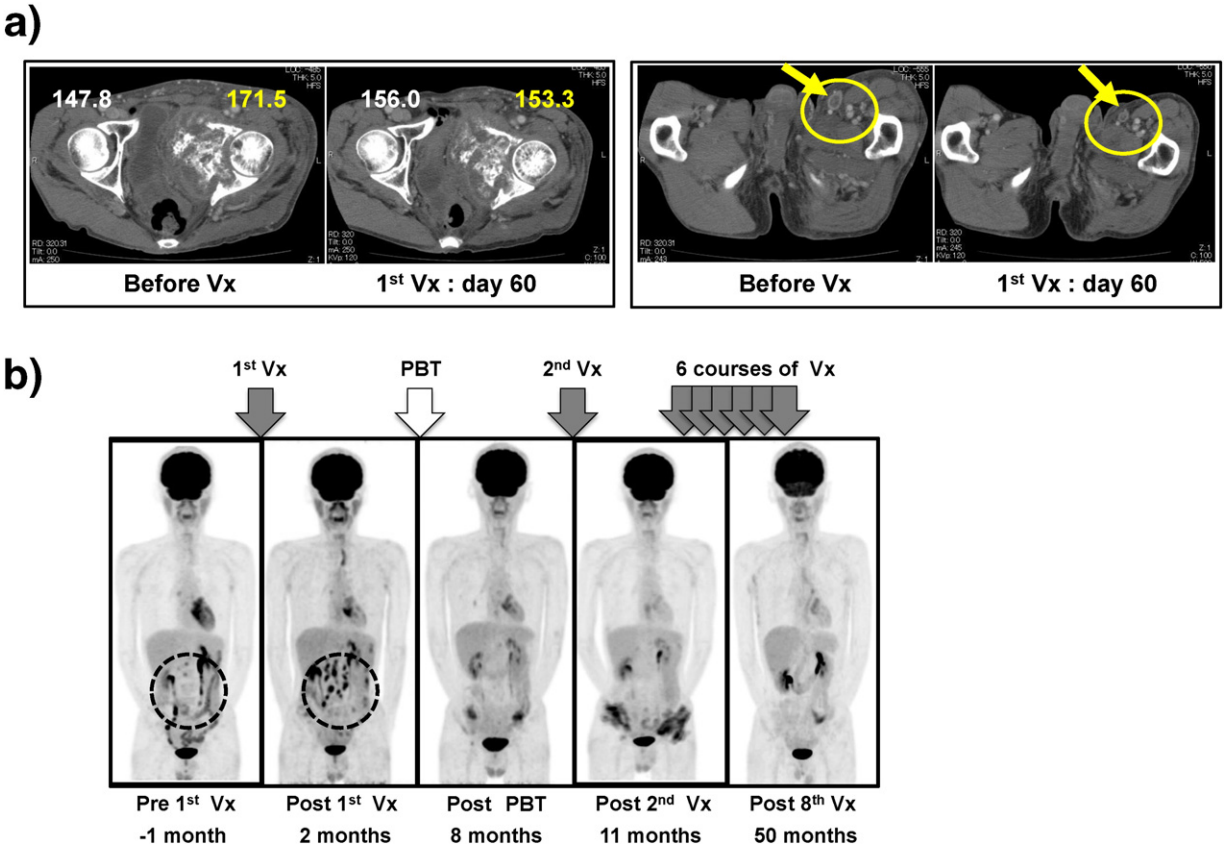

Fig. 1. Clinical responses in the patients. *a*) Clinical findings in Case 8. MRI findings of proximal femurs (left figures) and pelvis (right figures) before vaccine and 60 days after first course of vaccination. Each number in the left figures represents diameter of the femur (mm). Arrows and circles in the right figures indicate a left inguinal lymph node metastasis. Vx, vaccination. *b*) Clinical findings in Case 17. Time course of PET-CT image for Case 17. Dot circles show that multiple metastases of celiac lymph nodes occurred after postoperative adjuvant chemoradiation of the primary site and the PBT for metastatic site of descending thoracic aorta. Signals in pelvis after 8 months show inflammatory changes in both sides of the inguinal injection sites. Vx, vaccination; PBT, proton beam therapy; LNs, lymph nodes.

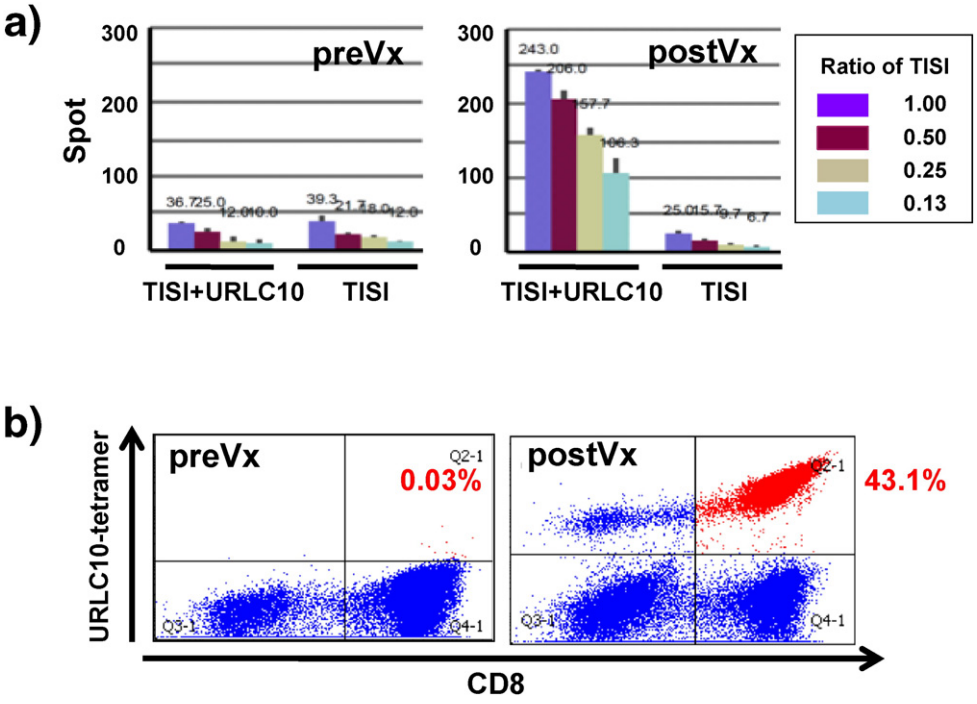

Fig. 2. TAA-specific CD8 T-cell responses in Case 17. *a*) Representative results of ELISPOT assays for Case 17. Results of pre-vaccination and post-vaccination are compared to demonstrate the significant vaccine-induced immune response against URLC10. TISI (lymphoblast cells positive for HLA-A*2402) + URLC indicates stimulators with an URLC peptide pulse. TISI indicates stimulators alone, without the URLC peptide pulse. Vx, vaccination. *b*) Representative results of multimer assays for Case 17. The numbers in the multimer analysis indicate the ratio (%) of URLC peptide-specific CD8 + T cells among all CD8 + T cells.

five peptides (RNF43, TOMM34, KOC1, VEGFR1, and VEGFR2) confirmed a survival benefit (OS 13.5 months) and induction of T cells with broader antigenic repertoires that were associated with survival [40]. Although those authors also used multiple-peptide vaccine with five TAAs, only KOC1 overlapped with our vaccine. The frequencies of ELISPOT positivity for KOC1 were 50% in our CRC patients and 68% in Hazama's study, and the rate of KOC1 expression was 86% in our CRC patients and 77% in Hazama's study, showing no significant dissociation.

Using vaccination with three peptides, Kono et al. found that more than one TAA was expressed in tumor tissues from all patients [14]. Corresponding to the data showing that the five TAAs used in our study were highly and specifically expressed in gastrointestinal, lung and cervical cancers, retrospective pathological analyses in primary lesions confirmed expression of all five TAAs (except for DEPDC1) in almost all patients in this study. These results suggest that absence of TAA expression might not explain the lack of TAA-specific T-cell responses. Rather, as Kono et al. proposed, loss of MHC class I expression is more likely to explain the absence of CTL induction; however, we did not monitor expression of this protein. As another possibility, the presence of immune tolerance such as Treg also might explain lack of TAA-specific T cell responses because immune responders pretreated with CPM survived longer [11].

Current studies of adoptive immune cell transfer showed that antigen-specific CD8 + T cells with high proliferative potential and fewer differentiation markers, including CD27 and CD28, should be induced preferentially in order to yield clinical benefit [5]. On the other hand, it is not known which immune profile favors antitumor peptide vaccination. A prior clinical study of immunotherapy against breast cancer using peptide-loaded DCs found that a lower frequency of naïve CD8 + T cells correlated with disease progression, suggesting that a predominantly naïve subset of CD8 + T cells helped to induce immune responses [41]. We analyzed the frequency of naïve CD8 + T cells in relation to both antitumor immune responses and survival of patients who received peptide vaccine immunotherapy. Although the association of frequencies of naïve CD8 + T cells to immune responses could not be determined due to the small sample size, we observed a

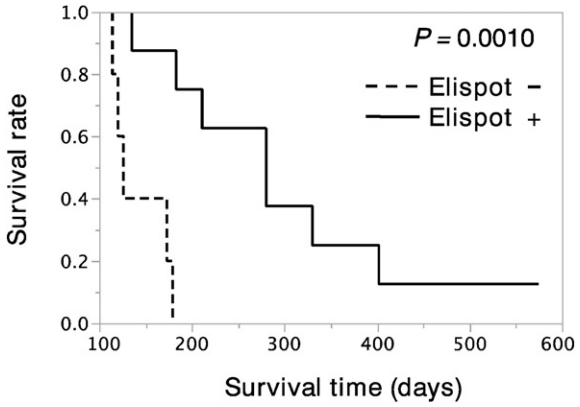

Fig. 3. Survival days between ELISPOT-positive (+) and negative (−) groups. Survival curves (overall survival: days) comparing ELISPOT-positive (+) (n=9, solid line) and negative (−) (n = 4, dotted line) groups.

Table 5
Expression of five tumor associated antigens (TAAs) in tumor samples from patients enrolled in this clinical trial.

	DEPDC1	MPHOSPH1	URLC10	KOC1	TTK
Colorectal cancer	2/7	7/7	7/7	5/7	5/7
Non-small cell lung carcinoma	1/2	2/2	2/2	2/2	2/2
Small cell lung carcinoma	0/1	1/1	0/1	1/1	1/1
Cholangiocellular carcinoma	0/2	2/2	1/2	2/2	2/2
Esophageal cancer	0/1	1/1	1/1	1/1	1/1
Gastric cancer	1/1	1/1	1/1	1/1	1/1
Uterine cervical cancer	1/1	1/1	1/1	1/1	1/1

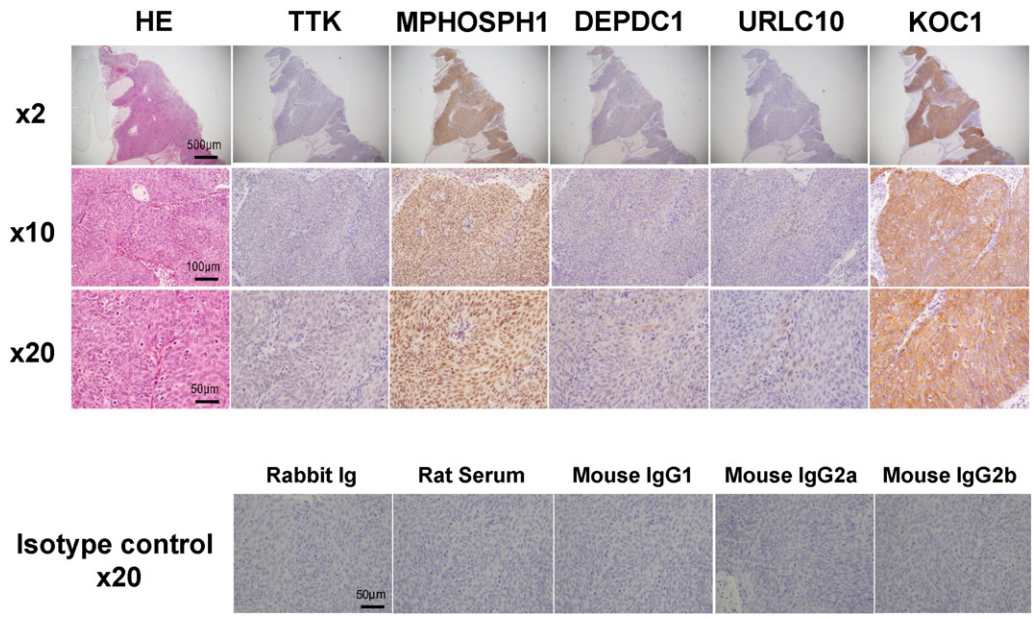

Fig. 4. Expressions of TAA examined by immunohistochemistry (IHC) in primary cervical cancer tissue of case 15. Representative IHC results are shown in photos powered x2, x10 and x20. Scale bar is 500 μm in x2, 100 μm in x10, 50 μm in x20. The images of negative controls using isotype control antibodies are shown below the positive results. Scores of IHC signals of each TAA in patients' cancer tissue are demonstrated in Supplementary Table 2.

significant association between frequencies of naïve CD8 + T cells before vaccination and longer survival, and this observation was reproducible among the nine CRC patients ($P = 0.001$). These results suggested that the frequencies of the naïve subset of CD8 + T cells might be correlated with TAA-specific immune responses, i.e., it may reflect capacity in the diverse antigenic repertoire.

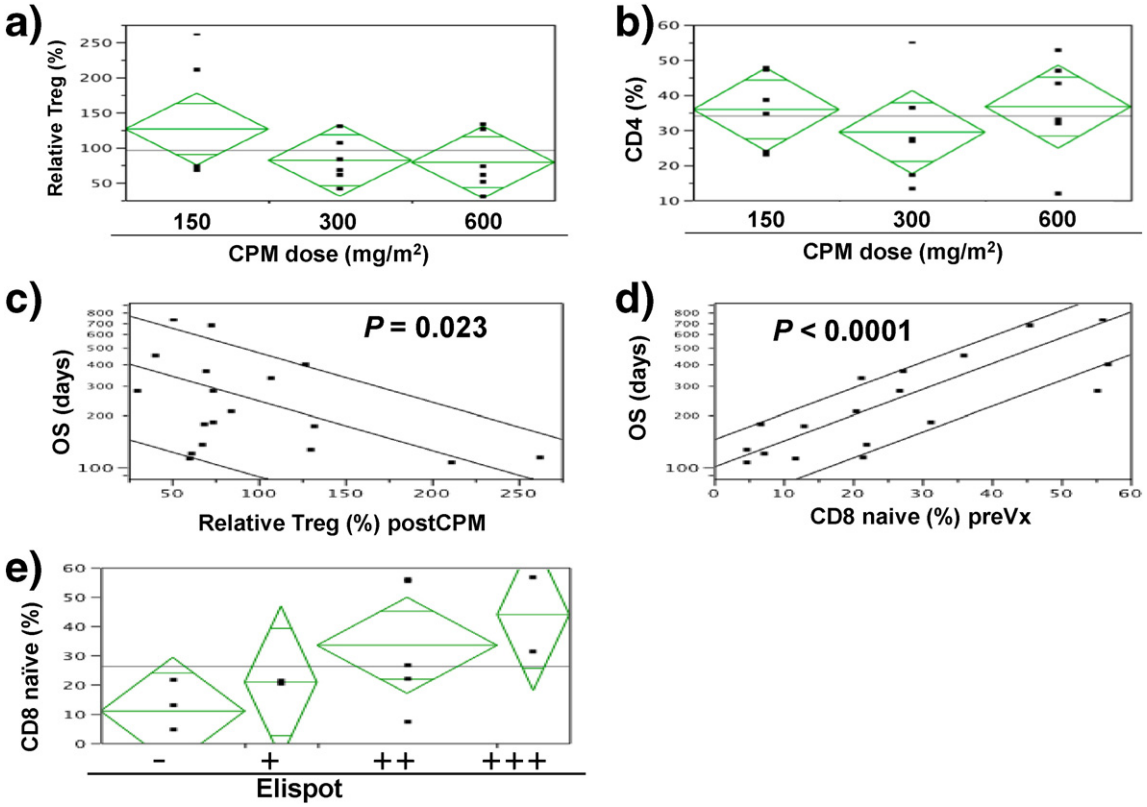

Fig. 5. Treg cells and other immune parameters with survival. *a*) Three parameters (CD4 +, $CD25^{hi}$, and Foxp3 +) were analyzed to detect Treg cells after gating lymphocytes. We counted these triple-positive cells as Treg cells. Relative changes in the number of Treg cells (absolute Treg count/mm^3) from baseline after administration of cyclophosphamide (post-CPM) are shown. We defined relative change (%) of the number of Treg cells as [(post-CPM Treg count/pre-CPM Treg count) × 100(%)]. *b*) Relationship between % of CD4 T cells and the dose of CPM. *c*) Weibull parametric model to evaluate effects of relative change in the number of Treg cells on survival. *d*) Weibull parametric model to evaluate effects of % of CD8 + naïve T cells to overall survival (OS). 'Naïve' was defined as the T-cell population doubly positive for CD45RA and CD62L. Effector memory T-cell population was doubly negative for CD45RA and CD62L. Central memory population was negative for CD45RA and positive for CD62L. *e*) The relationship between % of CD8 + naïve T cells and immune responses in ELISPOT assays. The number of "+" corresponds to the number of TAA-specific responses among the five antigens.

Our case 17, a long-term survivor with esophageal cancer, received PBT at celiac node metastases after 1st course of vaccination (Fig. 1b). Then he continued vaccination every 6 months, and has maintained CR for 5 years. Median survival time for patients with locoregional recurrence of squamous cell carcinoma of the thoracic esophagus after surgery was 25.5 months [42]. A clinical study of PBT for locoregionally advanced esophageal cancer (T1-4 N0-1) showed that median survival time was 20.5 months and median local control time was 25.5 months [43]. Taken together, it is difficult to explain only by the effect of PBT what led this patient to CR state from the second relapse involving multiple celiac nodes metastases, suggesting some contribution of the cancer vaccine. Release of DAMPs by RT-induced immunogenic cell death, the facilitation of tumor antigen uptake by DCs, and cross-presentation on MHC class I are molecular mechanisms by which RT modifies the tumor microenvironment and enhances antitumor immune responses. Thus, the combination of immunotherapy and radiation induces synergistic effects, and is therefore a reasonable option for antitumor therapy [12,44].

5. Conclusion

In this study, we showed a phase I clinical trial of a five-peptide cancer vaccine combined with CPM in patients with advanced solid tumors. Treatment was well tolerated without any therapy-associated adverse events above grade 3. We have confirmed expression of TAA targeted by the cancer vaccine immunohistochemically in the primary lesion and demonstrated that TAA-specific T cell responses induced by vaccine were associated significantly with longer survival. In addition, a higher degree of reduction of the number of Treg cells just after administration of CPM correlated with longer survival. This phase I clinical trial demonstrated safety and promising immune responses. Therefore, this approach warrants further clinical studies. The dogma that myelosuppressive effects prevent combination of chemotherapy with immunotherapy has been challenged by a growing body of experimental data [45]. Chemotherapies may enhance antitumor immune responses by depleting immunosuppressive immune cells (as CPM does in the case of Treg cells) and by inducing immunogenic cell death (e.g., anthracyclins) [46]. Immune checkpoint inhibitors prove that eliminating immune tolerance is an appropriate strategy for immunotherapy. Although immune checkpoint inhibitors may be used predominantly in this context, CPM should still be investigated as an option to eliminate Treg cells, because of its wide availability. The next clinical challenge is combination of our method with immune checkpoint inhibitors. It will be applied according to the data of pre-existing antitumor T-cell responses and mutation load in tumor by whole-exome sequencing [47].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2016.03.015.

Conflicts of interest

Yoshida K and Tsunoda T were employees of OncoTherapy Science, Inc. Yoshida K and Tsunoda T are current employees of AstraZeneca K.K. and Merck Serono Co., Ltd., respectively. Nakamura Y reports receiving a commercial research grant from and has ownership interest (including patents) in OncoTherapy Science, Inc. Tani K reports receiving a commercial research grant from OncoTherapy Science, Inc. Y. in 2007. No potential conflicts of interest were disclosed by the other authors.

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