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

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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 carcinoma5 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.
### 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  |
| KIF20A 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 (≥ Grade 2/ < Grade 2)| 0.046  | 0.122  |

**Abbreviations:** CRP, C-reactive protein; CTL, cytotoxic T lymphocyte.
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*2402/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 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.
robust responses to VEGFR1-derived peptides. The ability of the vaccine to induce a strong T-cell response seemed to be linked not only to the nature of the epitope but also to 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 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 succumbed 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...
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. Many Phase III clinical trials testing anticancer vaccines have failed for a variety of reasons. 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. 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. 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. Another method to circumvent this issue, based on the antineoplastic agent denileukin diftitox, has also been examined in animal and human models. 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, CTLA-4), as well as antibodies targeting programmed cell death 1 (PD1, best known as PD-1) and its major ligand (CD274, best known as PD-L1) 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.

Several peptides derived from tumor-associated antigens have already been tested in clinical trials. In the present study, we selected peptides from 4 distinct antigens, inducing strong immune responses in vivo. KIF20A is a conserved motor domain that binds to microtubules, while CDCA1 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 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/mm³, hemoglobin > 8 mg/dL, platelet counts > 100,000/mm³, 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 (KVYLVRPPLL), CDCA1 (VYGIRLEHF), VEGFR1 (DYLNEWGSRF), and VEGFR2 (RFVPDGHNRI). 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 axillary 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 (CLIA) 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 × 10⁶ 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 × 10⁵ cells/mL) were cultured in the presence of 10 μg/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 × 10⁶ 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 × 10⁵ 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 cytometer (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-aminoacynominocycin 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 \( p < 0.05 \) 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.

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Supplemental Materials

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

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