Clinical Trial of a 7-Peptide Cocktail Vaccine with Oral Chemotherapy for Patients with Metastatic Colorectal Cancer

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Abstract. Aim: The combination of a peptide vaccine and tegafur-uracil plus leucovorin (UFT/LV) were evaluated in patients with metastatic colorectal cancer refractory to standard chemotherapy. Patients and Methods: Thirty human leukocyte antigen (HLA)-A2402-positive patients were enrolled in the study. In a cycle of treatment, a vaccine comprising of seven synthetic peptides (five tumor antigen-derived and two vascular endothelial growth factor receptor-derived) was injected weekly, and oral chemotherapy, UFT/LV was given daily for four weeks followed by one week of rest. The immunological and clinical responses were evaluated at the end of every five weeks. Results: Notable adverse events included grade 1 injection site redness/induration in 25 patients. Tumor imaging showed partial response in three patients, stable disease in 15, and progressive disease in 12. Survival analysis indicated that patients who exhibited positive cytotoxic T lymphocyte responses to all seven peptides had longer overall survival compared to other patients. Conclusion: A 7-peptide vaccine used with UFT/LV is safe and is recommended for further trials in patients with metastatic colorectal cancer.

A number of genes are frequently up-regulated in colorectal cancer (CRC) cells. Some of these genes have been identified by genome-wide exploration using cDNA microarray profiling. This strategy has also shown that certain proteins encoded by these genes are essential for the proliferation or survival of CRC cells\textsuperscript{(1)}. Several of these proteins are tumor-associated antigens (TAAs) as they are highly expressed in CRC, with limited expression in normal tissues.

In a previous trial, we studied the effect of a 2-peptide vaccine, derived from two TAAs, together with oral chemotherapeutic drugs, tegafur-uracil plus leucovorin (UFT/LV), in the treatment of advanced or recurrent CRC. Vaccination with the 2-peptide vaccine in combination with UFT/LV induced antigen-specific T-cell responses. The patients with multiple-antigen-specific T-cell responses also had a longer overall survival. However, no remarkable clinical responses (complete response, or partial response) were observed in that trial\textsuperscript{(2)}.

In an effort to improve clinical efficacy, we formulated a 7-peptide cocktail vaccine for use with UFT/LV in a clinical trial for patients with metastatic CRC refractory to standard chemotherapy. The peptides were derived from proteins ring finger protein-43 (RNF43), translocase of the outer mitochondrial membrane-34 (TOMM34), forkhead box M1 (FOXM1), maternal embryonic leucine zipper-kinase (MELK), Holliday junction-recognizing protein (HJURP), vascular endothelial growth factor receptor-1 (VEGFR1) and vascular endothelial growth factor receptor-2 (VEGFR2) (Table I). Five of the proteins were identified as cancer-testis antigens and two were vascular endothelial growth factor receptors (VEGFRs). HLA-A2402-restricted epitope peptides from these antigens had already been identified for use in vaccination of patients with CRC. The cancer-testis antigens are expressed only on the cell surface of the testicles among all normal tissues tested. Since the cells of the testicles do not express the human leukocyte antigen (HLA) molecule on their surface, the immune system is not expected to attack these cells. The binding of VEGF to VEGFR stimulates angiogenesis and tumor growth. Anticancer drugs targeting VEGFR have already proven efficacious clinically, and vaccination against VEGFR has resulted in an antitumor action in clinical settings\textsuperscript{(3)}.

Patients and Methods

Patients and eligibility criteria. The study protocol was approved by the Institutional Ethical Review Boards of Kinki University (approval no. 23-59) and was registered at the UMIN Clinical Trials Registry as UMIN000007801 (http://www.umin.ac.jp/ctr/index.htm). Complete written informed consent was obtained from all patients at the time of
enrollment. All patients (n=30) were required to have histologically confirmed mCRC unsuitable for surgical resection and to be HLA-A*2402-positive. This HLA genotype is expressed in the majority of the Japanese population. In addition, all patients had failed to respond to their prior standard chemotherapy. Patients were required to have completed prior chemotherapy at least four weeks before trial enrollment and to have recovered from any adverse event with a toxicity of grade 3 or higher by the Common Terminology Criteria for Adverse Event (CTCAE) scale (11). All patients were also required to have an Eastern Cooperative Oncology Group performance status (PS) of 0-2, to be older than 20 years of age, and to have a life expectancy of at least three months. Adequate bone marrow (white blood cell count ≥3,000/mm³, hemoglobin ≥10 g/dl and platelet count ≥75,000/mm³), renal function (serum creatinine ≤1.4 mg/dl), and liver function (bilirubin ≤1.5 mg/dl and transaminase within 2.5× of the Institution’s upper limit of normal range) were required for acceptance into the trial. Patients were excluded if they were pregnant or if they had detectable hepatitis B or C virus antigens or human immunodeficiency virus antigens.

Peptides and drugs. Peptides: The peptides used in this trial are shown in Table I. The synthetic peptides were manufactured sterilely in accordance with good manufacturing practice standards, and preclinical trials confirmed that the peptides did not produce acute toxicity.

Montanide ISA-51VG: Montanide is a sterile vaccine adjuvant manufactured by SEPPIC Co. (Puteaux, France) in accordance with good manufacturing practice standards and is also known as incomplete Freund’s adjuvant. Montanide is currently used as an adjuvant in vaccine therapies worldwide, and no serious adverse events due to Montanide have been reported.

UFT/LV: UFT® and UZEL® are oral anticancer drugs marketed in Japan and approved for the treatment of CRC. UFT/LV leads to the same response rates as fluorouracil plus LV. UFT/LV inhibit DNA synthesis and RNA function in cancer cells and have anticaner action clinically (12). Moreover, we previously demonstrated that the standard dose of UFT/LV did not impede the immunological responses of patients with advanced CRC to peptides administered in cancer vaccination (13).

Clinical protocol. This trial was an open-label phase Ib study of a vaccine consisting of seven peptides (1 mg of each peptide) derived from five cancer-testis antigens that are highly expressed in CRC and two VEGFRs. These seven peptides were mixed with Montanide ISA 51VG (SEPPIC, Puteaux, France), and administered to patients subcutaneously once every seven days five times. In addition, all patients received daily doses of UFT (UFT®: 300 mg/m²/day) plus LV (UZEL®: 75 mg/day) orally for 28 days. Each cycle of treatment was followed by 1 week of rest (Figure 1). Patients continued multiple cycles of treatment unless their disease deteriorated, but no treatment was discontinued for any patient based solely on the occurrence of adverse events.

Evaluation of safety. Adverse events resulting from the peptide vaccine were evaluated using the National Cancer Institute’s Common Terminology Criteria for Adverse Events (NCI-CTCAE) v 4.0 (11).

Evaluation of antitumor action and endpoints. The primary endpoints of the trial were safety and feasibility, and the secondary endpoints were overall survival (OS), tumor size as determined by imaging studies in accordance with the RECIST Guidelines (14), and peptide-specific activities of cytotoxic T-lymphocytes (CTLs) as measured by the ELISPOT assay.

Enzyme-linked immunospot (ELISPOT) assay. Peptide-specific CTL responses were estimated by the ELISPOT assay following in vitro sensitization (2). Frozen peripheral blood mononuclear cells (PBMCs)
obtained from each patient were thawed at the same time, and the viability was confirmed to be more than 90%. PBMCs (5×10^5/ml) were then cultured with 10 mg/ml of each peptide and 100 IU/ml of interleukin-2 (Novartis, Emeryville, CA, USA) at 37˚C for two weeks. Peptides were added to the cultures at day 0 and day 7. Following CD4+ cell depletion by the Dynal CD4 positive isolation kit (Invitrogen, Carlsbad, CA, USA), interferon-γ (IFN-γ) ELISPOT assays were performed using the 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, USA) were incubated with 20 mg/ml of vaccine peptides overnight, then the residual peptides in

Figure 1. Clinical protocol. The 7-peptide vaccine mixed with adjuvant were injected weekly, and oral chemotherapy, UFT/LV, was given daily for 4 weeks followed by 1 week of rest. The cycle was repeated twice. The immunological and clinical responses were evaluated at the end of every 5 weeks. After the 2nd cycle, patients continued multiple cycles of treatment unless their disease deteriorated.

Figure 2. Imaging of a typical partial response (PR). All 4 lesions of lung metastases (black and white arrows) reduced in size after two cycles of peptide vaccination.
the media were washed out to prepare the peptide-pulsed TISI cells as stimulator cells. Prepared CD4− cells from the patients were cultured with peptide-pulsed stimulator cells (2×10⁴ cells/well) at 1:1, 1:2, 1:4, and 1:8 mixture ratios of responder cells and stimulator cells (R:S ratio) in 96-well plates (Millipore, Bedford, MA, USA) at 37˚C overnight. Non-peptide-pulsed TISI cells were used as negative control stimulator cells. To assess IFN-γ production, responder cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (66 ng/ml) and ionomycin (3 mg/ml) overnight, then applied to IFN-γ ELISPOT assay (2.5×10³ cells/well) without stimulator cells. All ELISPOT assays were performed in triplicate. The plates were analyzed by the automated ELISPOT reader, ImmunoSPOT S4 (Cellular Technology Ltd., Shaker Heights, OH, USA) and ImmunoSpot Professional Software Version 5.0 (Cellular Technology Ltd.). The number of peptide-specific spots was calculated by subtracting the spot number in control wells from that in wells with peptide-pulsed stimulator cells. The peptide-specific T-cell responses were classified into four grades (−, +, ++, and ++++) according to the algorithm flow chart described in our previous report (15). Sensitivity of our ELISPOT assays was estimated as approximately the average level by the ELISPOT panel of the Cancer Immunotherapy Consortium [CIC (http://www.cancerresearch.org/consortium/assay-panels/)] (16).

Statistical analysis. OS rates were analyzed by the Kaplan–Meier method, and survival was calculated in days from the first vaccination to death. All statistical analyses were performed with SPSS statistics 17.0 (SPSS, Chicago, IL, USA).

Results

Patients’ characteristics. Between November 2011 and May 2012, 30 patients with mCRC refractory to standard chemotherapy were enrolled in this study (Table II). All patients underwent resection of their primary CRC, but their metastatic sites were unresectable. They also had undergone several standard chemotherapy regimens, but either their disease had continued to progress, or their standard chemotherapy was discontinued because of unacceptable toxic side-effects. All enrolled patients had a PS of 0, 1 or 2.

Adverse events. All adverse events during the trial are shown in Table III. The most frequent adverse event observed was injection-site reaction. The pattern of other toxicities resembles that of the accompanying UFT/LV chemotherapy. Grade 3 seizures may be considered a result of the deterioration due to brain metastasis, blood bilirubin increase to liver metastasis, and hypercalcemia due to bone metastasis. One patient developed anaphylaxis on the 26th vaccine injection. Although she recovered immediately after transfusion and rest, she received no further vaccination after this event.

Immunological responses and clinical responses. We observed three partial responses (PR) by the RECIST criteria. Another three patients showed tumor shrinkage, i.e. objective response (OR), but did not reach the PR criteria. The overall response rate (CR+PR) was 10%, and the disease control rate (CR+PR+SD) was 60%. In a typical PR case, multiple lung metastases reduced in size after two cycles of peptide vaccination (Figure 2). For immunological responses, we measured the patients’ peptide-specific T-cell responses by the ELISPOT assay. Nine out of 30 patients showed measurable CTL responses to all seven peptides. It is noteworthy that all nine patients are long-term survivors in this study, including two PR and five SD cases (Pt. # 1, 3, 5, 6, 16, 21, 22, 28 and 30) (Table IV).

Correlation of CTL responses to the number of vaccine peptides with OS. The OS was analyzed in all 30 patients. The median survival time (MST) of OS was 10.63 months (Figure 3). The Kaplan–Meier analysis indicated a correlation of OS with positive CTL responses to the number
of peptides used in the vaccine. Nine patients with positive CTL responses to all seven peptides are long-term survivors in this study compared to patients who had detectable CTL responses to only six peptides or fewer (Figure 4).

**Discussion**

We report that a 7-peptide cocktail vaccine and UFT/LV induced antigen-specific CTL responses in patients with mCRC refractory to standard chemotherapy. The treatment also produced a good disease control rate of 60%, including 3 PR and 15 SD. More importantly, the patients with positive CTL responses to all seven peptides showed the longest long-term survival rate in this study.

In our earlier trial of a 2-peptide vaccine with UFT/LV, the patients who showed immunological responses to both peptides were long-term survivors when compared to the patients who showed response to only one peptide or to none. However, no remarkable clinical responses, PR or CR were observed in that study (2).
be significantly associated with clinical benefits. The results suggest that targeting multiple antigens in a vaccine formulation may increase clinical efficacy. Our data do not show whether positive CTL responses to all seven peptides are required for long-term survival or if certain subsets of 'key CTL responses' to the 7 peptides are the major contributors. Neither total white blood cell counts or peripheral blood lymphocyte counts before vaccination, nor ELISPOT peptide-specific IFN-γ production before vaccination by ELISPOT assay correlated with peptide-specific CTL responses (data not shown).

The advantages of multi-antigen vaccines have been discussed in the Food and Drug Administration Guidance, which raised the possibility that multi-antigen vaccines not only induce multiple tumor-specific immunological responses, but also hinder potential tumor-escape mechanisms (17). Moreover, Walter et al. demonstrated that multiple tumor-associated peptides composed of 11 peptides induced potent immune responses and resulted in long-term survival of patients with renal cancer in several clinical trials (IMA901) (18).

While the data presented in this report are promising for the treatment of mCRC using a multi-peptide vaccine and UFT/LV, the therapeutic outcome achieved thus far is still not optimal. Potential reasons for the limited success in this trial include immune regulation mediated by cancer cells and leukocyte populations through a variety of cell-surface and secreted molecules, including regulatory T-cells, myeloid-derived suppressor cells, and activated (type 2) macrophages (M2).
Walter et al. reported that cyclophosphamide pre-treatment before multi-peptide vaccination successfully reduced the numbers of regulatory T-cells, as determined by immunophenotyping, and resulted in long-term survival of patients with advanced renal cell cancer in a randomized trial (18). Therefore, further clinical trials directed at the blockade of suppressive immune responses, including immune checkpoint antibodies such as to programmed death-1(PD-1)/programmed death ligand-1(PD-L1) and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), are attractive options for improving clinical responses in conjunction with this peptide vaccine and UFT/LV (19).

Finally, regorafenib is currently the only available treatment for recurrent CRC when standard chemotherapy has failed. Regorafenib is a novel oral multi-kinase inhibitor that blocks the activities of several protein kinases, including kinases involved in the regulation of tumor angiogenesis (VEGFR1, VEGFR2, VEGFR3, TIE2) and oncogenesis (KIT, RET, RAF1, BRAF and BRAFV600E). In a recent multicenter, randomized, placebo-controlled CORRECT trial, the MST of the regorafenib group was reported to be 6.4 months, while the MST of the placebo group was 5.0 months (20). In comparison, the patients in our trial with almost the same background as that of the CORRECT trial had a MST of 10.63 months with peptide vaccination, although our trial was a preliminary pilot study for HLA-A24-positive patients and had a far smaller sample size. We are planning to undertake a randomized placebo-controlled multipeptide trial for HLA-A24-positive patients with mCRC refractory to standard chemotherapy to further explore this form of cancer vaccine.

Conflicts of Interest

The Authors declare no conflicts of interest.

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