Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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

Running title: Multiple-peptide vaccination for biliary tract cancer

Atsushi Aruga[1,2], Nobuhiro Takeshita[1], Yoshihito Kotera[1], Ryuji Okuyama[1], Norimasa Matsushita[1], Takehiro Ohta[1], Kazuyoshi Takeda[3] and Masakazu Yamamoto[1]

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

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

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

The authors have no conflict of interest to report.

Contact Information
Atsushi Aruga: aruga@abmes.twmu.ac.jp
Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan
81-3-5367-9945
Nobuhiro Takeshita: nobutks@yahoo.co.jp
Yoshihito Kotera: kodera@ige.twmu.ac.jp
Ryuji Okuyama: ryujioku@gmail.com
Norimasa Matsushita: matsushita0710@hotmail.com
Takehiro Ohta: otakehiro72@yahoo.co.jp
Masakazu Yamamoto: yamamoto@ige.twmu.ac.jp

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Department of Gastroenterological Surgery, Tokyo Women's Medical University, Tokyo, Japan
81-3-3353-8111
Kazuyoshi Takeda: ktakeda@juntendo.ac.jp
Department of Immunology, Juntendo School of Medicine,
2-1-1 Hongo, Bunkyo-ku, Tokyo
81-3-3813-3111

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. 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 biliary tract cancer.

Abstract

Purpose: The prognosis of patients with advanced biliary tract cancer is extremely poor and there are only a few standard treatments. We performed a phase I trial to investigate the safety, immune response and anti-tumor 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 biliary tract cancer 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 (IGF)-II mRNA binding protein 3 and DEP domain containing 1 were vaccinated subcutaneously once a week 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 7 of 9 patients and clinical responses were observed in 6 of 9 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 appeared to provide some clinical benefit to some patients. These immunologic and clinical responses were maintained over the long-term through continuous vaccinations.

Key words

peptide vaccine, cancer-testis antigen, immunotherapy, biliary tract cancer

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

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

reported (3, 4). Cancer peptide vaccines are capable of inducing antigen-specific cytotoxic T cells *in vivo* (5). In this study, we selected four 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 performed as a Phase I study to assess the safety and antigen-specific immune response of a four-peptide vaccination in patients with advanced biliary tract cancer. Patients were vaccinated on a continuous basis over the long-term until their disease had progressed, at which time 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) 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 2000 and 10000/mm^3, hemoglobin > 8 mg/dL, platelet count > 100,000/mm^3, AST and ALT < 100IU/L, and total bilirubin< 2 mg/dl. Performance status measured by the ECOG scale was 0 to 2. It was required that there be an 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

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

an HLA-A type of A*2402 were enrolled. Four peptides were used for the vaccine, LY6K-177 (RYCNLEGPPI) (6), TTK-567 (SYRNEIAYL) (7), IMP3-508 (KTVNELQNL) (8) and DEPDC1(EYYELFVNI) (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 analytical 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., San Diego, CA). These peptides were mixed with incomplete Freund's adjuvant (IFA; Montanide ISA51, SAEPPIC), which has been proven safe and used in many clinical studies, and injected subcutaneously into the inguinal or the axicilla site. Each of the four peptides at doses of 0.5 mg, 1 mg or 2 mg was injected subcutaneously into three patients once a week until the 8th vaccination and once or twice a week after the 9th vaccination as a monotherapy until the patient was judged to exhibit disease progression. This dose escalation design was chosen based on 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 immunological response, tumor response, progression-free survival (PFS) and overall survival (OS) from the 1st dose given. For the image analysis, CT scan or ultrasound was performed during the pre-vaccination period and every 4th vaccination until the disease had progressed. 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, 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

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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. PBLs were obtained from the patients at the pre-vaccination period and after every 4th vaccination. Peripheral blood was taken by venipuncture, collected in an EDTA tube and transferred to the center laboratory within 24 hrs at room temperature. Within 24 hrs of blood collection, PBLs were isolated using Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Piscataway, NJ) density gradient solution and were stored at -80°C in cell stock media (Juji Field, Tokyo) without serum at $5X10^6$ cells/ml. After thawing, the cell viability was confirmed to be more than 90% by trypan-blue dye staining.

Enzyme-linked immunospot (ELISPOT) assay. The peptide-specific CTL response was estimated by ELISPOT assay following *in vitro* sensitization. Frozen PBMCs derived from the same patient were thawed at the same time, and the viability was confirmed to be more than 90%. PBMCs ($5X10^5$/ml) were cultured with 10 mg/ml of the respective peptide and 100IU/mL of IL-2 (Novartis, Emeryville, CA) at 37ºC for two weeks. The peptide was added to the culture at day 0 and day 7. Following CD4+ cell depletion by a Dynal CD4 Positive Isolation Kit (Invitrogen, Carlsbad, CA), an IFN-γ ELISPOT assay was performed using a Human IFN-γ ELISpot PLUS kit (MabTech, Nacka Strand, Sweden) according to the manufacturer's instructions. Briefly, HLA-A*2402-positive B-lymphoblast TISI cells (IHWG Cell and Gene Bank, Seattle, WA) 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 ($2X10^4$ 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, Bedford, MA) at 37ºC overnight. Non-peptide-pulsed TISI cells were used as negative control stimulator cells. To confirm IFN-γ productivity, responder cells were stimulated with PMA and ionomycin (3 μg/ml) overnight, then applied to an IFN-γ ELISPOT assay ($2.5X10^3$ cells/well) without stimulator cells. All ELISPOT assays were performed in triplicate wells. The plates were analyzed by an automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology, Ltd., Shaker

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Heights, OH) 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, San Jose, CA) using LY6K-derived epitope peptide-MHC pentamer-PE (ProImmune, Ltd., Oxford, UK), TTK or DEPDC1-derived epitope peptide-MHC dextramer-PE (Immudex, Copenhagen, Denmark) 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 FITC-conjugated anti-human CD8 mAb, APC-conjugated anti-human CD3 mAb, PE-Cy7-conjugated anti-human CD4 mAb, and 7-AAD (BD Pharmingen, San Diego, CA) at 4ºC for 20 minutes. Conventional two-color phenotypical analysis was also performed with FITC-conjugated anti-human CD3, CD4, and CD8 mAb plus PE-conjugated anti-human CD28, CD57, and CD62L mAb (BD Pharmingen, San Diego, CA) and CXCR3 plus CCR4 mAb (R&D Systems, Minneapolis, MN) in order to assess the change of Th1/Th2 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 SSPS statistics software.

Results

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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 3 cases, the extrahepatic bile duct in 3 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 (GEM), cisplatin (CDDP), tegafur-gimeracil-oteracil potassium (TS-1), carboplatin (CBDCA) or etoposide (VP-16). Two patients dropped out after the 1st follow-up study and 1 patient dropped out after 2nd 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 1st 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 four 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 Figure S1). Table 1 summarizes the responses to each antigen in each patient based on the algorithm given in Supplementary Figure S2. The number of peptide-specific IFN-γ spots per section increased with the number of vaccinations (Fig. 1A, 1B), and the number of LY6K-specific CTLs also increased (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, since 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

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

standard chemotherapy plus the autologous formalin-fixed tumor vaccine (AFTV) (10) at approximately one year before enrolling in this study.

Clinical response

Two patients exhibited a clinical activity (CA) indicating tumor regression in some targets but did not achieve a CR or PR, 4 had stable disease (SD) and 3 had progressive disease (PD) as judged after the 8th vaccination. The 6 patients who were judged to have CA or SD continued to be administered the vaccination until their disease was judged to be PD. Although SD was achieved through long-term vaccination, all of the patients eventually showed disease progression, and all had died within 3 years of the 1st vaccination. The median progression-free survival (PFS) of all patients after the first vaccination was 156 days (Fig. 3A) and the median overall survival (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 1 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 for BTC, but unfortunately most of these lesions are not found until the late stage. There are only a few standard chemotherapies for this disease-i.e., GEM, GEM plus CDDP and/or S-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 complete remission (CR) or partial response (PR) was seen, but long-term stable disease (SD) was seen in some patients, and thus the overall survival (OS) appeared to improve. This is a special characteristic of cancer vaccine therapy; therefore we should plan a phase II study to assess the PFS and/or

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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 vaccine therapies in particular have been investigated for a long time. We previously reported the clinical utilization 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 employ injection of a mixture of four peptides into one site, and our results showed that each of the peptide-specific CTLs was induced *in vivo*. The immune responses to the four peptides were not equal. Each of the four 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 four peptides. This result is meaningful in part because a single vaccination of mixed peptides would be less painful for a patient than four separate vaccinations of the individual peptides. In our previous study, these four 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 four 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 over 1500 tended to

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

show a better prognosis.

Although peptide vaccines are a hopeful candidate for cancer therapy, their clinical efficacy is currently limited. In order 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. Non-myeloablative chemotherapy to deplete the regulatory T cells is a promising technique to overcome these problems (19). A CCR4 antagonist or anti-CCR4 monoclonal antibody (mAb) which has already been approved in Japan might be a useful tool, because the regulatory T cells express CCR4 (20, 21). Another method using denileukin diftitox 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 (Ipilimimab) 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 anti-cancer strategy for cancer immunotherapy in the future.

At this stage, there is only one cancer vaccine, Sipuluecel-T, which was approved by the 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 demonstrated 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 biliary tract cancer. In order 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 demonstrated the safety, immune response and clinical utilization of a peptide vaccine in patients with advanced biliary tract cancer. We anticipate that this immunotherapy will eventually be established as the standard therapy for biliary tract cancer. 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

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

would be the 1st choice therapy along with standard chemotherapy to verify our hypothesis.

Conclusions

We have shown that a cancer peptide vaccine therapy using a mixture of four peptides was well tolerated, induced peptide-specific CTLs and appeared to provide some clinical benefit in some patients with advanced BTC throughout the long-term vaccination. Based on 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.

Disclosure of Potential Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

Author Contributions

Conception and design: A. Aruga
Development of methodology: A. Aruga
Acquisition of data: A. Aruga, N. Takeshita, N. Matsushita
Analysis and interpretation of data: K. Takeda
Writing, review, and/or revision of the manuscript: A. Aruga
Administrative, technical, or material support: Y. Kotera, R. Okuyama
Study supervision: M. Yamamoto, T. Ohta

Acknowledgments

The authors would like to thank Prof. Yusuke Nakamura, Dr. Takuya Tsunoda, and Dr. 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 funds from the Nakayama Cancer

Research Institute.

References

1. Okusaka T, Nakachi K, Fukutomi A, Mizuno N, Ohkawa S, Funakoshi A, et al. Gemcitabine alone or in combination with cisplatin in patients with biliary tract cancer: a comparative multicenter study in Japan. British J Cancer 2010;103,469-474.
2. Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, et al. Cisplatin plus Gemcitabine versus Gemcitabine for Biliary Tract Cancer. N Engl J Med 2010;362,1273-1281.
3. Sangha R, Butts C. L-BLP25: A Peptide Vaccine Strategy in Non-Small Cell Lung Cancer. Clin Cancer Res 2007;13(15Suppl):4652s-4654s.
4. Schwartzentruber DJ, Lawson DH, Richards JM, Conry RM, Miller DM, Treisman J, et al. gp100 Peptide Vaccine and Interleukin-2 in Patients with Advanced Melanoma. N ENGL J MED 2011;364(22):2119-2127.
5. Sawada Y, Yoshikawa T, Nobuoka D, Shirakawa H, Kuronuma T, MotomuraY, et al. Phase I Trial of a Glypican-3-Derived Peptide Vaccine for Advanced Hepatocellular Carcinoma: Immunologic Evidence and Potential for Improving Overall Survival. Clin Cancer Res 2012;18(13),3686-3696.
6. Ishikawa N, Takano A, Yasui W, Inai K, Nishimura H, Ito H, et al. Cancer-Testis Antigen Lymphocyte Anteigen 6 Complex Lolus K Is a Serologic Biomarker and a Therapeutic Target for Lung and Esophageal Carcinomas. Cancer Res 2007;67(24),11601-11611.
7. Suda T, Tsunoda T, Daigo Y, Nakamura Y, Tahara H. Identification of human leukocyte antigen-A24-restricted epitope peptides derived from gene products upregulated in lung and esophageal cancers as novel targets for immunotherapy. Cancer Sci. 2007;98(11),1803-1808.
8. Mizukami Y, Kono K, Daigo Y, Rakano A, Tsunoda T, Kawaguchi Y, et al. Detection of novel cancer-testis antigen-specific T-cell responses in TIL, regional lymph nodes, and PBL in patients with esophageal squamous cell carcinoma. Cancer Sci. 2008;99(7),1448-1454.
9. Kanehira M, Harada Y, Takata R, Shuin T, Miki T, Fujioka T, et al. Involvement of upregulation of DEPDC1 (DEP domain containing 1) in

bladder carcinogenesis. Oncogene 2007;26,6448-6455.

10. Muragaki Y, Maruyama T, Iseki H, Tanaka M, Shinohara C, Takakura K, et al. Phase I/II trial of autologous formalin-fixed tumor vaccine concomitant with fractionated radiotherapy for initially-diagnosed Glioblastoma. J NeuroSurgery 2011;115(2),248-255.
11. Shimizu K, Kotera Y, Aruga A, Takeshita N, Takasaki K, Yamamoto M. Clinical utilization of postoperative dendritic cell vaccine plus activated T-cell transfer in patients with intrahepatic cholangiocarcinoma. J HepatobiliaryPancreatSci 2012;19,171-178.
12. De Vleeschouwer S, Fieuws S, Rutkowski S, Van Calenbergh F, Van Loon J, Goffin J, et al. Postoperative adjuvant dendritic cell-based immunotherapy in patients with relapsed glioblastoma multiforme. Clin Cancer Res 2008;14(10):3098-3104.
13. Kono K, Mizukami Y, Daigo Y, Takano A, Masuda K, Yoshida K, et al. Vaccination with multiple peptides derived from novel cancer-testis antigens can induce specific T-cell responses and clinical responses in advanced esophageal cancer. Cancer Sci 2009;100(8),1502-1509.
14. Kono K, Iinuma H, Akutsu Y, Tanaka H, Hayashi N, Uchikado Y, et al. Multicenter, phase II clinical trial of cancer vaccination for advanced esophageal cancer with three peptides derived from novel cancer-testis antigens. J Translational Med 2012;10,141.
15. Obara W, Ohsawa R, Kanehira M, Takata R, Tsunoda T, Yoshida K, et al. Cancer Peptide Vaccine Therapy Developed from Oncoantigens Identified through Genome-wide Expression Profile Analysis for Bladder Cancer. Jpn J ClinOncol 2012;42(7),591-600.
16. Miyazawa M, Ohsawa R, Tsunoda T, Hirono S, Kawai M, Tani M, et al. Phase I clinical trial using peptide vaccine for human vascular endothelial growth factor receptor 2 in combination with gemcitabine for patients with advanced pancreatic cancer. Cancer Sci 2010;101(2):433-439.
17. Ohno S, Okuyama R, Aruga A, Sugiyama H, Yamamoto M. Phase I Trial of Wilm's Tumor 1 (WT1) Peptide Vaccine with GM-CSF or CpG in Patients with Solid Malignancy. Anticancer Res 2012;32,2263-2270.
18. Noguchi M, Kakuma T, Uemura H, Nasu Y, Kumon H, Hirao Y, et al. A randomized phase II trial of personalized peptide vaccine plus low dose estramustine phosphate (EMP) versus standard dose EMP in patients

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

with castration resistant prostate cancer. Cancer Immunol Immunother 2010;59:1001-1009.

19. Koike N, Pilon-Thomas S, Mule JJ. Nonmyeloablative chemotherapy followed by T-cell adoptive transfer and dendritic cell-based vaccination results in rejection of established melanoma. J Immunother 2008;31(4):402-412.
20. Pere H, Montier Y, Bayry J, Quintin-Colonna F, Merillon N, Dransart E, et al. A CCR4 antagonist combined with vaccines induces antigen-specific CD8+ T cells and tumor immunity against self antigens. Blood 2012;118(18):4853-4862.
21. Ishida T, Joh T, Uike N, Yamamoto K, Utsunomiya A, Yoshida S, et al. Defucosylated Anti-CCR4 Monoclonal Antibody (KW-0761) for Relapsed Adult T-Cell Leukemia-Lymphoma: A Multicenter Phase II Study. J Clin Oncol 2012;30(8):837-842.
22. Matsushita N, Pilon-Thomas SA, Martin LM, Riker AI. Comparative methodologies of regulatory T cell depletion in a murine melanoma model. J Immunol Methods 2008;333(1-2):167-179.
23. Morse MA, Hobeika AC, Osada T, Serra D, Niedzwiechi D, Lyerly DH, et al. Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. Blood 2010; 112(3):610-618.
24. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus Dacarbazine for Previously Untreated Metastatic Melanoma. N ENGL J MED 2011;3646(26),2517-2526.
25. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, Activity, and Immune Correlates of Anti-PD-1 Antibody in Cancer. N ENGL J MED 2012;366(26),2443-2454.
26. Brahmer JR, Tykodi SS, Chow LQM, Hwu W-J, Topalian SL, Hwu P, et al. Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer. N ENGL J MED 2012;366(26),2455-2465.
27. Cheever MA, Higano CS. PROVENGE (Sipuleucel-T) in Prostate Cancer: The First FDA-Approved Therapeutic Cancer Vaccine. Clin Cancer Res 2011;17(11):3520-3526.

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure legends

Fig. 1 Immunologic monitoring of LY6K peptide-specific T-cell responses in case 8.

Fig. 2 Immunologic and clinical response assessment in case 3. A. LY6K peptide-specific IFN-γ ELISPOT assay (bar) and pentamer analysis (line). The responder/stimulator 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 mm to 21 mm. Several lymph node metastases regressed markedly, but not all achieved a CR or PR.

Fig. 3 Progression-free survival and overall survival in all enrolled patients. A. Progression-free survival after 1st vaccination. The MST was 5.2 months and the 1-year PFS ratio was 33.3%. B. Overall survival after 1st vaccination. The MST was 12.7 months and the 1-year OS ratio was 55.6%.

Table 1. Patient Characteristics

No.	Age/sex	Tumor site: Primary[a]	Tumor site: Metastases	Previous therapy[b]	Peptide dose (mg)	Number of vaccines	Clinical response[c]	PFS (days)	OS (days)	TM[d]	ISR[e] (Grade)	Lymphocyte number (%)	Peptide-specific CTL[f]: 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	1390(17.3)	NA	NA	NA	NA
3	67/M	GB	Lymph nodes	Ope, GEM, AFTV	0.5	54	CA	491	639	Decrease	2	1801(39.4)	3+	3+	2+	3+
4	65/F	IBD	Lung/Lymph nodes/Bone	Ope, GEM	1.0	29	SD	372	1044	ND	2	1521(34.1)	3+	1+	1+	3+
5	59/F	GB	Liver/Lymph nodes	Ope, GEM, TS-1	1.0	19	SD	176	380	Decrease	2	2124(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	1450(30.2)	3+	1+	1+	3+
7	69/F	IBD	Liver	Ope, GEM, TS-1	2.0	35	SD	428	764	NC	2	1216(23.2)	3+	2+	1+	3+
8	74/M	IBD	Lung	Ope, GEM, TS-1	2.0	16	SD	156	466	ND	2	2399(38.2)	3+	3+	1+	3+
9	78/F	EBD	Liver/Peritoneum	GEM, TS-1	2.0	16	CA	137	179	Decrease	1	1249(36.1)	3+	1+	1+	3+

[a]Primary tumor site: IDB, intrahepatic bile duct; GB, gallbladder; EBD, extrahepatic bile duct.

[b]Previous therapy: CBDCA, Carboplatin; GEM, Gemcitabine; CDDP, Cisplatin; TS-1,Tegafur-Gimeracil-Oteracil potassium; AFTV, autologous formalin-fixed tumor vaccine.

[c]Clinical response: PD, progressive disease; SD, stable disease; PD, progressive disease; CA, clinical activity. CA means that CR or PR was not achieved and tumor regression occurred.

[d]TM, tumor marker; NT, not tested; ND, not detected; NC, no change.

[e]ISR: injection site reaction evaluated according to CTCAE v3.0.

[f]CTL: CTLs were assessed by the algorithm shown in Supplementary Figure S2. NA, not analyzed.

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Table 2. Adverse events assessed by CTCAE v3.0

Adverse Events	Total (%)	Grade1(%)	Grade2(%)	Grade3(%)	Grade4(%)
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

Hemoglobin and lymphopenia were observed before the 1st vaccination.

No other adverse events were seen throughout the period of peptide vaccination.

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592

Table 3. Prognostic factors of PFS or OS

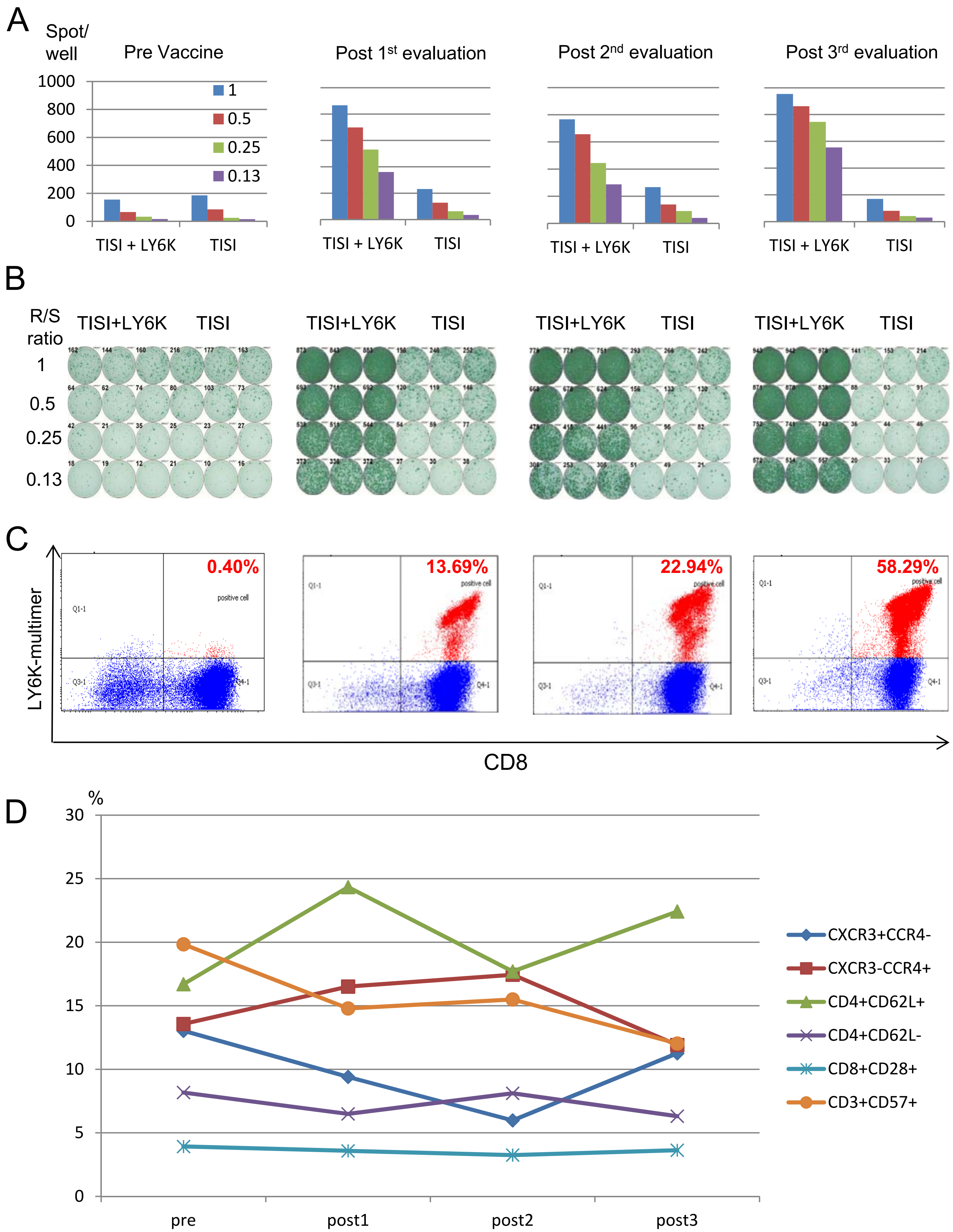
Factors	PFS	OS
Sex (male/female)	0.954	0.297
Age (≥65/<65)	0.728	0.544
Primary tumor site (I/G, I/E, G/E)[a]	0.679, 0.207, 0.364	0.235, 0.207, 0.364
LY6K CTL Spots (≥25/<25)	0.002	0.002
TTK CTL Spots (≥25/<25)	0.017	0.005
DEPDC1 CTL Spots (≥25/<25)	0.002	0.002
LY6K multimer+ CTLs (≥10%/<10%)	0.113	0.840
CXCR3+CCR4- (≥8%/<8%)	0.017	0.005
Skin reaction of vaccine site (≥G2/<G2)	0.003	0.003
Vaccine dose (0.5mg/1mg, 0.5mg/2mg, 1mg/2mg)	0.988, 0.988, 0.694	0.343, 0.343, 0.832
Lymphocyte (%) (≥30%/<30%)	0.545	0.423
Lymphocyte (number) (≥1500/<1500)	0.155	0.155

[a] Primary tumor site: I, intrahepatic bile duct; E, extrahepatic bile duct; G, gallbladder.

Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592

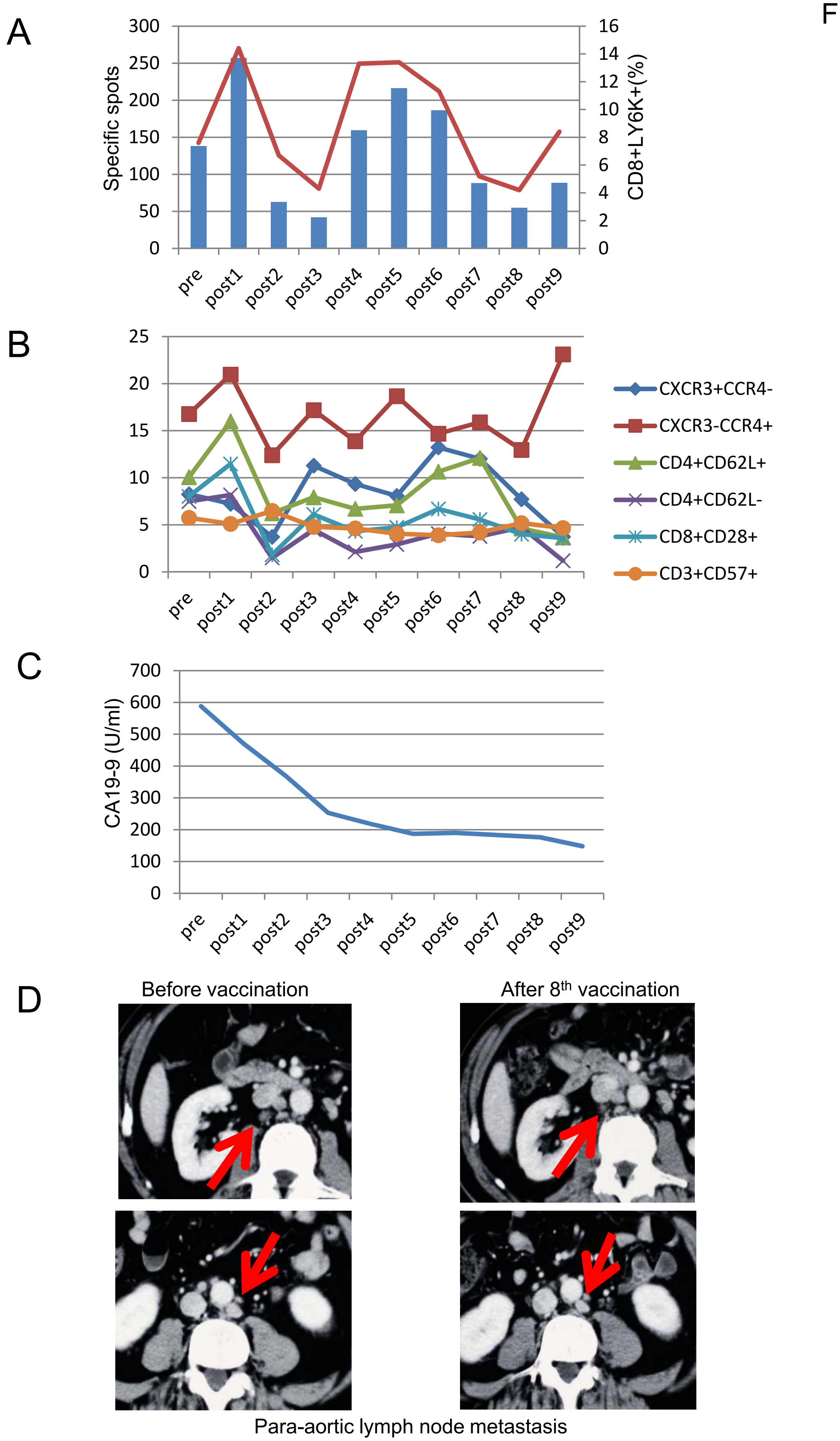
Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Fig. 1



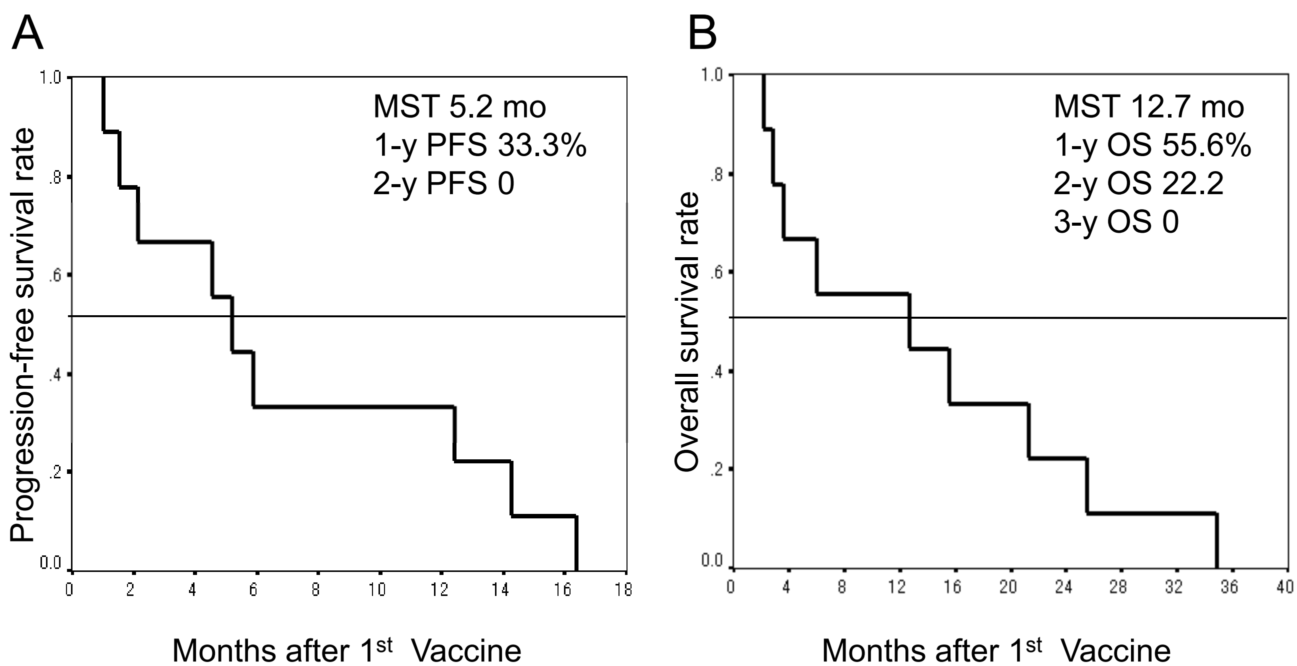
Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Fig. 2



Author Manuscript Published OnlineFirst on March 11, 2013; DOI: 10.1158/1078-0432.CCR-12-3592
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Fig. 3



American Association for Cancer Research

Clinical Cancer Research

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, Nobuhiro Takeshita, Yoshihito Kotera, et al.

Clin Cancer Res Published OnlineFirst March 11, 2013.