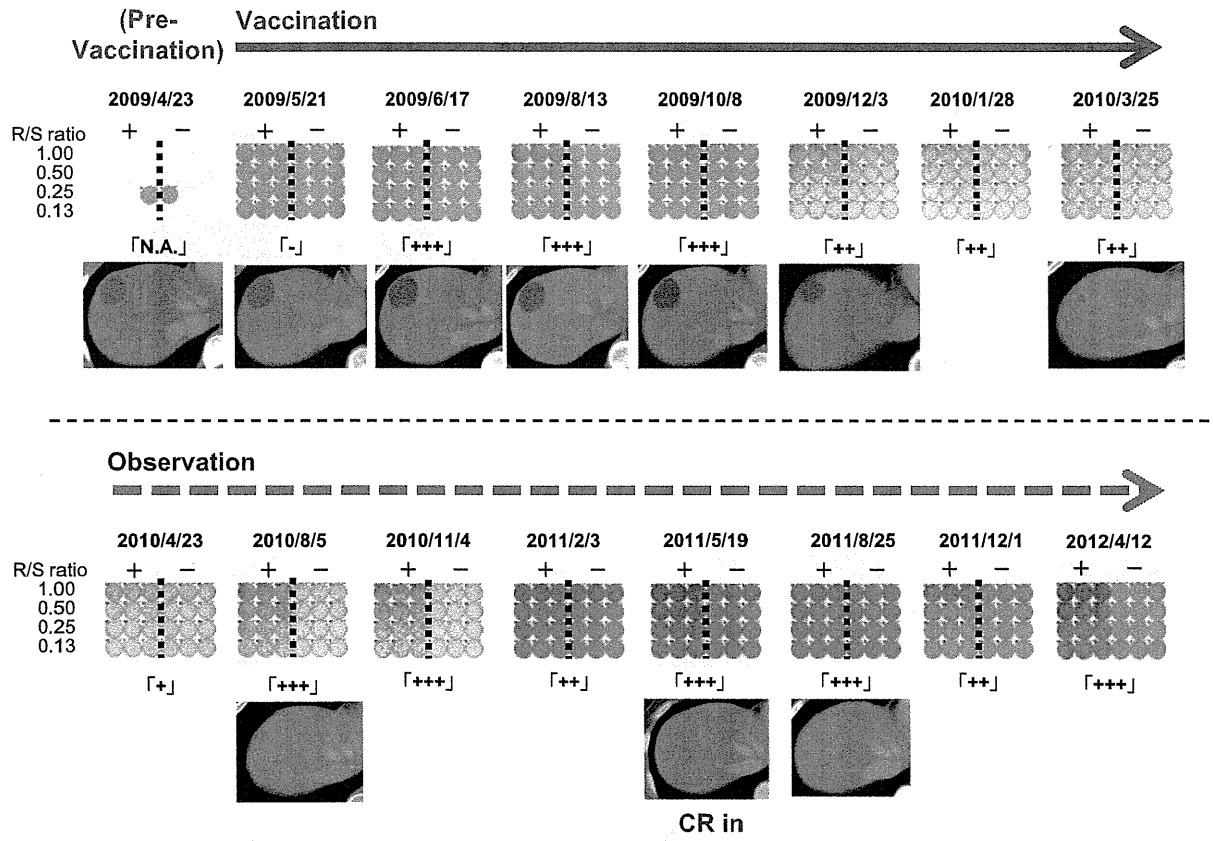

Figure 2 Peptide specific CTL response in case 9. Strong CTL responses specific to KIF20A-66 peptide were obtained at the time of 2 months after vaccination. The responses were kept strong positive during 2 years of the observation period. The number of the spots specific to peptide was calculated by subtracting the spot number in control wells from that in peptide-pulsed TISI cells.

intensity of CTL response was increased (Table 2), determined by the algorithm flow chart [25]. Of note, strong CTL response specific to KIF20A-66 was observed two months after the start of the vaccination in the patient of case 9, who achieved CR. This response kept strong for one year, and it was detectable even 2 years after the drug was discontinued (Figure 2). A flow cytometry assay demonstrated that the number of KIF20A-66 specific TCR in CD8-positive T cells was consistent with the grades classified according to our algorithm flow chart [25] (Figure 3a), compared to the negative control stain utilizing HIV-dextramer (Figure 3b). Also, injection site reactions were observed in 23 patients. MST of the patients with positive skin reaction was 182 days, while that of the patients with negative reaction was 42 days (Figure 5). These results demonstrate that CTL response and ISRs could be employed as biological markers to rapidly diagnose the efficacy of the peptide vaccination. Consistent with these results, when the 29 patients were classified into two groups in regard to the content ratio of lymphocyte (more than 16% (n = 23) vs. less than 16% (n = 6)), the group with higher number of lymphocyte yielded better prognosis with statistical significance (p = 0.0296). This result suggests that the number of lymphocyte is positively associated with the survival of the patients.

Discussion

Currently, there is no therapeutic strategy effective for the patients, whose pancreatic cancer is refractory to gemcitabine and TS-1. Combination therapy utilizing a couple of cytotoxic agents with gemcitabine has been investigated, but it has been failed to prove their clinical benefit so far [6-15]. We conducted an expression screening of proteins that were highly up-regulated in tumor cells, and not in normal cells, as a candidate of the target to develop novel anti-cancer drugs [20]. We successfully identified a member of kinesin super family protein 20A (KIF20A). Subsequently, we established an epitope peptide that were likely to be presented as an antigen in a HLA-A*2402- or HLA-A*0201-restricted manner [23,24,27]. In this report, we demonstrated that the KIF20A-derived peptide could improve the prognosis of the patients with advanced pancreatic cancer, suggesting that the KIF20A peptide vaccination is a promising approach as cancer immunotherapy.

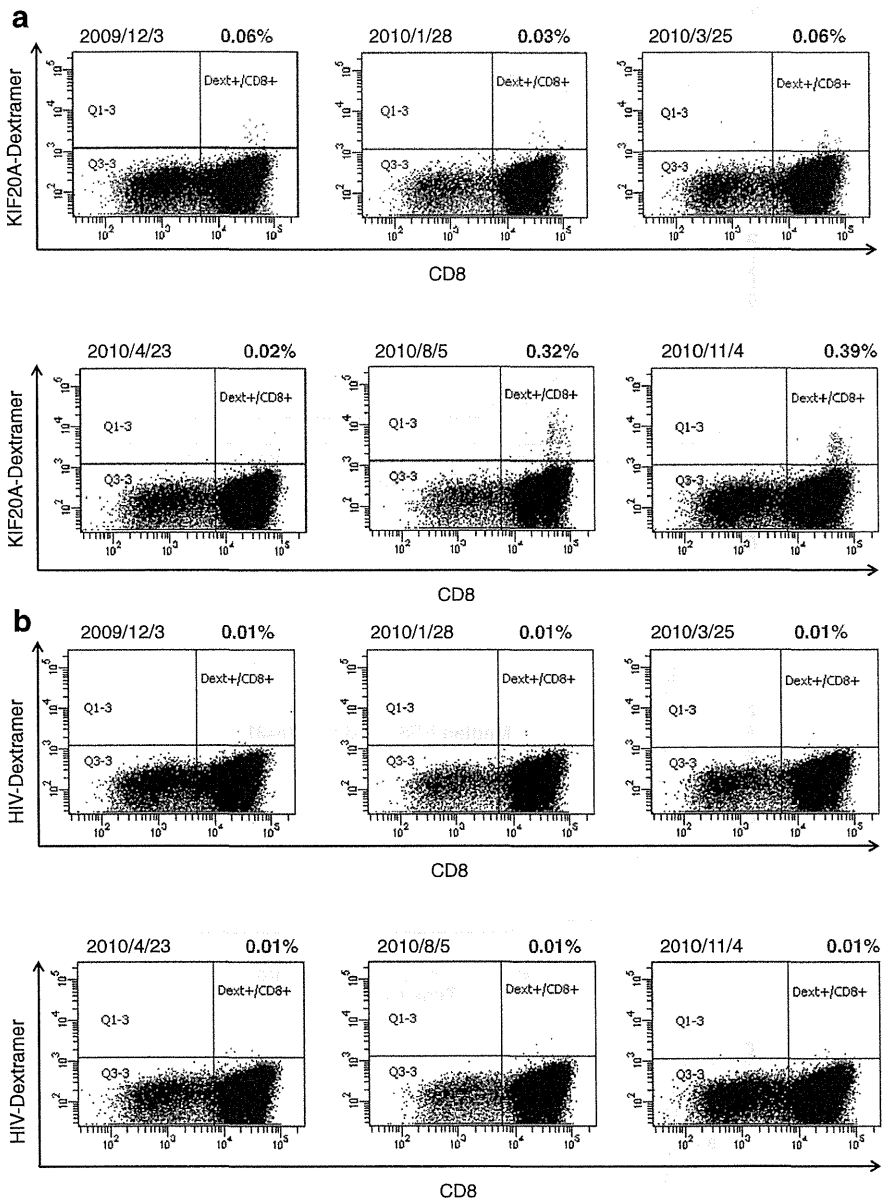

Figure 3 Flow cytometry analysis of KIF20A-66 specific TCR expression in $CD8^+$ cells in case 9. Cells were stained with either KIF20A-dextramer **(a)** or HIV-dextramer **(b)** after IVS as described in Methods section. The content rates of KIF20A-dextramer positive or HIV-dextramer positive cells (red dots) in $CD3^+$ $CD4^-$ $CD8^+$ cells are shown above panels in red.

In this clinical trial, we evaluated the safety and efficacy of KIF20A-66 peptide vaccine monotherapy for the patients with HLA-A*2402. This vaccine was well tolerated in the doses of 1.0 mg and 3.0 mg/body, although we do not exclude the possibility of two adverse events related to vaccination. The MST of 31 patients was 142 days in this phase I/II trial, indicating that vaccine treatment utilizing KIF20A-66 peptide provides survival benefit. Therefore, we concluded that the peptide vaccination improved overall survival period of the patients with advanced pancreatic cancer, who were resistant to chemotherapy. A placebo-controlled clinical trial should be required to further establish this peptide vaccine as a standard immunotherapy against pancreatic cancer.

We realized, during the course of peptide vaccination, that an induction of peptide-specific CTL and positive skin reaction were observed in the majority of the patients. We assure that these reactions could be employed as biomarkers of preferable clinical responses. Therefore, the number of CTL induced by peptide injection and the skin reaction at an injection site were analyzed. As we expected,

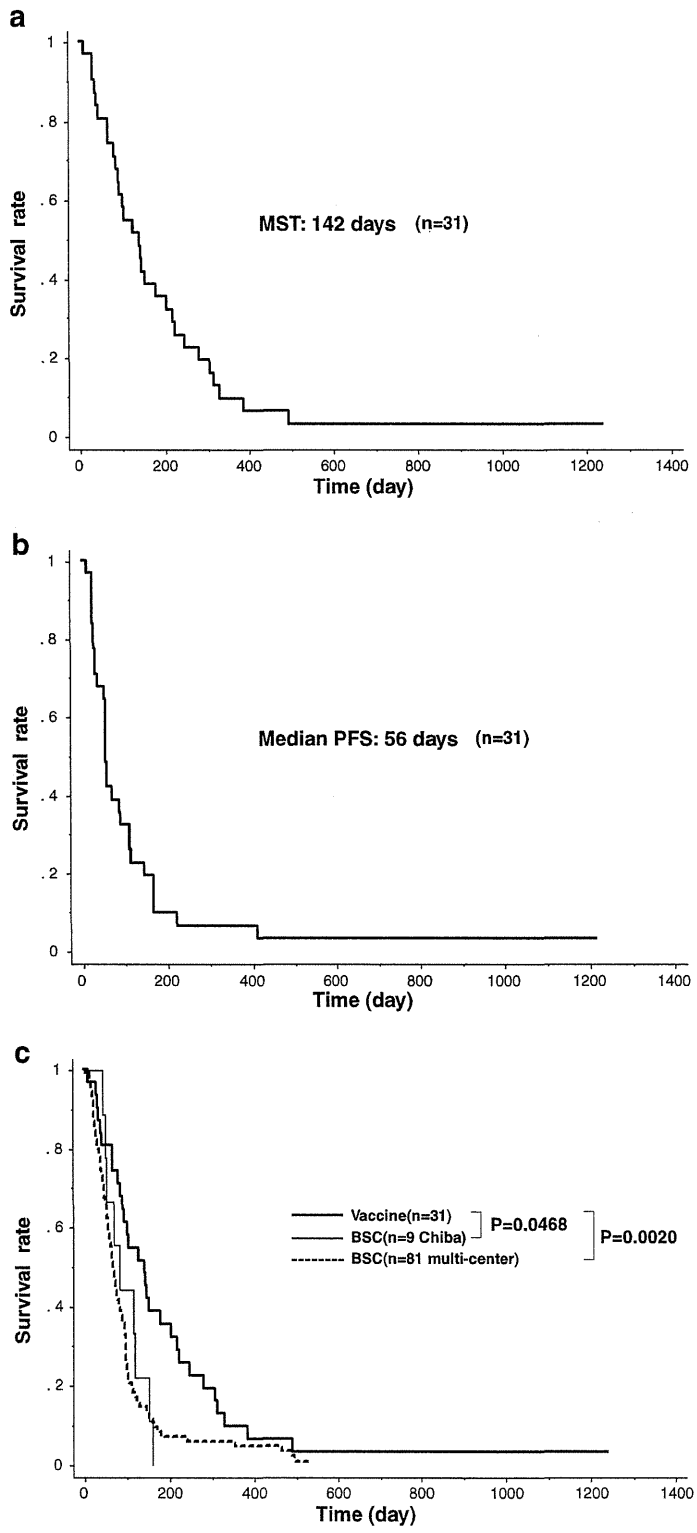

Figure 4 (See legend on next page.)

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Figure 4 Overall survival and progression free survival in phase I/II trial. Overall survival of the patients was shown in Kaplan-Meier plots (n = 31) **(a)**. MST of the patients with peptide vaccine was 142 days. PFS of the patients with peptide vaccine was 56 days **(b)**. In comparison with the control patients who were treated with best supportive care in Chiba Tokushukai Hospital (n = 9), overall survival of the patients with the KIF20A-peptide vaccination was fairly improved (p = 0.0468, MST: 142 vs. 83 days). In comparison with the BSC patients (n = 81), overall survival of the vaccinated patients in Chiba Tokushukai Hospital was significantly improved (p = 0.0020, MST: 142 vs. 63 days) **(c)**.

high level of CTL response specific to KIF20A-66 peptide resulted in CR in case 9. The liver metastasis continuously shrunk even after the peptide vaccination was discontinued (Figure 1a), and there was no sign of recurrence or metastasis at the time of 40 months after the vaccination started. Since biopsy of the tumor lesion was not performed during or after the vaccination, there is no information regarding the tumor infiltrating lymphocyte (TIL). This example indicates that positive correlation between tumor shrinkage and immunological reactions is of clinically interest (Figure 2). On the other hand, there is no CTL induction detected in Case No. 4, 27, and 28, while objective shrinkages were observed in these patients during the course of treatment. Since the number of CTL is usually low in peripheral blood, the CTL induction is measured after the stimulation utilizing respective peptide and IL-2 to yield higher detection limit. Despite this procedure, it is assumed that the intensity of CTL induction and the efficacy of vaccine treatment are not necessarily correlated according to a linear function, possibly due to the high expression levels of MHC Class I and/or targeted antigen KIF20A in tumor cells. Therefore, development of sensitive and reliable methods to detect CTL is required to evaluate the results of peptide vaccine treatment in the patients.

The US FDA published the guidance for the therapeutic cancer vaccine [28], describing that it is hard to expect clinical benefit of the vaccine treatment for the patients after multiple chemotherapy regimens due to very poor immune status. However, unlike many trials tested so far utilizing other peptide vaccines, this clinical study was quite successful. Our results clearly demonstrate that therapeutic cancer vaccination is still a promising approach for advanced pancreatic cancer after the failure of standard chemotherapy. In general, patients with relapsed or recurrent metastatic disease receive multiple treatments for their cancer. These therapies may be detrimental to the immune system, and adequate time is required for the cancer vaccine to elicit a detectable immune response. Given such therapeutic conditions affect the results of peptide vaccination, the use of adjuvant setting and the cohort study during an early treatment of the vaccine may be necessary to better understand a cause-and-result relationship of cancer immunotherapy. Furthermore, it is important to develop the peptides with the higher immunogenicity against active oncoproteins. Indeed, we have examined several peptides derived from a variety of cancer-testis antigens that have the oncogenic activity, including KIF20A, DEPDC1, MPHOSPH1, URLC10(LY6K),TTK, KOC1(IMP3),

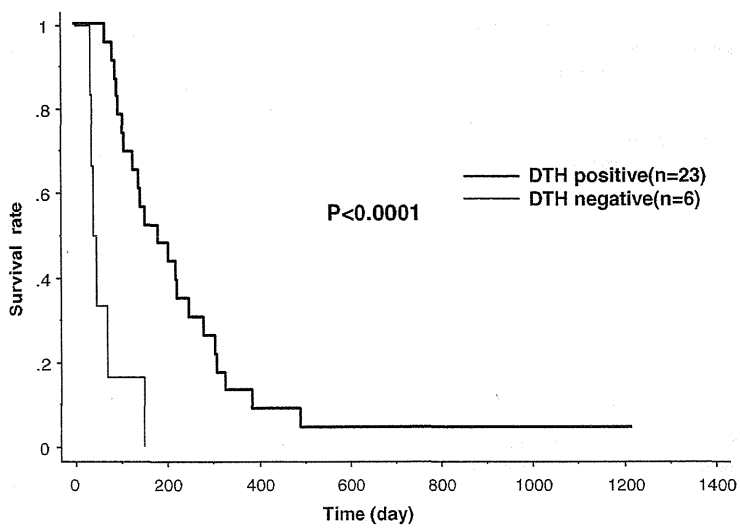

Figure 5 Correlation between OS and ISR. The local immune reactions at the site of injection were observed in 23 patients. MST of the patients who had injection site reaction was 182 days, while MST of the patients without such reaction (n = 6) was 42 days (p < 0.0001).

CDCA1, RNF43, and TOMM34 [16,17,20,22-25,27,29]. We propose that the trial of the cocktail vaccine of these high immunogenic peptides including KIF20A-66 will provide with better treatment and cure for cancer.

Abbreviations

HLA: Human leukocyte antigen; CR: Complete response; SD: Stable disease; PD: Progressive disease; MST: Median survival time; CTL: Cytotoxic T lymphocyte; 5-FU: 5-fluorouracil; ECOG: Eastern cooperative oncology group; RECIST: Response evaluation criteria in solid tumors; OS: Overall survival; PFS: Progression free survival; ISRs: Injection site reactions; IFA: Incomplete freund's adjuvant; ELISPOT: Enzyme-linked immunospot; PBMC: Peripheral blood mononuclear cell; IFN: Interferon; CIC: Cancer immunotherapy consortium; SAE: Severe adverse event; PR: Partial response; TIL: Tumor infiltrating lymphocyte.

Competing interests

The authors declare that they have no financial competing interest.

Authors' contribution

SA designed, performed, and evaluated clinical study. KT participated as the main coordinator and investigator regarding the immunological data analysis and evaluation. KY, HM, and HY analyzed control studies in their hospitals. SA wrote the manuscript. All authors read and approved the final manuscript.

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RESEARCH PAPER

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Immunological responses to a multi-peptide vaccine targeting cancer-testis antigens and VEGFRs in advanced pancreatic cancer patients

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Keywords: cancer-testis antigens, clinical trial, immunotherapy, multi-target vaccine, pancreatic cancer, peptide, VEGFR

Abbreviations: CDCA1, cell division cycle-associated 1; CT, cancer-testis; CTL, cytotoxic T lymphocyte; KIF20A, kinesin family member 20A; OS, overall survival; PFS, progression-free survival; VEGFR, vascular endothelial growth factor receptor

The prognosis of patients with advanced pancreatic cancer is extremely poor and there are only a few standard treatments. Here, we report the results of a Phase I clinical trial to investigate the safety, immunostimulatory effects, and antineoplastic activity of a multi-target vaccine composed of four distinct peptides derived from cancer-testis (CT) antigens and vascular endothelial growth factor receptors (VEGFRs). Nine patients with unresectable, advanced pancreatic cancer who were refractory to standard chemotherapy were enrolled. Each patient was vaccinated with HLA-A*2402-restricted peptides derived from the CT antigens kinesin family member 20A (KIF20A) and cell division cycle-associated 1 (CDCA1) as well as from VEGFR1 and VEGFR2 subcutaneously once a week, and disease progression was evaluated up to 6 mo later. Adverse events were assessed using the Common Terminology Criteria for Adverse Events v. 3.0. Immunological responses were monitored by ELISPOT assays and flow cytometry based on peptide-specific dextramers. The clinical outcomes that were measured were tumor response, progression-free survival (PFS) and overall survival (OS). In general, the multi-peptide vaccine was well-tolerated, and no grade 3 or 4 adverse events were observed upon vaccination. Peptide-specific T-cell responses were detected in all 9 patients, and clinical benefits were observed in four of them. Median PFS and OS were 90 and 207 d, respectively. The elicitation of multiple and robust peptide-specific T-cell responses as well as the status of host lymphocytes may be useful prognostic factors among patients with advanced pancreatic cancer treated with peptide-based anticancer vaccines.

Introduction

Pancreatic cancer is a common disease worldwide and its incidence is gradually increasing. Pancreatic cancer is associated with a high mortality rate because most cases are not diagnosed until they are advanced and inoperable.[1] Nowadays, very few standard treatments have been established for the treatment of this deadly disease,[2] implying that new therapeutic modalities are urgently needed. Anticancer vaccines based on synthetic peptides have been developed several laboratories worldwide, and their safety and clinical efficacy are documented by an abundant literature.[3,4] We have previously reported that peptide-based anticancer vaccines are capable of inducing antigen-specific cytotoxic T lymphocyte (CTL) responses in vivo and of providing clinical benefits to some patients with advanced colorectal carcinoma[5] or biliary tract cancer.[6] In the present study, we selected 4 peptides, 2 of which deriving from cancer-testis (CT) antigens and 2 of which deriving from vascular endothelial growth factor receptors (VEGFRs), that were identified by cDNA microarray technology coupled with laser microdissection to be overexpressed by close to 100% of pancreatic cancer cells and the associated endothelium. In particular, we performed a Phase I clinical study to assess the safety, immunostimulatory potential, and therapeutic profile of a multi-peptide vaccine in patients with advanced pancreatic cancer. Patients were vaccinated on a continuous basis over a long-term until their disease had progressed, at which time we assessed the safety, immunological and clinical parameters. Here, we report the immunological responses to such a multi-peptide vaccine in anticipation of a Phase II clinical trial that will evaluate the clinical profile of this immunotherapeutic anticancer intervention.

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Table 1. Patient characteristics

Patients	Age/Sex	Tumor site		Prior therapy	Peptide (mg)
		Primary	Metastases		
1	69/M	Head	Liver	GEM, TS-1, CDDP, RX	1
2	71/M	Body	Liver	GEM, TS-1	1
3	52/M	Head	Liver	GEM, TS-1	1
4	66/F	Body	Peritoneum	GEM, TS-1	2
5	78/F	Head	Liver	GEM, TS-1	2
6	61/M	Body		GEM, UFT	2
7	58/F	Body	Liver, LN	GEM, TS-1	3
8	73/M	Tail	Liver	GEM, TS-1, CDDP	3
9	64/M	Head	Liver	GEM, TS-1	3

Abbreviations: CDDP, cisplatin; GEM, gemcitabine; LN, lymph node; RX, radiation therapy; TS-1, tegafur, gimeracil oteracil potassium; UFT, uracil, tegafur.

Table 2. Clinical outcomes and immunological responses

Patients	No. of vaccine	Clinical response	PFS (days)	OS (days)	ISR (grade)	Peptide-specific CTL responses			
						KIF20A	CDCA1	VEGFR1	VEGFR2
1	24	SD	189	231	2	1+	1+	1+	3+
2	24	PD	91	207	2	3+	3+	2+	1+
3	10	PD	63	76	2	3+	1+	1+	1+
4	8	PD	21	51	2	1+	2+	0	0
5	5	PD	42	54	1	1+	3+	2+	1+
6	26	SD	161	371	2	3+	3+	3+	3+
7	23	SD	90	244	2	3+	3+	2+	3+
8	22	SD	168	826	2	3+	3+	1+	3+
9	6	PD	36	168	1	1+	1+	0	1+

Abbreviations: CTL, cytotoxic T lymphocyte; ISR, injection site reaction; SD: stable disease; PD, progressive disease.

Table 3. Prognostic factors for progression-free and overall survival

Factors	PFS	OS
Gender (male/female)	0.065	0.235
Age (≥ 66/ < 66)	0.372	0.084
CRP (≥ 0.33/ < 0.33)	0.002	0.068
Hemoglobin (≥ 12/ < 12)	0.777	0.132
Lymphocyte (%) (≥ 18/ < 18)	0.003	0.003
Lymphocyte (number) (≥ 1100/ < 1100)	0.501	0.017
KFI20A CTL spots (≥ 3+/ < 3+)	0.729	0.059
CDCA1 CTL spots (≥ 3+/ < 3+)	0.832	0.084
VEGFR1 CTL spots (≥ 3+/ < 3+)	0.747	0.465
VEGFR2 CTL spots (≥ 3+/ < 3+)	0.017	0.005
CTL 3+ (≥ 1/ < 1)	0.002	0.068
CTL 3+ (≥ 2/ < 2)	0.501	0.017
CTL 3+ (≥ 3/ < 3)	0.514	0.011
Injection site reaction (≥ Grade2/ < Grade2)	0.046	0.122

Abbreviations: CRP, C-reactive protein; CTL, cytotoxic T lymphocyte.

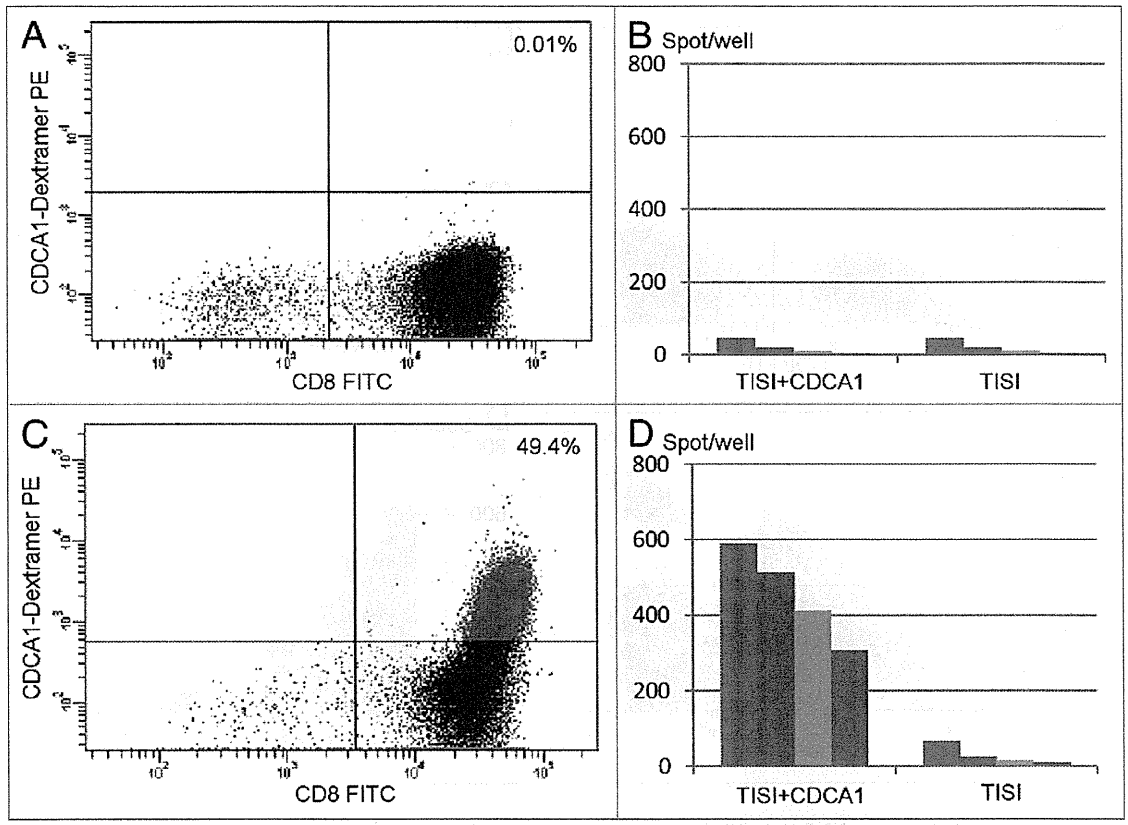

Figure 1. Immunological monitoring of the response of one patient to CDCA1-targeting vaccination. (**A**) Pre-vaccination lymphocytes were analyzed by flow cytometry using HLA-A2402/CDCA1 dextramers in combination with anti-CD8 monoclonal antibodies. (**B**) Interferon γ (IFNγ) secretion by lymphocytes isolated from patient n° 2 before vaccination and exposed to TISI cells pulsed with CDCA1-derived peptides, as monitored by ELISPOT assays. (**C**) Lymphocytes isolated from patient n° 2 after the 1st cycle of vaccination were analyzed by flow cytometry using HLA*A2402/CDCA1 dextramers in combination with anti-CD8 monoclonal antibodies. (**D**) IFNγ secretion by lymphocytes isolated from patient n° 2 after the 1st cycle of vaccination and exposed to TISI cells pulsed with CDCA1-derived peptides, as monitored by ELISPOT assays. In **B** and **D**, responder-to-stimulator (R/S) cell ratios were 1, 0.5, 0.25, and 0.13.

Results

Patient characteristics

Nine patients (6 men and 3 women; median age: 65.8 y; age range: 52–78 y) whose HLA type was A*2402 were enrolled in this study (**Table 1**). Their primary tumor site was the pancreas head in 4 cases, the pancreas body in 4 cases, and the pancreas tail in 1 case. All patients had several metastases to the liver, lymph nodes, or peritoneum. The previous therapies received by these individuals consisted of gemcitabine (GEM), tegafur plus gimeracil plus oteracil potassium (TS-1), cisplatin (CDDP), or uracil plus tegafur (UFT). One patient was also exposed to radiation therapy.

Assessment of toxicity

We assessed toxicity using Common Terminology Criteria for Adverse Events (CTCAE) v3.0. Two of the patients developed a grade 1 injection site reaction while 7 developed a grade 2 injection site reaction. Low hemoglobin, white blood cell, neutrophil, and platelet counts were observed before the 1st vaccination, but did not worsen throughout the study period, and no other severe adverse events over grade 3 were seen in this time frame. Thus, the multi-peptide vaccine that we employed was well-tolerated up to a dose of 3 mg per peptide (9 mg total) during the 6 mo of the study.

Antigen-specific immune responses

Peptide-specific CTL responses were documented by ELISPOT assays in all 9 patients enrolled in the study. We determined the response to each specific antigen in every patient using the algorithm described in **Figure S1**. The results of this study are summarized in **Figure S2**. The number of peptide-specific interferon γ (IFNγ) spots per section increased with the number of vaccinations, a trend that continued for the entire duration of the study. CDCA1-specific CTLs were shown to increase upon vaccination by HLA*A2402/CDCA1 dextramers and flow cytometry. (**Fig. 1A and C**). The number of CDCA1-specific IFNγ spots increased according to a similar trend (**Fig. 1B and D**). The same applied to VEGFR2-specific CTLs and IFNγ spots (**Fig. 2A–D**). The immune responses elicited by our multi-peptide vaccine were not the same for all antigens in a specific patient, nor for the same antigen across different patients. Strong CTL responses against KIF20A-, CDCA1-, and VEGFR2-derived peptides were indeed more frequent than

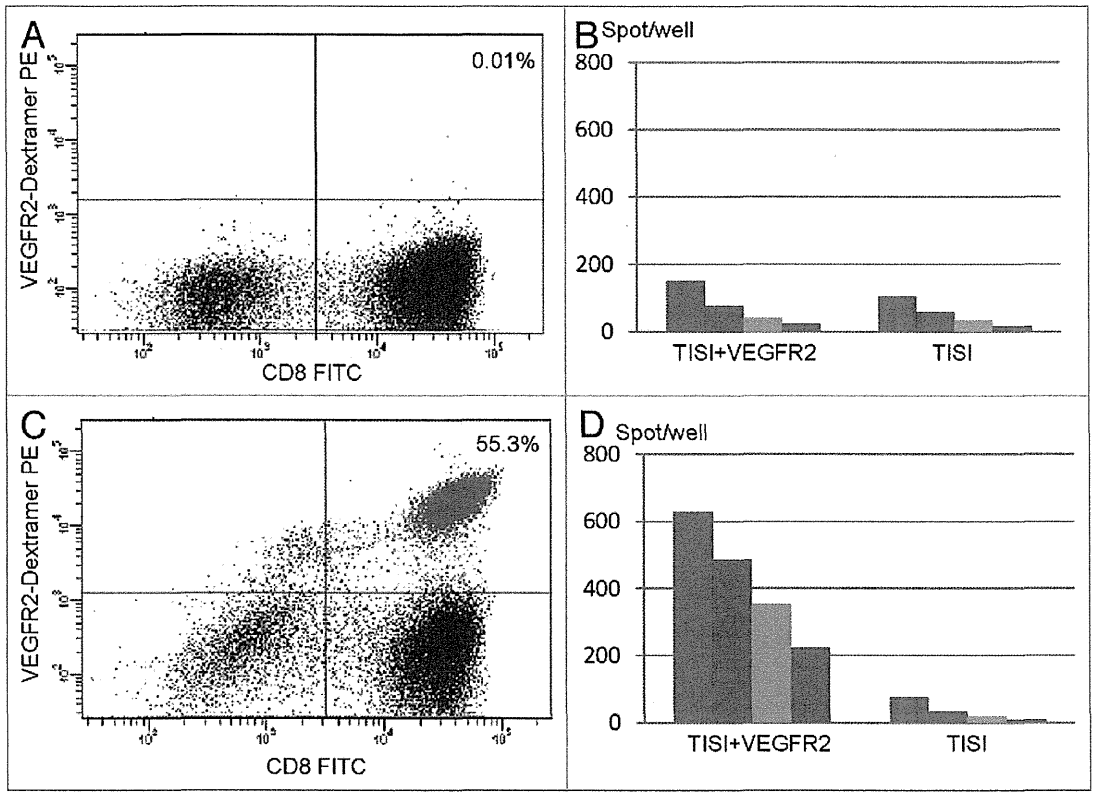

Figure 2. Immunological monitoring of the response of one patient to VEGFR2-targeting vaccination. (**A**) Pre-vaccination lymphocytes were analyzed by flow cytometry using HLA*A2402/VEGFR2 dextramers in combination with anti-CD8 monoclonal antibodies. (**B**) Interferon γ (IFNγ) secretion by lymphocytes isolated from patient n° 1 before vaccination and exposed to TISI cells pulsed with VEGFR2-derived peptides, as monitored by ELISPOT assays. (**C**) Lymphocytes isolated from patient n° 1 after the 2nd cycle of vaccination were analyzed by flow cytometry using HLA-A2402/VEGFR2 dextramers in combination with anti-CD8 monoclonal antibodies. (**D**) IFNγ secretion by lymphocytes isolated from patient n° 2 after the 2nd cycle of vaccination and exposed to TISI cells pulsed with VEGFR2-derived peptides, as monitored by ELISPOT assays. In **B** and **D**, responder-to-stimulator (R/S) cell ratios were 1, 0.5, 0.25, and 0.13.

robust responses to VEGFR1-derived peptides. The ability of the vaccine to induce a strong T-cell response seemed to linked not only to the nature of the epitope but also the status of the host immune system.

Clinical responses

The clinical responses of patients enrolled in this study are summarized in **Table 2**. Four patients manifested stable disease (SD) and 5 progressive disease (PD). The 4 patients who achieved SD plus of those who exhibited PD wished to receive optional rounds of vaccination and continued the study for up to 6 mo. Eventually, the disease progressed in all 9 patients and they all succumb to pancreatic cancer within 3 y. The median progression-free survival (PFS) of these patients upon vaccination was 90 d (95% CI: 11–169 d), while 1-y PFS was 0% (**Fig. 3A**). The median overall survival (OS) of this cohort was 207 d (95% CI: 93–321 d) and the 1-y OS was 22.2% (**Fig. 3B**). According to the univariate analysis of prognostic factors, patients who developed multiple and robust CTL responses to the vaccine exhibited an improved prognosis (**Table 3**). Patients with a relatively high lymphocyte counts also exhibited improved disease outcome as compared with individuals with a poor lymphocytic compartment.

Discussion

Pancreatic cancer is well known as a neoplasm associated with an extremely poor prognosis. Surgery in the early stages of disease is the only curative treatment for pancreatic cancer patients, but unfortunately most of these lesions are not found until late disease stages. There are only a few standard chemotherapeutic regimens employed in this setting: GEM, TS-1, or CDDP. The PFS and OS rates achieved with these treatments are similar to those obtained with the multi-peptide vaccine presented here, though our patients were enrolled after the failure of standard chemotherapy. This observation suggests that peptide-based anticancer vaccines might improve the PFS and OS of pancreatic cancer patients. Similarly to recent reports on the therapeutic activity of peptide-based anticancer vaccination, we observed no complete remissions or partial responses in the present study, but an apparent improvement in OS. We should now plan a Phase II clinical study to assess the therapeutic profile of our multi-peptide vaccine in a randomized setting.

Here we focused on the induction of CTL responses targeting not only CT antigens, but also VEGFRs, which are

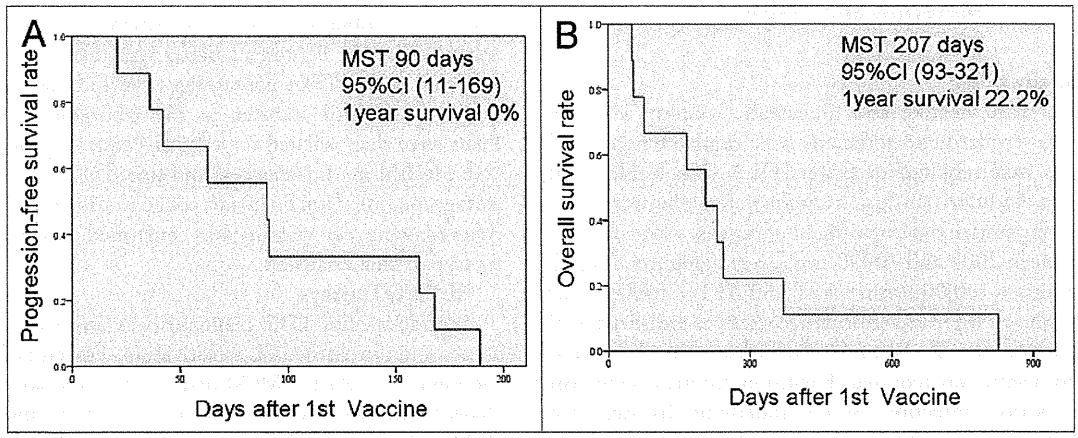

Figure 3. Progression-free and overall survival of the patients enrolled in this study. (**A**) Progression-free survival (PFS) after the 1st vaccination. The median survival time (MST) was 90 d (95% CI: 11–169 d) and the 1-y PFS ratio was 0%. (**B**) Overall survival (OS) after the 1st vaccination. The MST was 207 d (95% CI: 93–321 d) and the 1-y OS ratio was 22.2%.

highly expressed by cancer-associated endothelial cells. One of the crucial factors in the escape of neoplastic cells from immunosurveillance is the downregulation of HLA antigens. CTLs are not able to react against malignant cells that do not express HLA, and this frequently occurs in the course of oncogenesis or tumor progression. In our approach, CTLs are able to respond to VEGFR expressed by the tumor vasculature even if cancer cells do not express HLA molecules. Our multi-peptide vaccine should therefore work in any HLA situation. Our findings demonstrate that multi-peptide anticancer vaccines are able to elicit CTL responses specific for each of the vaccine components in all patients. Thus, multi-peptide vaccines might represent a valuable candidate for the treatment of pancreatic cancer.

So far, anticancer vaccination has been tested in several clinical trials, but only one vaccine, namely sipuleucel-T (trade name Provenge®) is available for clinical use. This preparation has been approved by the US FDA in 2011.[7] Many Phase III clinical trials testing anticancer vaccines have failed for a variety of reasons.[8] It is thought that the efficacy of therapeutic anticancer vaccines is largely influenced by the conditions of the host immune system, and that a new classification for candidate patients is therefore needed to ensure the clinical success of such an approach.[9,10] Our results indicate that pancreatic patients with relatively good lymphocyte counts achieve a better prognosis that patients with a poor lymphocyte status. Thus the conditions of the host immune system are crucial for anticancer vaccines to elicit robust immune responses and mediate clinically-relevant effects. In an attempt to further elucidate the relationship between immune parameters of the hosts and the therapeutic profile of anticancer vaccine, data from a Phase II study to be analyzed with a multivariate regression model is required.

Although multi-peptide vaccines are valuable candidate for the treatment of pancreatic cancer, their clinical efficacy is currently limited. One of the major obstacles against the efficacy of such an immunotherapeutic strategy is related to immunosuppression. Regulatory T cells are well known to play a critical role in this setting. Accordingly, non-myeloablative chemotherapy to deplete regulatory T cells is a promising approach to overcome immunosuppression.[11] Chemokine (C-C motif) receptor 4 (CCR4) antagonists as well as anti-CCR4 monoclonal antibodies, one of which have already been approved in Japan for use in cancer patients, might also constitute useful tool against immunosuppression, as regulatory T cells express CCR4.[12,13] Another method to circumvent this issue, based on the antineoplastic agent denileukin diftitox, has also been examined in animal and human models.[14,15] Finally, the blockade of immunological checkpoint is crucial for obtaining robust anticancer immune responses. Ipilimumab (an monoclonal antibodies specific for cytotoxic T lymphocyte-associated protein 4, CTL4),[16] as well as antibodies targeting programmed cell death 1 (PDCD1, best known as PD-1)[17,18] and its major ligand (CD274, best known as PD-L1)[19] showed very promising results in clinical studies. Combining these agents with an anticancer vaccine may constitute an efficient means of boosting the clinical activity of the latter.[20]

Several peptides derived from tumor-associated antigens have already been tested in clinical trials.[21-25] In the present study, we selected peptides from 4 distinct antigens, inducing strong immune responses in vivo. KIF20A[26] is a conserved motor domain that binds to microtubules, while CDCA1[27] is a molecular linker between the kinetochore attachment site and tubulin subunits. Both KIF20A and CDCA1 are overexpressed by pancreatic cancers. Conversely, VEGFR1 and VEGFR2[28] are expressed on the tumor endothelium. Some of these peptides have been used separately or in different combinations for the treatment of non-small cell lung carcinoma, renal cell carcinoma, or pancreatic cancer. Our study is the first to report on the use of a four-peptide vaccine that simultaneously target cancer cells and the tumor endothelium in pancreatic cancer patients. Before this approach can be considered as a candidate for the treatment of patients with pancreatic cancer, it will be necessary to test its therapeutic potential in randomized a Phase II clinical trial.

Materials and Methods

Patient eligibility

Patients with unresectable pancreatic cancer who were refractory to standard chemotherapy were eligible for this study. All patients were required to express HLA-A molecules of the A*2402 type. Additional inclusion criteria were age between 20 and 80 y, no severe functional impairment of organs, white blood cell counts between 2000 and 10000/mm^3, hemoglobin > 8 mg/dL, platelet counts > 100,000/mm^3, AST and ALT < 100IU/L, and total bilirubin < 2 mg/dL. Performance status as measured by the ECOG scale was 0 to 2. An interval of at least 4 weeks since the last chemotherapy was required. Exclusion criteria encompassed pregnancy, serious infections, severe underlying diseases, severe allergic diseases and a judgment of unsuitability by the principal investigator.

Study design and endpoints

This was a Phase I study. Patients who received standard chemotherapy under a diagnosis of inoperable pancreatic cancer between May 2009 and August 2009 were invited to participate after providing their informed consent. The HLA-A genotypes of these patients were examined, and 9 patients with HLA-A*2402 were enrolled. Four peptides were used for the vaccine, which were derived from KIF20A (KVYLRVRPLL), CDCA1 (VYGIRLEHF), VEGFR1 (DYLNEWGSRF), and VEGFR2 (RFVPDGNRI). These peptides were chosen among antigens identified by a cDNA microarray technology coupled with laser microdissection as highly overexpressed by pancreatic cancer cells or the associated endothelium. We determined the purity (> 97%) of the peptides by analytical high-performance liquid chromatography (HPLC) coupled to mass spectrometry. We tested both the endotoxin levels and bioburden of these peptides and found them to be within acceptable levels based on GMP grade vaccines (PolyPeptide or NeoMPS Inc.). Peptides were mixed with incomplete Freund's adjuvant (IFA, also known as Montanide ISA51, from SEPPIC) which has been used in many clinical studies, and were injected subcutaneously (at doses of 1, 2, or 3 mg per peptide) once a week into the inguinal or the axillar site before the judgment of disease progression, for up to 6 mo. The endpoints of the study were the assessment of toxicities caused by vaccination based on CTCAE v.3.0, immunological responses, tumor responses, progression-free survival (PFS) and overall survival (OS) from the first administration of the vaccine. Assessments were performed every 4 vaccinations. This study was approved by the institutional review board at Tokyo Women's Medical University and was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, 000004337). Informed consent was obtained from all patients, and all procedures were in accordance with the Declaration of Helsinki.

Lymphocyte preparation for immunomonitoring

Immunological assays were periodically standardized and validated by Clinical Laboratory Improvements Amendments (CLIAs) and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human use (ICH) guidelines. Peripheral blood lymphocytes (PBLs) were obtained from each patient before and after every 4th vaccination. Peripheral blood was taken by venipuncture, collected in an EDTA-containing tube and maintained at room temperature until transfer to the laboratory (within 24 h). PBLs were then isolated on a Ficoll-Paque Plus density gradient (GE Healthcare Bio-sciences) and stored at -80 °C in serum-free storage medium (Juji Field) at a concentration of 5×10^6 cells/mL. After thawing, cell viability was confirmed to be more than 90% by trypan blue exclusion.

ELISPOT assays

Peptide-specific CTL responses was estimated by ELISPOT assays upon in vitro CTL sensitization. Frozen peripheral blood mononuclear cells (PBMCs) derived from the same patient were thawed and their viability was confirmed to be more than 90%. PBMCs (at a concentration of 5×10^5 cells/mL) were cultured in the presence of 10 mg/mL of the respective peptide and 100 IU/mL interleukin-2 (IL-2, from Novartis, Emeryville, CA) at 37°C for 2 wks. Peptides were added to cell cultures on days 0 and 7. Following CD4$^+$ T-cell depletion by a Dynal CD4 Positive Isolation Kit (Invitrogen), an IFNγ ELISPOT assay was performed using Human IFNγ ELISpot PLUS kits (MabTech), according to the manufacturer's instructions. Briefly, HLA-A*2402$^+$ TISI B lymphoblasts (IHWG Cell and Gene Bank) were incubated with 20 μg/mL of peptides overnight, followed by the washout of residual peptides in media, resulting in the generation of peptide-pulsed TISI cells as stimulating cells. CD4$^-$ cells were then cultured with peptide-pulsed TISI cells (2×10^4 cells/well) at 1:1, 1:2, 1:4, or 1:8 responder to stimulator (R/S) cell ratios in 96-well plates (Millipore) at 37°C overnight. Unpulsed TISI cells were used as negative control for stimulation. To confirm IFNγ secretion, we stimulated responder cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin (3 μg/mL) overnight, and then tested them by an ELISPOT assay (2.5×10^3 cells/well) in the absence of stimulator cells. All ELISPOT assays were performed in triplicate wells. Plates were analyzed by the automated ELISPOT reader ImmunoSPOT S4 (Cellular Technology) and ImmunoSpot Professional Software v. 5.0 (Cellular Technology). The number of peptide-specific spots was calculated by subtracting the number of spots in control wells from the number of spots in each of the wells containing peptide-pulsed TISI cells. The sensitivity of our ELISPOT assay was estimated to be at an average level by an ELISPOT panel of the Cancer Immunotherapy Consortium (CIC, http://www.cancerresearch.org/consortium/assay-panels/).

Flow cytometry

We analyzed the expression of peptide-specific T-cell receptors on a FACSCantoII cytofluoromter (Becton Dickinson) using CDCA1-, VEGFR1-, or VEGFR2-derived peptide-HLA dextramers coupled to phycoerythrin (PE) (Immudex), according to the manufacturer's instructions. A PE-conjugated dextramer involving a HIV1-derived epitope (RYLRDQQLL) was used as a negative control. In brief, cells were incubated with peptide-HLA PE-conjugated dextramers for 10 min at room temperature, then treated with fluorescein isothiocyanate (FITC)-conjugated anti-CD8 antibodies, allophycocyanin (APC)-conjugated anti-CD3 antibodies, PE-Cy7-conjugated anti-CD4 antibodies,

and 7-aminoactinomycin D (7-AAD; from BD PharMingen) at 4°C for 20 min.

Statistical analyses

PFS and OS were analyzed done using the Kaplan-Meier method and statistical significance was evaluated by log-rank tests. A p value < 0.05 was considered as statistically significant. All statistical analyses were conducted using the SSPS statistics software v. Twenty-one (IBM).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Materials

Supplemental materials may be found here: http://www.landesbioscience.com/journals/oncoimmunology/article/27010/

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OPEN

A Phase I Clinical Trial of Vaccination With KIF20A-derived Peptide in Combination With Gemcitabine For Patients With Advanced Pancreatic Cancer

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Summary: KIF20A (RAB6KIFL) belongs to the kinesin superfamily of motor proteins, which play critical roles in the trafficking of molecules and organelles during the growth of pancreatic cancer. Immunotherapy using a previously identified epitope peptide for KIF20A is expected to improve clinical outcomes. A phase I clinical trial combining KIF20A-derived peptide with gemcitabine (GEM) was therefore conducted among patients with advanced pancreatic cancer who had received prior therapy such as chemotherapy and/or radiotherapy. GEM was administered at a dose of 1000 mg/m^2 on days 1, 8, and 15 in a 28-day cycle. The KIF20A-derived peptide was injected subcutaneously on a weekly basis in a dose-escalation manner (doses of 0.5, 1, and 3 mg/body; 3 patients/cohort). Safety and immunologic parameters were assessed. No severe adverse effects of grade 3 or higher related to KIF20A-derived peptide were observed. Of the 9 patients who completed at least one course of treatment, interferon-γ (IFN-γ)-producing cells were induced in 4 of 9 patients (P2, P3, P6, and P7), and IFN-γ-producing cells were increased in 4 of the 9 patients (P1, P5, P8, and P9). Four of the 9 patients achieved stable disease. The disease control rate was 44%. The median survival time after first vaccination was 173 days and 1-year survival rate was 11.1%. IFN-γ-producing cells were induced by the KIF20A-derived peptide vaccine at a high rate, even in combination with GEM. These results suggest that this combination therapy will be feasible and promising for the treatment of advanced pancreatic cancer.

Key Words: pancreatic cancer, peptide, KIF20A, phase I, immunotherapy

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Pancreatic cancer is the fourth leading cause of cancer mortality in the world. The prognosis for patients with pancreatic cancer is extremely poor, with an overall 5-year survival of only 5%.[1] The primary reason for this high mortality rate is the aggressive nature of the malignancy in the absence of early detection. There are few (if any) symptoms that offer an early indication of pancreatic cancer growth; therefore, most such cancers are diagnosed in the advanced stage. As a result, the majority of pancreatic cancers are unresectable. Other therapies, including radiation and chemotherapy, have limited effects in terms of increased survival. Consequently, median survival time (MST) after the diagnosis of pancreatic cancer is measured in months rather than years.[2,3] Gemcitabine (GEM) is currently one of the standard therapies for advanced pancreatic cancer, although many chemotherapeutic agents have been used in clinical trials over the past 2 decades.[4–6] Among these chemotherapeutic agents, GEM is clinically more effective, but the MST is still <6–9 months. The development of new treatment modalities, including specific immunotherapies, is thus required. Recent advances in molecular biology and cellular immunology in the field of tumor immunology have resulted in the identification of a large number of antigens and epitopes recognized by human leukocyte antigen (HLA) class I restricted cytotoxic T lymphocytes (CTL) from melanomas and epithelial cancers.[7–12] Using cDNA microarray technology coupled with laser microdissection, we recently identified novel HLA-A24-restricted epitope peptides as targets for cancer vaccination for patients with pancreatic cancer.[13–15] KIF20A (RAB6KIFL) belongs to the kinesin superfamily of motor proteins, which have critical functions in the trafficking of molecules and organelles.[16] Immunotherapy using a new epitope peptide for KIF20A is expected to improve clinical outcomes. A phase I clinical trial combining KIF20A-derived peptide with GEM was therefore conducted for patients with advanced pancreatic cancer who had received prior therapy such as chemotherapy and/or radiotherapy.

MATERIALS AND METHODS

Peptides

The KIF20A-10-66 peptide (KVYLRVRPLL) was synthesized by BCN Peptides (Barcelona, Spain) according to a standard solid-phase synthesis method, thereafter purified by reversed-phase high-performance liquid chromatography (HPLC). The purity (> 90%) and identity of peptides were determined by analytical HPLC and mass spectrometry analysis, respectively. Endotoxin levels and the bioburden of these peptides were tested and determined to be within acceptable levels as Good Manufacturing Practice grade for vaccines.

Patient Eligibility

The institutional review board at Yamaguchi University approved this clinical protocol. Complete written informed consent was obtained from all patients at the time of enrollment. According to the protocol, patients were

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required to show positive results for HLA-A*2402. Nine patients diagnosed with metastatic and/or unresectable pancreatic cancer who had received prior therapy such as chemotherapy and/or radiotherapy were enrolled in this trial between January and December 2009 at Yamaguchi University Hospital. Eligibility criteria were as follows: age ≥20 years; life expectancy ≥3 months; and adequate hepatic, renal, and bone marrow function (serum creatinine level, <2.0 mg/dL; bilirubin level, <3.0 g/dL; platelet count, ≥75,000/mL; total white blood cell count ≥3000/mL and ≤15,000/mL). All patients were untreated for ≥4 weeks before enrolling into the study and had to have an Eastern Cooperative Oncology Group performance status of 0-2 at the time of enrollment.

Study Design and End-points

This study was a nonrandomized, open-label, phase I clinical trial with dose escalation of the KIF20A-derived peptide combined with GEM for patients with advanced unresectable pancreatic cancer. The primary end-point in this trial was the safety of peptide vaccination combined with GEM. Secondary end-points were clinical outcome, immunologic responses, and determination of the optimal dose of peptide for further clinical trials. The MST is calculated as time after first vaccination. Immunologic responses were assessed by measuring levels of interferon (IFN)-γ production from antigen-specific T cells responding to the KIF20A-derived peptide.

Adverse Events and Clinical Responses

Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (CTCAE). Dose-limiting toxicity was defined as a hematological toxicity of ≥grade 4 and nonhematological toxicity of ≥grade 3. Clinical response was evaluated based on clinical observations and radiologic findings. All known sites of disease were evaluated on a monthly basis by computed tomography (CT) or magnetic resonance imaging before vaccination and after each course. Tumor size was estimated by direct measurement of the region of abnormal enhancement observed on CT or magnetic resonance imaging. Patients were assigned a response category according to the Response Evaluation Criteria in Solid Tumors. Overall survival (OS) was estimated from the date of initial vaccination to the date of death.

Treatment Protocol

Dose was escalated from 0.5 to 1 to 3 mg/body of the vaccinated peptide. The KIF20A-derived peptide was administered emulsified with incomplete Freund's adjuvant (Montanide ISA-51VG; SEPPIC, Paris, France) by subcutaneous injection on days 1, 8, 15, and 22 in a 28-day treatment course. GEM was administered intravenously at a dose of 1000 mg/m^2 on days 1, 8, and 15. Administration of KIF20A and GEM was performed repeatedly for at least one course until satisfying the criteria for treatment cessation. We injected peptide vaccine biweekly after 8 times weekly injection (2 courses) to avoid the risk of exhaustion of the immune response and we chose right inguinal lesion or left inguinal lesion alternately as injection site.

Enzyme-linked ImmunoSpot (ELISPOT) Assay

Antigen-specific T-cell response was estimated by ELISPOT assay following in vitro sensitization.[17] Immunologic response of all cases is shown in Table 3. Representative data are shown in Figure 1. Frozen peripheral blood mononuclear cells (PBMCs) derived from the patient were thawed at the same time, and viability was confirmed as >90%. PBMCs (5×10^5/mL) were cultured with 10 μg/mL of the candidate peptide and 100 IU/mL of interleukin (IL)-2 (Novartis, Emeryville, CA) at 37°C for 2 weeks. Peptide was added into the culture on days 0 and 7. Following CD4$^+$ cell depletion using a Dynal CD4-positive isolation kit (Invitrogen, Carlsbad, CA), IFN-γ ELISPOT assay was performed with vaccinated peptide-pulsed or HIV-Env peptide-pulsed (as the control) HLA-A*2402-positive TISI cells (IHWG Cell and Gene Bank, Seattle, WA) using Human IFN-γ ELISpot PLUS kit (MabTech, Cincinnati, OH) and MultiScreen-IP 96-plate (Millipore, Bedford, MA). Briefly, HLA-A*2402-positive TISI cells were incubated overnight with 20 μg/mL of respective peptides; thereafter, residual peptides in the media were washed out to prepare peptide-pulsed TISI cells as stimulator cells. Prepared CD4$^-$ cells were cultured overnight with peptide-pulsed stimulator cells (2×10^4 cells/well) at 1:1, 1:2, 1:4, and 1:8 mixture ratios of responder cells to stimulator cells (R/S ratio) on 96-well plates (Millipore) at 37°C. To confirm IFN-γ productivity, responder cells were stimulated overnight with phorbol 12-myristate 13-acetate (66 ng/mL) and ionomycin (3 μg/mL), then applied to IFN-γ ELISPOT assay (2.5×10^3 cells/well) without stimulator cells. All ELISPOT assays were performed in triplicate wells. Plates were analyzed using an automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology, Shaker Heights, OH), and ImmunoSpot Professional Software version 5.0 (Cellular Technology). The number of peptide-specific spots was calculated by subtracting the spot number in the control well from the spot number of a well with vaccinated peptide-pulsed stimulator cells. Antigen-specific T-cell response was classified into 4 grades (−, +, ++, or +++) according to the algorithm flow chart described in our previous report (+++: IFN-γ-producing cell is contained >0.2%, ++: IFN-γ-producing cell is contained 0.02%–0.2%, +: IFN-γ producing cell is contained 0.01%–0.02%, −: IFN-γ producing cell is contained <0.01% in the sample applied for ELISPOT).[18] Sensitivity of our ELISPOT assay was estimated as approximately average level by the ELISPOT panel of the Cancer Immunotherapy Consortium [CIC (http://www.cancerresearch.org/consortium/assay-panels/)].[19]

Statistical Analysis

Statistical analysis was performed using the unpaired Student t test for the ELISPOT assay. A value of $P < 0.05$ was considered statistically significant. OS curves were estimated using Kaplan-Meier methodology. Any correlations with clinical outcomes were estimated using the Wilcoxon rank sum test.

RESULTS

Feasibility and Adverse Reactions

No severe adverse effects of grade 4 or higher were observed. Nine patients satisfying the eligibility criteria were enrolled in this study. Patient characteristics are shown in Table 1. All patients developed grade 1 or 2 local skin reactions with redness and induration at the injection sites. In particular, all 9 patients completed at least 1 course of treatment and all 9 developed immunologic reactions at

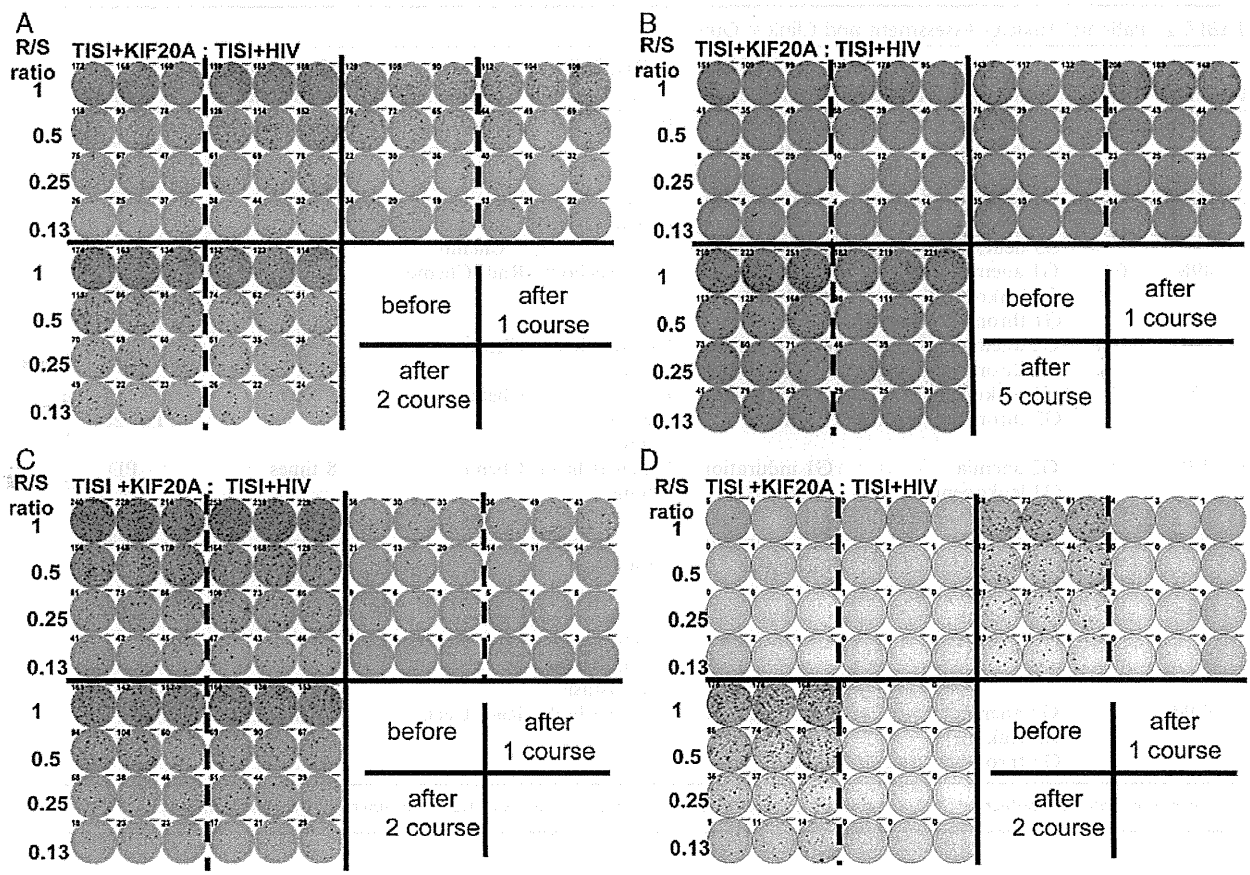

FIGURE 1. Representative immunologic monitoring assays detecting antigen-specific T-cell responses in patient 2 (A), 3 (B), 6 (C), and 7 (D), which were induced interferon-γ (IFN-γ)-producing cells. Positivity of antigen-specific T-cell response was quantitatively defined according to the evaluation tree algorithm.[18] In brief, the peptide-specific spots (SS) were the average of triplicates by subtracting the HIV peptide-pulsed stimulator well from the immunized peptide-pulsed stimulator well. The %SS means the percentage of SS among the average spots of the immunized peptide-pulsed stimulator well. The positivity of antigen-specific T-cell response were classified into four grades (−, +, ++, and +++) depending on the amounts of peptide-specific spots and invariability of peptide-specific spots at different responder/stimulator ratios.

the injection sites. G2/G3 leukopenia and neutropenia and G1/G2 thrombocytopenia appeared to be caused by GEM itself. G1–G3 anemia appeared attributable to the progression of pancreatic cancer, although GEM is known to cause anemia as well. No febrile neutropenia was recorded during the course of this study. High-grade fever, fatigue, diarrhea, headache, rash, and itching were not observed in any patients. No hematologic, cardiovascular, hepatic, or renal toxicity was observed during or after vaccination (Table 2). The vaccination protocol was well tolerated in all patients enrolled.

TABLE 1. Patients' Characteristics

	Peptide (n = 3)		
Characteristics	**0.5 mg**	**1.0 mg**	**3.0 mg**
Age (y)		62 (48–74)	
Sex			
Male/female	1/2	2/1	1/2
Performance status (ECOG)			
0/1	2/1	1/2	1/2
Disease stage			
III/IV	1/2	2/1	1/2
Prior therapy			
Radical operation	1	0	0
Chemotherapy	3	3	3
Radiotherapy	1	0	1

UICC-TNM classification of malignant tumors was used for determination of clinical stage.
ECOG indicates Eastern Cooperative Oncology Group.

Immunologic Monitoring

The KIF20A-specific T-cell (IFN-γ-producing cells) response was determined using the IFN-γ ELISPOT assay. Representative antigen-specific T-cell responses are shown in Figure 1. In which, PBMC from patients 2, 3, 6, and 7 produced higher level of IFN-γ after vaccine than the level of pre-vaccination (Fig. 1). Positive antigen-specific T-cell (IFN-γ producing cells) responses specific to the vaccinated peptide were determined as described in the Materials and methods section. IFN-γ-producing cells were induced in 4 of 9 patients (P2, P3, P6, and P7), and IFN-γ producing cells were increased in 4 of the 9 patients (P1, P5, P8, and P9) (Table 3). Antigen-specific T-cell responses were seen in all 3 patients receiving 0.5 mg vaccination; in 2 of the 3 patients receiving 1 mg; and in all 3 patients receiving 3 mg.

TABLE 2. Patients' Toxicity Assessment and Clinical Outcome

Patients	Peptide (mg)	Hematologic Toxicity	Local Adverse Effect	RECIST Lesion	Prior Therapy	Frequency of Vaccination	Evaluation	Prognosis (d)
1 61F	0.5	G2 anemia G3 leukopenia	G2 induration redness	Pancreas uncus tumor	Palliative operation, Chemo	16 times	SD PFS:175 d	218
2 53F	0.5	G2 leukopenia G2 thrombocytopenia G3 neutropenia	G1 induration redness	Liver metastasis	Distal pancreatectomy, Chemo	8 times	PD	366
3 49M	0.5	G1 anemia G3 leukopenia G1 thrombocytopenia	G2 induration redness	Pancreas body tumor	Rad, Chemo	22 times	SD PFS:170 d	251
4 70M	1	G2 anemia G1 thrombocytopenia	G0 induration	Pancreas body tumor	Chemo	7 times	PD	71
5 72M	1	G2 leukopenia G2 thrombocytopenia	G1 induration redness	Pancreas uncus tumor	Chemo	8 times	SD PFS: 28 d	208
6 53F	1	G2 anemia G3 leukopenia G2 thrombocytopenia	G1 induration redness	Pancreas head tumor	Chemo	8 times	PD	173
7 74F	3	G3 anemia G2 leukopenia G2 neutropenia	G2 induration redness	Pancreas head tumor	Chemo	8 times	PD	120
8 64F	3	G1 anemia G2 leukopenia	G2 induration redness	Pancreas head tumor Multiple liver metastasis	Chemo	8 times	PD	94
9 60M	3	G2 anemia G3 leukopenia G2 thrombocytopenia	G2 induration redness	Pancreas body tumor	Rad, Chemo	11 times	SD PFS: 85 d	126

Chemo indicates chemotherapy; PD, progression disease; PFS, progression-free survival; Rad, radiotherapy; SD, stable disease.

Antigen-specific T-cell response (IFN-γ-producing cells) could therefore be induced by the KIF20A peptide vaccine at a high rate, even in combination with GEM.

Clinical Responses and OS

Four of the 9 patients achieved stable disease (SD), with the other 5 patients showing progression disease (PD). The disease control rate was 44%. Achievement of SD was seen in 2 of the 3 patients receiving 0.5 mg vaccination, 1 of 3 patients receiving 1 mg, and 1 of 3 patients receiving 3 mg (Table 2). Images from CT of a patient with SD are shown in Figure 2. All 4 patients who achieved SD showed induction of the antigen-specific T-cell responses at a level of 2 + or more (+ + or + + +) for the KIF20A peptide (Table 3). In contrast, 3 of the 5 patients who showed PD displayed induction of antigen-specific T-cell responses from negative (–) to reaction (+). No relationship between peptide doses and the antigen-specific T-cell responses or clinical outcome was identified. The MST calculated as time after first vaccination was 173 days and 1-year survival rate was 11.1% (Fig. 3). The MST calculated as time after first diagnosis was 18 months and 1-year survival rate was 78%.

DISCUSSION

The only cure for pancreatic cancer is surgical resection, although this malignancy is difficult to detect early. At the time of diagnosis, approximately 60% of patients are already beyond the possibility of surgical resection.[20–23] GEM is currently used as the standard therapy for unresectable pancreatic cancer. Noninferiority of S-1 compared with GEM was shown in GEST study conducted in Japan, but the superiority of the combination of GEM and S-1 over GEM monotherapy has not yet been conclusively proven.[24] The establishment of combination therapy with GEM has been performed many times to date. One large randomized controlled phase III trial with erlotinib showed significantly prolonged survival time ($P = 0.038$),[25] but the difference was only about 10 days. In another study, MST was 11.1 months for the FOLFIRINOX group, compared with 6.8 months in the GEM group, showing a significant difference ($P < 0.001$). However, markedly more adverse events were noted in the FOLFIRINOX group.[26] Taking into account toxicity and economic aspects, the development of new drugs for advanced pancreatic cancer is urgently required.

The present study investigated a novel cancer vaccine therapy for pancreatic cancer using a KIF20A-derived peptide in combination with GEM. To the best of our knowledge, this is the first report to use the KIF20A-derived peptide in a clinical trial. We observed no severe adverse events related to the treatment in this trial (Table 2). Specific adverse events caused by this vaccine treatment were local redness and induration at the injection site; however, no events > grade 3 were observed. In several papers we have examined—their authors show that the intradermic administration of vaccine has proven superior to subcutaneous administrations.[27]

We tried to administer the KIF20A-derived peptide emulsified with incomplete Freund's adjuvant as close as possible to the dermis—so as to activate the dendritic cells.

Because the volume was 2 mL, it was too much to inject the intradermic administration. We think the data of this study were able to prove that IFN-γ-producing cells

TABLE 3. Immunologic Response

Dose of Peptides (mg)	Case Number	Course	CTL Reaction		Clinical Response	HLA Typing
			KIF20A	CMV		
0.5	1	Pre	+ +	+ + +	SD	A*2402/A*3303
		Post 1	+	+ +		
		Post 2	+	+ + +		
		Post 3	+ +	+ +		
	2	Pre	–	+	PD	A*2402/A*0201
		Post 1	+	+ +		
		Post 2	+ +	+ + +		
	3	Pre	–	+ + +	SD	A*2402
		Post 1	–	+ + +		
		Post 2	+	+ + +		
		Post 3	+ +	+ + +		
		Post 4	+ + +	+ + +		
		Post 5	+ +	+ + +		
1	4	Pre	–	+ +	PD	A*2402/A*1101
		Post 1	–	+ + +		
	5	Pre	+ +	+ +	SD	A*2402/A*1101
		Post 1	+ +	+ +		
		Post 2	+ +	+		
	6	Pre	–	+	PD	A*2402/A*3303
		Post1	–	+		
		Post2	+	+ +		
3	7	Pre	–	–	PD	A*2402/A*0206
		Post 1	+ + +	+		
		Post 2	+ + +	+ +		
	8	Pre	+	+ + +	PD	A*2402/A*0206
		Post 1	+	+ + +		
		Post 2	NT	+ + +		
	9	Pre	+	+ + +	SD	A*2402/A*2601
		Post 1	–	+ + +		
		Post 2	+ +	+ + +		

CMV indicates cytomegalovirus; CTL, cytotoxic T lymphocytes; HLA, human leukocyte antigen; PD, progression disease; SD, stable disease.

could be enhanced by this message. Immunologic responses in this trial were measured by local redness and induration at the injection site and antigen-specific T-cell responses against the vaccinated peptide. No dose-limiting toxicity was observed in any dose cohort. We injected peptide vaccine biweekly after 8 times weekly injection (2 courses) to avoid the risk of exhaustion of the immune response. We chose right inguinal lesion or left inguinal lesion alternately as injection site. Local redness and induration as CTCAE grade 2 at the injection site were observed in all 3 patients with the 3 mg vaccination (Table 2). However, achievement of SD was seen in 2 of the 3 patients receiving 0.5 mg vaccination, 1 of 3 patients receiving 1 mg, and 1 of 3 patients receiving 3 mg (Table 2). In this study, we consider that the optimum peptide dosage for future clinical trials could be set at a level of at least 0.5 mg or more.

As a point of immunologic monitoring, IFN-γ-producing cells were induced in 4 of 9 patients (P2, P3, P6, and P7), and IFN-γ-producing cells were increased in 4 of the 9 patients (P1, P5, P8, and P9). Patient 4 in whom IFN-γ-producing cells response was absent was suffering from acute cholangitis during vaccination. Prior to vaccination, the proportion of lymphocyte in this patient was only 13%. Yamamoto et al[28] previously reported that peptide-reactive cellular and humoral responses to vaccinated peptides in postvaccination PBMCs and sera were lower for advanced pancreatic cancer patients than for patients with other solid cancers. They commented that these results suggest that immunity in advanced pancreatic cancer is more depressed than in other epithelial cancers. Alternatively, a more suitable peptide repertoire might be provided for pancreatic cancer patients. Miyazawa et al[29] reported that VEGFR2-169 peptide-specific positive CTL responses were observed in 11 of 18 patients who received at least one course of vaccination. Ishikawa et al[30] reported URLC10-177 peptide-specific positive CTL responses in 4 of 7 patients. KIF20A peptide vaccine therefore induced or further increased peptide-specific T-cell responses at a higher rate compared with these reports. Four of the 9 patients achieved SD, whereas the other 5 patients showed PD (Table 2). Achievement of SD was seen in 2 of the 3 patients receiving 0.5 mg vaccination, 1 of 3 patients receiving 1 mg, and 1 of 3 patients receiving 3 mg (Table 2). There is no evidence that the SD was mediated by the vaccine. This could simply be the natural history of this disease, but it is interesting to note that all 4 patients who achieved SD showed antigen-specific T-cell response of + + or + + + reactions for KIF20A peptide. In contrast, 3 of the 5 patients who experienced PD showed antigen-specific T-cell response from negative to 1 + reaction. A tendency toward a correlation between antigen-specific T-cell response and clinical outcome was suggested, but no significant relationship was proved ($P = 0.074$). However, the population was too small to be evaluated in this clinical trial. Many prior peptide vaccine studies have demonstrated significant immunogenicity against the peptides utilized in the vaccine without translating into significant clinical benefits. This will be our next focus but

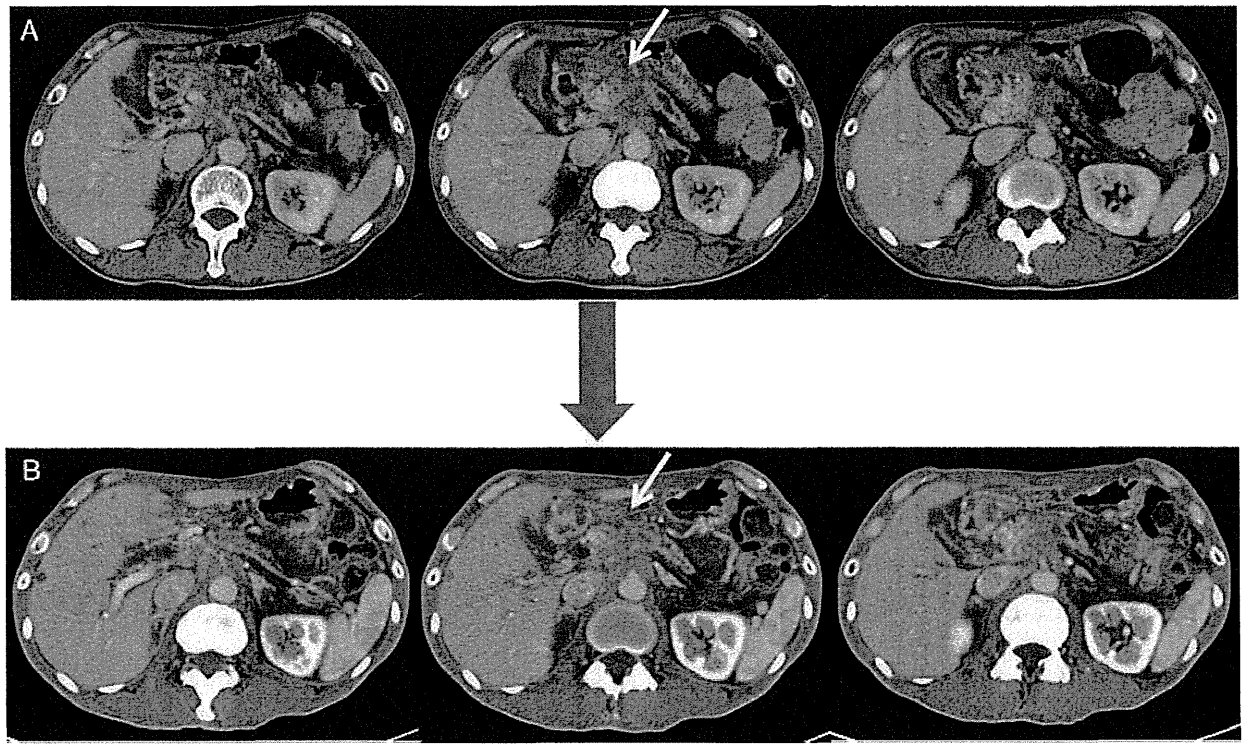

FIGURE 2. Axial contrast-enhanced computed tomography (CT) scans of patient 3 who showed SD. A, Axial contrast-enhanced CT showing locally advanced tumor of the pancreatic body before vaccination (arrow). B, Axial contrast-enhanced CT after 4 months shows SD of the pancreatic body mass (arrow). SD indicates stable disease.

prior to that the important thing is to identify a new peptide that possesses high immunogenicity. This protocol was well tolerated, and peptide-specific IFN-γ-producing cells were found to be induced or increased by the KIF20A-derived peptide vaccine at a high rate, even in combination with the anticancer agent, GEM. Although safety and immunogenicity are promising, no conclusions can be made about efficacy at this level of study. We are proceeding on to conduct a phase II clinical trial among patients with advanced pancreatic cancer by combining KIF20A-derived peptide with GEM as the first line. Therefore, additional efficacy data would be required before committing to a large randomized controlled trial.

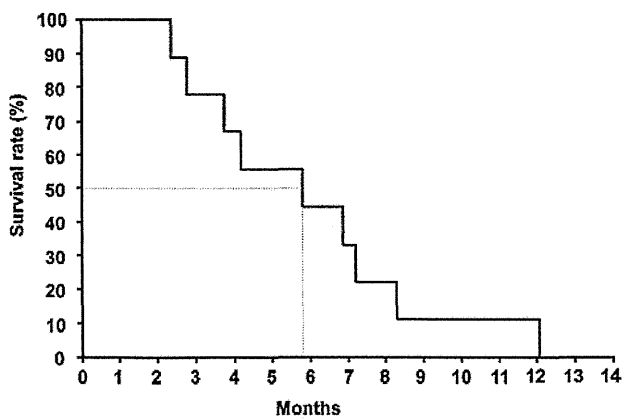

FIGURE 3. Overall survival measured using the Kaplan-Meier method. The median survival time after first vaccination was 173 days. One-year survival rate was 11.1%.

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CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

All authors have declared there are no financial conflictsof interest with regard to this work.

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