

Long-term Vaccination with Multiple Peptides Derived from Cancer-Testis Antigens Can Maintain a Specific T-cell Response and Achieve Disease Stability in Advanced Biliary Tract Cancer

Atsushi Aruga^{1,2}, Nobuhiro Takeshita¹, Yoshihito Kotera¹, Ryuji Okuyama¹, Norimasa Matsushita¹, Takehiro Ohta¹, Kazuyoshi Takeda³, and Masakazu Yamamoto¹

Abstract

Purpose: The prognosis of patients with advanced biliary tract cancer (BTC) is extremely poor and there are only a few standard treatments. We conducted a phase I trial to investigate the safety, immune response, and antitumor effect of vaccination with four peptides derived from cancer-testis antigens, with a focus on their fluctuations during long-term vaccination until the disease had progressed.

Experimental Design: Nine patients with advanced BTC who had unresectable tumors and were refractory to standard chemotherapy were enrolled. HLA-A*2402-restricted epitope peptides, lymphocyte antigen 6 complex locus K, TTK protein kinase, insulin-like growth factor-II mRNA-binding protein 3, and DEP domain containing 1 were vaccinated subcutaneously once a week at doses of 0.5, 1, or 2 mg and continued until disease progression. The adverse events were assessed by Common Terminology Criteria for Adverse Events and the immune response was monitored by an enzyme-linked immunospot assay or by flow cytometry. The clinical effects observed were tumor response, progression-free survival (PFS), and overall survival (OS).

Results: Four-peptide vaccination was well tolerated. No grade 3 or 4 adverse events were observed. Peptide-specific T-cell immune responses were observed in seven of nine patients and clinical responses were observed in six of nine patients. The median PFS and OS were 156 and 380 days. The injection site reaction and CTL induction seemed to be prognostic factors of both PFS and OS.

Conclusions: Four-peptide vaccination was well tolerated and seemed to provide some clinical benefit to some patients. These immunologic and clinical responses were maintained over the long term through continuous vaccinations. *Clin Cancer Res*; 19(8); 2224–31. ©2013 AACR.

Introduction

Biliary tract cancer (BTC) is not a common disease worldwide, but is prevalent in East Asia and Latin America. The occurrence rate is gradually increasing and there is a high mortality rate because most cases of BTC are not diagnosed until advanced and inoperable. At this time, very few standard treatments have been established for BTC (1, 2), and thus development of new treatment modalities is urgently needed. Recently, cancer vaccines using synthetic peptides have been undergoing development throughout the world,

and their safety and clinical efficacy have been reported (3, 4). Cancer peptide vaccines are capable of inducing antigen-specific CTLs *in vivo* (5). In this study, we selected 4 cancer-testis antigens that were overexpressed in nearly 100% of BTC cancer cells, as revealed by cDNA microarray technology coupled with laser microdissection in a previous study. Patients were enrolled on the basis of unresectable BTC refractory to standard chemotherapy, and no additional diagnostic procedures were needed, except for genotyping for HLA-A*2402. This study was conducted as a phase I study to assess the safety and antigen-specific immune response of a 4-peptide vaccination in patients with advanced BTC. Patients were vaccinated on a continuous basis over the long term until their disease had progressed, a time when we assessed the safety of the vaccination by CTCAE v3.0 as a primary endpoint and the antigen-specific immune response and clinical benefit as secondary endpoints.

Materials and Methods

Patient eligibility

Patients with unresectable BTC (intrahepatic bile duct cancer, extrahepatic bile duct cancer, or gallbladder cancer)

Authors' Affiliations: ¹Department of Gastroenterological Surgery; ²Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University; and ³Department of Immunology, Juntendo School of Medicine, Tokyo, Japan

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Corresponding Author: Atsushi Aruga, Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan. Phone: 81-3-5367-9945; Fax: 81-3-3359-6046; E-mail: aruga@abmes.twmu.ac.jp

doi: 10.1158/1078-0432.CCR-12-3592

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Translational Relevance

Numerous clinical reports have shown that peptide vaccines can induce peptide-specific CTLs to mediate tumor-specific responses *in vivo*. However, there is currently no suitable peptide vaccine for biliary tract cancer (BTC). In addition, the immunologic and clinical responses of peptide vaccines injected over the long term have not been sufficiently investigated. In this phase I clinical study, we investigated the safety, antitumor effect, and immunologic response of a multiple-peptide vaccination administered until the signs of disease progression. Our results showed that a four-peptide vaccine induced each of the respective peptide-specific CTLs, and these responses lasted throughout a long-term vaccination without any serious adverse events. These observations suggest that multiple-peptide vaccination could be a novel and promising therapy for patients with BTC.

who were refractory to standard chemotherapy were eligible for this study. All patients were required to have an HLA-A type of A*2402. Additional inclusion criteria consisted of age between 20 and 80 years, absence of severe organ function impairment, white blood cell count between 2,000 and 10,000/mm³, hemoglobin >8 mg/dL, platelet count >100,000/mm³, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) <100 IU/L, and total bilirubin <2 mg/dL. Performance status measured by the Eastern Cooperative Oncology Group (ECOG) scale was 0 to 2. It was required that there should be at least 4-week interval since the last chemotherapy. The exclusion criteria consisted of pregnancy, serious infections, severe underlying disease, severe allergic disease, 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 BTC between April 2008 and March 2009 were invited to participate after providing their informed consent. The HLA-A genotypes of these patients were examined, and the 9 patients with an HLA-A type of A*2402 were enrolled. Four peptides were used for the vaccine, lymphocyte antigen 6 complex locus K (LY6K)-177 (RYCNLEGPPI; ref. 6), TTK protein kinase (TTK)-567 (SYRNEIAYL; ref. 7), insulin-like growth factor-II mRNA-binding protein 3 (IMP3)-508 (KTVNELQNL; ref. 8), and DEP domain containing 1 (DEPDC1; EYYELFVNI; ref. 9). These peptides were chosen from a large number of antigens identified by using cDNA microarray technology coupled with laser microdissection because they were the most highly overexpressed in BTC samples in a previous study. The purity (>97%) of the peptides was determined by analytic high-performance liquid chromatography (HPLC) and mass spectrometry analysis. The endotoxin levels and bioburden of these peptides were tested and determined to be acceptable based

on the GMP grade for the vaccines (NeoMPS Inc.). These peptides were mixed with incomplete Freund's adjuvant (IFA; Montanide ISA51, SEPPIC), which has been proven safe and used in many clinical studies, and injected subcutaneously into the inguinal or the axilla site. Each of the 4 peptides at doses of 0.5, 1, or 2 mg was injected subcutaneously into 3 patients once a week until the eighth vaccination and once or twice a week after the ninth vaccination as a monotherapy until the patient was judged to exhibit disease progression. This dose escalation design was chosen on the basis of limitations in the production of the emulsion component. The primary endpoint in this study was the assessment of toxicities caused by the vaccination based on the Common Terminology Criteria for Adverse Events version 3 (CTCAE v.3). The secondary endpoint was the assessment of the immunologic response, tumor response, progression-free survival (PFS), and overall survival (OS) from the first dose given. For the image analysis, computed tomography (CT) scan or ultrasound was conducted during the prevaccination period and every fourth vaccination until the disease had progressed. This study was approved by the Institutional Review Board at Tokyo Women's Medical University (Tokyo, Japan) and was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, 000003207). Informed consent was obtained from all the patients and the procedures followed were in accordance with the Declaration of Helsinki.

Measurement of immunologic response

Lymphocyte preparation for immunologic monitoring. The performance of the immunologic assay at the central laboratory was 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 (PBL) were obtained from the patients at the prevaccination period and after every fourth vaccination. Peripheral blood was taken by venipuncture, collected in an EDTA tube, and transferred to the center laboratory within 24 hours at room temperature. Within 24 hours of blood collection, PBLs were isolated using Ficoll-Paque Plus (GE Healthcare Bio-Sciences) density gradient solution and were stored at -80°C in cell stock media (Juji Field) without serum at 5 × 10⁶ cells/mL. After thawing, the cell viability was confirmed to be more than 90% by Trypan-blue dye staining.

Enzyme-linked immunospot assay. The peptide-specific CTL response was estimated by enzyme-linked immunospot (ELISPOT) assay following *in vitro* sensitization. Frozen peripheral blood mononuclear cells (PBMC) derived from the same patient were thawed at the same time, and the viability was confirmed to be more than 90%. PBMCs (5 × 10⁵/mL) were cultured with 10 μg/mL of the respective peptide and 100 IU/mL of interleukin (IL)-2 (Novartis) at 37°C for 2 weeks. The peptide was added to the culture at day 0 and 7. Following CD4⁺ cell depletion by a Dynal CD4

Table 1. Patient characteristics

No.	Age/sex	Tumor site		Previous therapy ^b	Peptide dose, mg	Number of vaccines	Clinical response ^c	PFS, d	OS, d	TM ^d	ISR ^e (Grade)	Lymphocyte number (%)	Peptide-specific CTL ^f				
		Primary ^a	Metastases										LY6K	TTK	IMP3	DEPDC1	
1	64/M	IBD	Liver/peritoneum	GEM, CDDP, TS-1	0.5	4	PD	47	87	NT	1	955 (8.3)	1+	1+	0	1+	
2	75/M	GB	Liver/peritoneum	GEM, TS-1	0.5	4	PD	31	66	NT	0	1,390 (17.3)	NA	NA	NA	NA	
3	67/M	GB	Lymph nodes	Ope, GEM, AFTV	0.5	54	CA	491	639	Decrease	2	1,801 (39.4)	3+	3+	2+	3+	
4	65/F	IBD	Lung/lymph nodes/bone	Ope, GEM	1.0	29	Stable disease	372	1,044	ND	2	1,521 (34.1)	3+	1+	1+	3+	
5	59/F	GB	Liver/lymph nodes	Ope, GEM, TS-1	1.0	19	Stable disease	176	380	Decrease	2	2,124 (22.1)	3+	1+	1+	3+	
6	76/F	EBD	Liver/lymph nodes	Ope, GEM, CBDCA, VP-16	1.0	9	PD	64	109	Increase	1	1,450 (30.2)	3+	1+	1+	3+	
7	69/F	IBD	Liver	Ope, GEM, TS-1	2.0	35	Stable disease	428	764	NC	2	1,216 (23.2)	3+	2+	1+	3+	
8	74/M	IBD	Lung	Ope, GEM, TS-1	2.0	16	Stable disease	156	466	ND	2	2,399 (38.2)	3+	3+	1+	3+	
9	78/F	EBD	Liver/peritoneum	GEM, TS-1	2.0	16	CA	137	179	Decrease	1	1,249 (36.1)	3+	1+	1+	3+	

^aPrimary tumor site: EBD, extrahepatic bile duct; GB, gallbladder; IDB, intrahepatic bile duct.

^bPrevious therapy: CBDCA, carboplatin; GEM, gemcitabine; CDDP, cisplatin.

^cClinical response: CA, clinical activity. CA means that CR or PR was not achieved and tumor regression occurred.

^dNC, no change; ND, not detected; NT, not tested; TM, tumor marker.

^eISR: injection site reaction evaluated according to CTCAE v3.0.

^fCTL: CTLs were assessed by the algorithm shown in Supplementary Fig. S2. NA, not analyzed.

Positive Isolation Kit (Invitrogen), an IFN-γ ELISPOT assay was conducted using a Human IFN-γ ELISpot PLUS kit (MabTech) according to the manufacturer's instructions. Briefly, HLA-A*2402-positive B lymphoblast TISI cells (IHWG Cell and Gene Bank) were incubated with 20 μg/mL of vaccinated peptides overnight, and then the residual peptide in the media was washed out to prepare peptide-pulsed TISI cells as the stimulation cells. Prepared CD4⁺ cells were cultured with peptide-pulsed TISI cells (2 × 10⁴ cells/well) at a 1:1, 1:2, 1:4, or 1:8 mixture ratio of responder cells to stimulator cells (R:S ratio) on a 96-well plate (Millipore) at 37°C overnight. Nonpeptide-pulsed TISI cells were used as negative control stimulator cells. To confirm IFN-γ productivity, responder cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (3 μg/mL) overnight, then applied to an IFN-γ ELISPOT assay (2.5 × 10³ cells/well) without stimulator cells. All ELISPOT assays were conducted in triplicate wells. The plates were analyzed by an automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology, Ltd.) and ImmunoSpot Professional Software Version 5.0 (Cellular Technology, Ltd.). The number of peptide-specific spots was calculated by subtracting the number of spots in the control well from the number of spots in the well with peptide-pulsed TISI cells. The sensitivity of our ELISPOT assay was estimated as an approximately average level by an ELISPOT panel of the Cancer Immunotherapy Consortium [CIC (<http://www.cancerresearch.org/consortium/assay-panels/>)].

Flow cytometry assay. The expression of peptide-specific T-cell receptors was analyzed on a FACS-Canto II flow cytometer (Becton Dickinson) using LY6K-derived epitope peptide-MHC pentamer-phycoerythrin (PE; ProImmune, Ltd.), TTK, or DEPDC1-derived epitope peptide-MHC dextramer-PE (Immudex) according to the manufacturer's instructions. HIV-derived epitope peptide (RYLRDQQLL)-MHC pentamer or dextramer-PE was used as a negative control. Briefly, the *in vitro* cultured T cells were incubated with peptide-MHC pentamer or dextramer-PE for 10 minutes at room temperature, then treated with fluorescein isothiocyanate (FITC)-conjugated anti-human CD8 monoclonal antibody (mAb), allophycocyanin (APC)-conjugated anti-human CD3 mAb, PE-Cy7-conjugated anti-human CD4 mAb, and 7-aminoactinomycin D (BD Pharmingen) at 4°C for 20 minutes. Conventional 2-color phenotypical analysis was also conducted with FITC-conjugated anti-human CD3, CD4, and CD8 mAb plus PE-conjugated anti-human CD28, CD57, and CD62L mAb (BD Pharmingen) and CXCR3 plus CCR4 mAb (R&D Systems) to assess the change of T-helper cell (T_H)1/T_H2 subsets, cytotoxic cell subset, and central memory/effector memory subsets.

Statistical analysis

Statistical analyses of prognostic factors of PFS or OS were done using the Kaplan-Meier method and evaluated by log-rank test. A P value less than 0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using SPSS statistics software.

Table 2. Adverse events assessed by CTCAE v3.0

Adverse events	Total (%)	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Grade 4 (%)
Hemoglobin	6 (66.7)	5 (55.6)	1 (11.1)	0	0
Lymphopenia	2 (22.2)	2 (22.2)	0	0	0
Injection site reaction	8 (88.9)	3 (33.3)	5 (55.6)	0	0

NOTE: Hemoglobin and lymphopenia were observed before the first vaccination. No other adverse events were seen throughout the period of peptide vaccination.

Results

Patient characteristics

Nine patients (4 males and 5 females; median age, 70 years; range, 59–78) whose HLA type was A*2402 were enrolled in this study (Table 1). Their primary tumor site was the intrahepatic bile duct in 4 cases, the extrahepatic bile duct in 2 cases, and the gallbladder in 3 cases. They had several metastases to the liver, lungs, lymph nodes, peritoneum, and bone. Previous therapies consisted of operation, gemcitabine, cisplatin, tegafur–gimeracil–oteracil potassium (TS-1), carboplatin, or etoposide (VP-16). Two patients dropped out after the first follow-up study and 1 patient dropped out after second study. Six patients were vaccinated more than 16 times, with the maximum number being 54 times.

Assessment of toxicity

Toxicity was assessed by CTCAE v3.0. Eight of 9 patients developed grade 1 or 2 injection site reactions. Low hemoglobin and lymphopenia were observed before the first vaccination and were not worsened throughout the vaccination term. No other adverse events were seen through peptide vaccination. Therefore, the multiple-peptide vaccine therapy was well tolerated without any adverse events of grade 3 or higher (Table 2) up to a dose of 2 mg for each peptide, or a total of 8 mg for all 4 peptides.

Antigen-specific immune response

In the ELISPOT assay, one or more wells showed 25 spots or more observed in 7 of 9 patients (Supplementary Fig. S1). Table 1 summarizes the responses to each antigen in each patient based on the algorithm given in Supplementary Fig. S2. The number of peptide-specific IFN- γ spots per section increased with the number of vaccinations (Fig. 1A and B), and the number of LY6K-specific CTLs also increased (Fig. 1C) gradually. These immune responses were not found for all antigens and were not found in all patients. In particular, the anti-LY6K and DEPDC1 responses were greater than the responses to TTK or IMP3. In the patient receiving vaccination for the longest period of time, patient 3, these immune responses were observed over the long term with vaccination (Fig. 2A). However, patient 3 might not be a representative case, as the immune responses to antigens were already elevated before vaccination in this patient. The reason for the early elevation of antigens in this

patient might be that he had received the standard chemotherapy plus the autologous formalin-fixed tumor vaccine (AFIV; ref. 10) at approximately 1 year before enrolling in this study. The phenotypical analysis was shown in Figs. 1D and 2B.

Clinical response

Two patients exhibited a clinical activity indicating tumor regression in some targets (Fig. 2C and D) but did not achieve a complete remission (CR) or partial response (PR), 4 had stable disease, and 3 had progressive disease (PD) as judged after the eighth vaccination. The 6 patients who were judged to have clinical activity or stable disease continued to be administered the vaccination until their disease was judged to be PD. Although stable disease was achieved through long-term vaccination, all of the patients eventually showed disease progression, and all had died within 3 years of the first vaccination. The median PFS of all patients after the first vaccination was 156 days (Fig. 3A) and the median OS was 380 days (Fig. 3B). In the univariate analysis of the prognostic factors, the patients who developed grade 2 local skin reaction at the vaccination site, peptide-specific CTLs (i.e., CTLs with over 25 IFN- γ spots), or a type I immune condition (i.e., a CXCR3⁺CCR4⁻ T-cell ratio of over 8%) showed a longer survival time than those with either PFS or OS (Table 3). These parameters were therefore considered prognostic factors.

Discussion

BTC is well known as a disease with an extremely poor prognosis. Operation in the early stage is the only curative treatment of BTC, but unfortunately most of these lesions are not found until the late stage. There are only a few standard chemotherapies for this disease, that is, gemcitabine, gemcitabine plus cisplatin, and/or TS-1. Both PFS and OS of the patients treated with the standard chemotherapies were almost the same as the data of the patients in this study although they were enrolled after the failure of the standard chemotherapies. This result indicated the potential of the peptide vaccine for improving PFS and OS in patients with BTC. In this study, no CR or PR was seen, but long-term stable disease was seen in some patients, and thus the OS seemed to improve. This

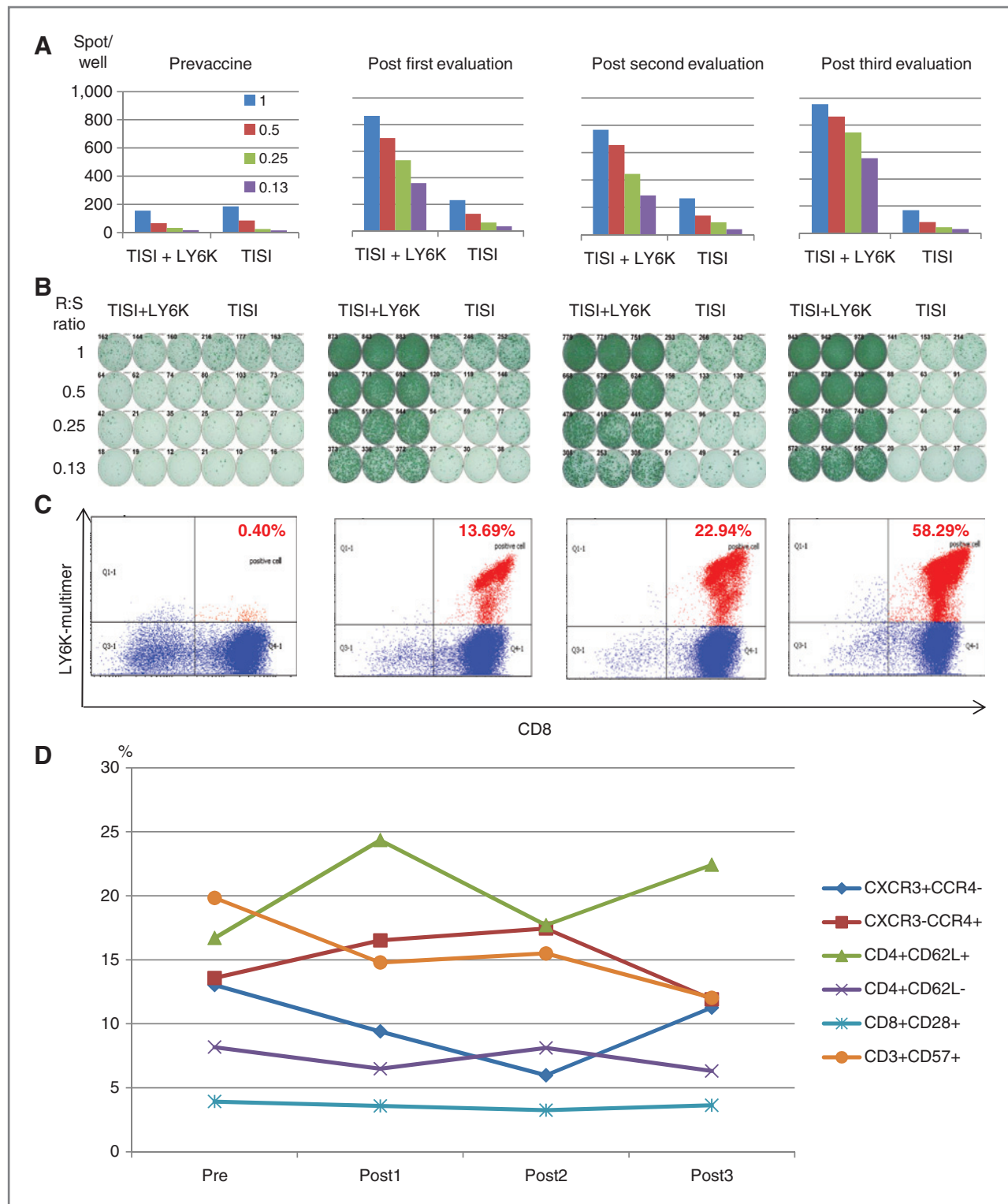


Figure 1. Immunologic monitoring in case 8. A, LY6K peptide-specific IFN- γ -positive spots at several R:S ratio. B, IFN- γ ELISPOT assay for LY6K. C, the value of LY6K-multimer positive/CD8 positive cells. D, phenotype analysis of lymphocytes by flow cytometry.

is a special characteristic of cancer vaccine therapy; therefore, we should plan a phase II study to assess the PFS and/or OS in a randomized study.

There have been numerous clinical trials on cancer vaccine therapy, and the safety, immune response, and clinical effects have already been reported. Dendritic cell

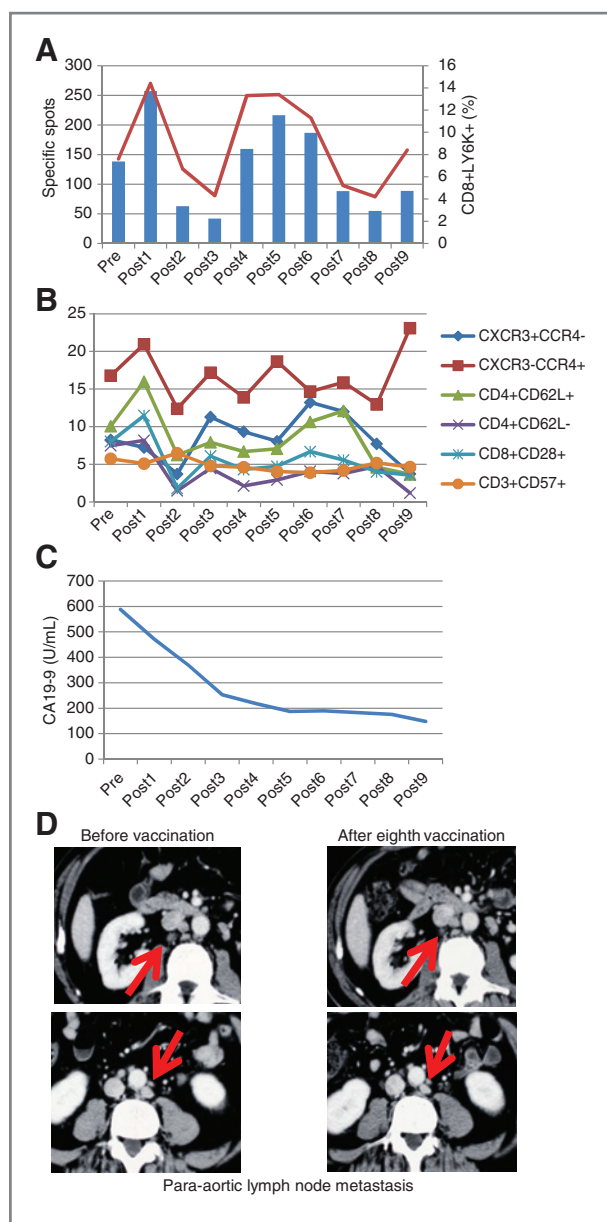


Figure 2. Immunologic and clinical response assessment in case 3. A, LY6K peptide-specific IFN- γ ELISPOT assay (bar) and pentamer analysis (line). The R:S ratio is 0.25. B, phenotype analysis of lymphocytes by flow cytometry. C, serum dosage of CA19-9. D, CT imaging of para-aortic lymph node metastases before and after vaccination. The tumor size was diminished from 40 to 21 mm. Several lymph node metastases regressed markedly, but not all achieved a CR or PR.

vaccine therapies in particular have been investigated for a long time. We previously reported the clinical use of a dendritic cell vaccine in an adjuvant setting for intrahepatic bile duct cancer (11), and a similar trial was also reported by another group (12). The dendritic cell vaccine seems to be a useful tool for adjuvant therapy, but it is difficult to harvest and induce the dendritic cells, and the high cost and severe regulations of the cell processing

are also major problems. Therefore, the peptide vaccine is expected to be developed as an attractive alternative for cancer vaccine therapy. The peptides used in this study have already been used in different combinations in other clinical trials for esophageal cancer (13, 14) or bladder cancer (15). These reports have shown the safety of these peptides and their ability to induce peptide-specific CTLs *in vivo* when injected individually. Our study is the first trial to use injection of a mixture of 4 peptides into one site, and our results showed that each of the peptide-specific CTLs was induced *in vivo*. The immune responses to the 4 peptides were not equal. Each of the 4 peptides was synthesized using the most immunogenic sequence measured in a previous *in vitro* study. There might be some differences in the immunogenic reaction among these 4 peptides. This result is meaningful in part because a single vaccination of mixed peptides would be less painful for a patient than 4 separate vaccinations of the individual peptides. In our previous study, these 4 antigens were expressed on almost all BTCs (data not shown). Therefore, it is not necessary to test the expression of antigens on each tumor. At present, there are very few trials to develop new therapeutics for BTC, and thus this peptide vaccine must be developed immediately.

There are many candidates for peptides that have already undergone clinical trials (16–18). The results of these previous studies suggest that peptide-specific CTL induction is needed to achieve a clinical effect by peptide vaccine therapy. The ability to induce peptide-specific CTLs is not equal among all peptides, and the 4 peptides that we used here were very effective. In particular, LY6K and DEPDC1 are very hopeful candidates for inducing a strong CTL response, and thereby improving the PFS and OS. In the blood examination, patients with a lymphocyte count more than 1,500 tended to show a better prognosis.

Although peptide vaccines are a hopeful candidate for cancer therapy, their clinical efficacy is currently limited. To obtain a good result in the clinical trials with immunotherapy, an important problem to be solved is the immune suppression in cancer patients. Regulatory T cells are one of the most critical factors in the suppression of immune response. Nonmyeloablative chemotherapy to deplete the regulatory T cells is a promising technique to overcome these problems (19). A CCR4 antagonist or anti-CCR4 mAb that has already been approved in Japan might be a useful tool, because the regulatory T cells express CCR4 (20, 21). Another method using denileukin difitox has also been examined in animal models and human models (22, 23). The regulation of the host immune condition is crucial for obtaining a good immune response in a clinical study. An anti-CTLA-4 mAb (ipilimumab) has also been approved for melanoma (24), and anti-PD-1 (25) or anti-PD-L1 (26) showed promising results in some clinical studies. A combination therapy could be a more successful anticancer strategy for cancer immunotherapy in the future.

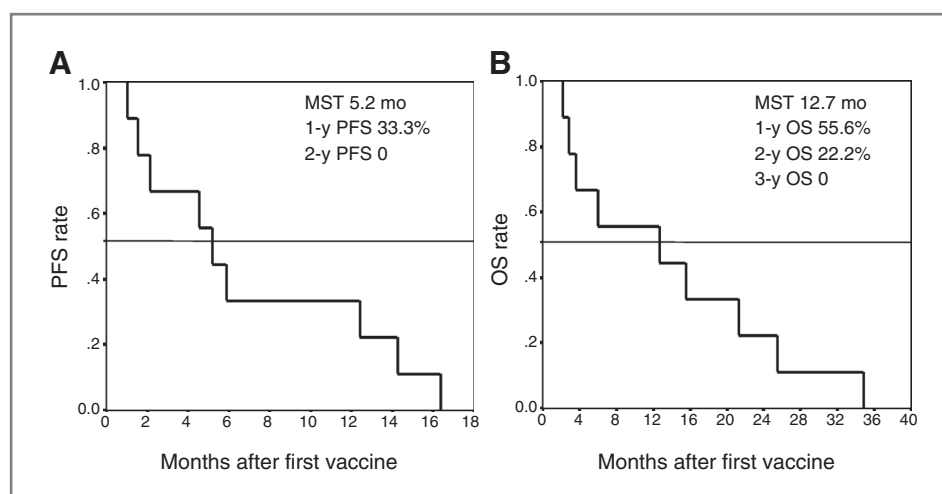


Figure 3. PFS and OS in all enrolled patients. A, PFS after first vaccination. The mean survival time (MST) was 5.2 months and the 1-year PFS ratio was 33.3%. B, OS after first vaccination. The MST was 12.7 months and the 1-year OS ratio was 55.6%.

At this stage, there is only one cancer vaccine, Sipuleucel-T, which was approved by the U.S. Food and Drug Administration (FDA) in 2011 (27). However, several phase III randomized trials of cancer peptide vaccines are ongoing throughout the world, and new candidates are coming soon. In this study, we showed that long-term vaccination with a multiple cancer peptide vaccine was feasible and resulted in the prolongation of PFS and OS in patients with advanced BTC. To obtain success in a clinical study, the next goal in the progress of cancer vaccines might be an adjuvant therapy after curative operation. Another possibility would be a combination with first-line chemotherapy, but we have not yet evaluated the ability of chemotherapy to induce antigen-specific CTLs *in vivo*. We should be careful when combining an immunotherapy and chemotherapy in order that these modalities do not counteract each other.

In this report, we showed the safety, immune response, and clinical use of a peptide vaccine in patients

with advanced BTC. We anticipate that this immunotherapy will eventually be established as the standard therapy for BTC. We are planning to advance to a phase II randomized study in an advanced cancer setting, an adjuvant setting after curative operation or a study in which the peptide vaccine would be the first choice therapy along with standard chemotherapy to verify our hypothesis.

Conclusions

We have shown that a cancer peptide vaccine therapy using a mixture of 4 peptides was well tolerated, induced peptide-specific CTLs, and seemed to provide some clinical benefit in some patients with advanced BTC throughout the long-term vaccination. On the basis of these results, a phase II clinical study with a suitable protocol is warranted along with subsequent clinical trials to verify the usefulness of the cancer peptide vaccine.

Table 3. Prognostic factors of PFS or OS

Factors	PFS	OS
Sex (male/female)	0.954	0.297
Age (≥ 65 / <65)	0.728	0.544
Primary tumor site (I/G, I/E, G/E) ^a	0.679, 0.207, 0.364	0.235, 0.207, 0.364
LY6K CTL spots (≥ 25 / <25)	0.002	0.002
TTK CTL spots (≥ 25 / <25)	0.017	0.005
DEPDC1 CTL spots (≥ 25 / <25)	0.002	0.002
LY6K multimer + CTLs ($\geq 10\%$ / $<10\%$)	0.113	0.840
CXCR3 ⁺ CCR4 ⁻ ($\geq 8\%$ / $<8\%$)	0.017	0.005
Skin reaction of vaccine site ($\geq G2$ / $<G2$)	0.003	0.003
Vaccine dose (0.5 mg/1 mg, 0.5 mg/2 mg, 1 mg/2 mg)	0.988, 0.988, 0.694	0.343, 0.343, 0.832
Lymphocyte (%; $\geq 30\%$ / $<30\%$)	0.545	0.423
Lymphocyte (number; $\geq 1,500$ / $<1,500$)	0.155	0.155

^aPrimary tumor site: E, extrahepatic bile duct; G, gallbladder; I, intrahepatic bile duct.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Aruga, T. Ohta

Development of methodology: A. Aruga, T. Ohta

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Aruga, N. Takeshita, N. Matsushita

Analysis and interpretation of data (e.g., statistical analysis, bio-statistics, computational analysis): K. Takeda

Writing, review, and/or revision of the manuscript: A. Aruga

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Kotera, R. Okuyama

Study supervision: M. Yamamoto

Acknowledgments

The authors thank Prof. Yusuke Nakamura and Drs. Takuya Tsunoda and Koji Yoshida of the Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo for their excellent advice and cooperation and for providing the peptides.

Grant Support

This study was supported in part by a grant from the Nakayama Cancer Research Institute to the authors.

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Received November 21, 2012; revised February 22, 2013; accepted February 26, 2013; published OnlineFirst March 11, 2013.

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