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

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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-A2402-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-A2402. 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)
Multiple-Peptide Vaccination for Biliary Tract Cancer

Translational Relevance

Numerous clinical reports have shown that peptide vaccines can induce peptide-specific CTLs to mediate tumor-specific responses in vitro. 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.

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 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.

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 immuno-spot (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 × 10⁷/mL) were cultured with 10μg/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

<table>
<thead>
<tr>
<th>No.</th>
<th>Age/sex</th>
<th>Primary tumor site</th>
<th>Previous therapy</th>
<th>Peptide</th>
<th>Dose, mg</th>
<th>No. of vaccines</th>
<th>Lymphocyte number (%</th>
<th>Clinical response</th>
<th>Peptide-specific CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64/M</td>
<td>IBD, Liver/peritoneum</td>
<td>GEM, CDDP, TS-1</td>
<td>TTK, IMP3, DEPDC1</td>
<td>0.5</td>
<td>4</td>
<td>PD</td>
<td>CD8+ 47</td>
<td>1,955 (8.3)</td>
</tr>
<tr>
<td>2</td>
<td>75/M</td>
<td>GB, Liver/peritoneum</td>
<td>GEM, TS-1</td>
<td>TTK, IMP3, DEPDC1</td>
<td>0.5</td>
<td>4</td>
<td>PD</td>
<td>CD8+ 31</td>
<td>1,390 (17.3)</td>
</tr>
<tr>
<td>3</td>
<td>67/M</td>
<td>GB, Lymph nodes</td>
<td>Ope, GEM, AFTV</td>
<td>IMP3, DEPDC1</td>
<td>0.5</td>
<td>5</td>
<td>PD</td>
<td>Stable decrease</td>
<td>1,801 (39.4)</td>
</tr>
<tr>
<td>4</td>
<td>65/F</td>
<td>IBD, Lung/lymph nodes</td>
<td>GEM</td>
<td>IMP3, DEPDC1</td>
<td>1.0</td>
<td>2</td>
<td>CD8+ 372</td>
<td>Stable increase</td>
<td>1,521 (29.4)</td>
</tr>
<tr>
<td>5</td>
<td>59/F</td>
<td>GB, Liver/peritoneum</td>
<td>Ope, GEM, TS-1</td>
<td>IMP3, DEPDC1</td>
<td>1.0</td>
<td>1</td>
<td>PD</td>
<td>Stable decrease</td>
<td>2,124 (22.1)</td>
</tr>
<tr>
<td>6</td>
<td>76/F</td>
<td>EBD, Liver/peritoneum</td>
<td>GEM, TS-1</td>
<td>IMP3, DEPDC1</td>
<td>1.0</td>
<td>9</td>
<td>PD</td>
<td>Stable decrease</td>
<td>1,450 (30.2)</td>
</tr>
<tr>
<td>7</td>
<td>69/F</td>
<td>IBD, Lung nodes</td>
<td>IMP3, DEPDC1</td>
<td>2.0</td>
<td>35</td>
<td>CD8+ 176</td>
<td>PD</td>
<td>Stable decrease</td>
<td>2,399 (38.2)</td>
</tr>
<tr>
<td>8</td>
<td>74/M</td>
<td>GB, Liver/peritoneum</td>
<td>GEM, TS-1</td>
<td>IMP3, DEPDC1</td>
<td>2.0</td>
<td>16</td>
<td>PD</td>
<td>Stable decrease</td>
<td>1,216 (22.3)</td>
</tr>
<tr>
<td>9</td>
<td>69/F</td>
<td>IBD, Liver/peritoneum</td>
<td>GEM, TS-1</td>
<td>IMP3, DEPDC1</td>
<td>2.0</td>
<td>16</td>
<td>PD</td>
<td>Stable decrease</td>
<td>1,249 (23.1)</td>
</tr>
</tbody>
</table>

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.

Flow cytometry assay. The expression of peptide-specific T-cell receptors was analyzed on a FACSCanto II flow cytometer (Becton Dickinson) using LYT6-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 (RVLQDQQL)-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 CD8 mAb and 7-aminoactinomycin D (BD Pharmingen) at 4°C for 20 minutes. Conventional 2-color phenotypic analysis was also conducted with FITC-conjugated anti-human CD3 mAb, PE-Cy7–conjugated anti-human CD4 mAb, and 7-aminoactinomycin D (BD Pharmingen) for 4°C for 20 minutes. Conventional 2-color phenotypic analysis was also conducted with FITC-conjugated anti-human CD3 mAb, PE-Cy7–conjugated anti-human CD4 mAb, and 7-aminoactinomycin D (BD Pharmingen) at 4°C for 20 minutes.

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.
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 (AFTV; 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

Table 2. Adverse events assessed by CTCAE v3.0

<table>
<thead>
<tr>
<th>Adverse events</th>
<th>Total (%)</th>
<th>Grade 1 (%)</th>
<th>Grade 2 (%)</th>
<th>Grade 3 (%)</th>
<th>Grade 4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>6 (66.7)</td>
<td>5 (55.6)</td>
<td>1 (11.1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>2 (22.2)</td>
<td>2 (22.2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Injection site reaction</td>
<td>8 (88.9)</td>
<td>3 (33.3)</td>
<td>5 (55.6)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: Hemoglobin and lymphopenia were observed before the first vaccination. No other adverse events were seen throughout the period of peptide vaccination.
is a special characteristic of cancer vaccine therapy; there-fore, 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
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 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 anticancer strategy for cancer immunotherapy in the future.
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

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

<sup>a</sup>Primary 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. Koteria, R. Okuyama
Study supervision: M. Yamamoto

References
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