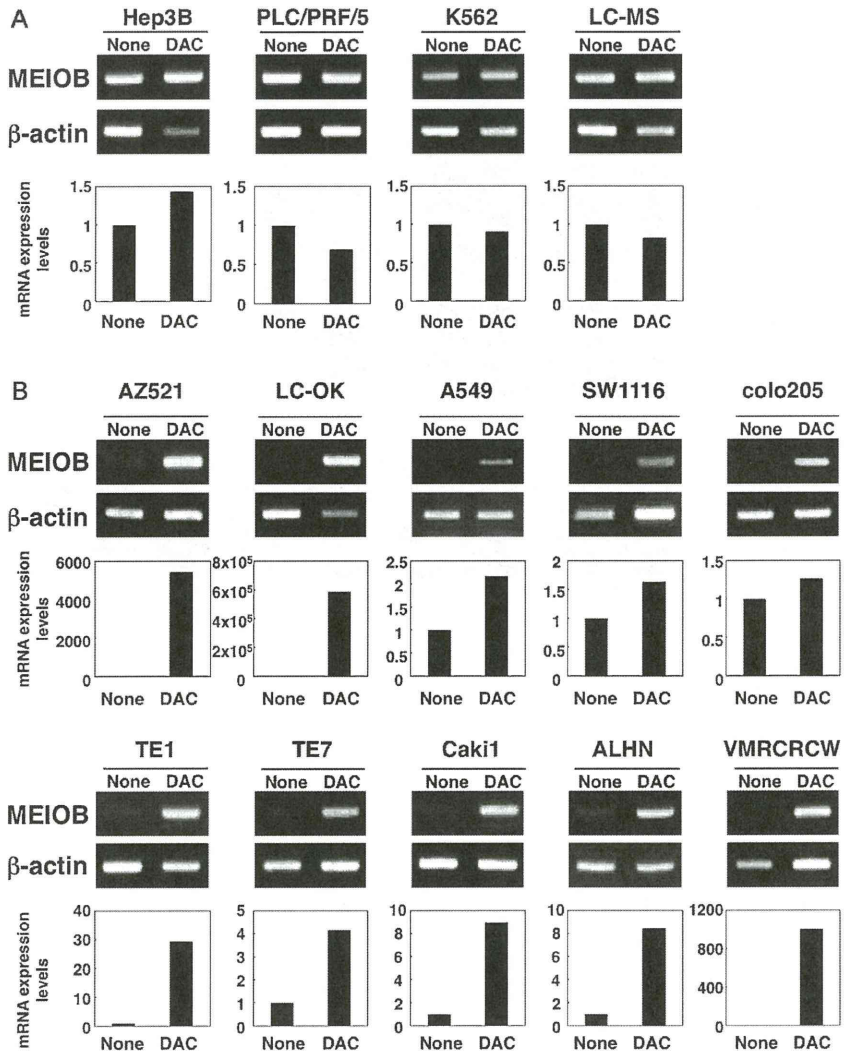


Fig. 2. Gene expression levels of MEIOB in DAC-treated cancer cell lines. Cancer cell lines, which (A) spontaneously expressed or (B) did not express the MEIOB gene before DAC treatment, were analyzed by quantitative-PCR and RT-PCR. For the gene expression analysis in each cancer cell line, the gene expression in the non-DAC-treated cancer cell line was assigned a value of 1, and the fold increases in the expression levels were determined by comparison. Three independent experiments were performed, and the representative result is indicated in the figure.

3.2. Overlapping peptides of MEIOB antigen induces antigen-specific type-1 helper T cells

Using MEIOB-overlapping peptides, we induced IFN-γ-producing helper T (Th) cells from purified $CD4^+$ T cells of 4 healthy donors and then identified the helper epitopes of the MEIOB antigen. We successfully established Th cells that could respond to various peptides from the donors as follows: donor 1: MIX1 (#3–5), MIX2 (#6–8), MIX3 (#11, #15), MIX4 (#16–20), MIX5 (#21–22, #24), MIX6 (#26–31); donor 2: MIX1 (#1–5), MIX2 (#6–10), MIX3 (#11–13), MIX4 (#16–20), MIX5 (#22–25), MIX7 (#32–35), MIX8 (#38–41), MIX9 (#46), MIX10 (#47); donor 3: Mix1 (#2, #4), MIX2 (#7–8), MIX3 (#11–14), MIX4 (#18–20), MIX5 (#24–25), MIX7 (#32, #34–35), MIX8 (#38–39, #41), MIX9 (#46), MIX10 (#47), MIX11 (#52–53), MIX12 (#60–61); and donor 4: MIX7 (#33–36), MIX8 (#38–39, #41), MIX9 (#42–46), MIX10 (#50), MIX11 (#52–54), MIX12 (#59–61) (Fig. 3A). Thus, it was notable that almost all regions of the MEIOB antigen contained helper epitopes (Fig. 3B). In the screening for helper epitopes, peptides #4, #7, #19, and #54 indicated that HLA restriction occurred frequently in the Japanese population (Fig. 3). Therefore, we selected these 4 peptides for the subsequent HLA-restriction analysis.

3.3. Determination of HLA restriction for the helper epitopes recognized by MEIOB-specific $CD4^+$ T cells

To determine the HLA restriction of the helper epitopes, we induced MEIOB-specific $CD4^+$ T cells from purified $CD4^+$ T cells by using peptide #4, #7, #19, or #54, and further established $CD4^+$ T cell clones from the MEIOB-#7-specific $CD4^+$ T cells. Then, we evaluated the reactivity of the $CD4^+$ T cells or the T cell clones against peptide-pulsed allogeneic EBV-B cell lines with different types of HLA-DP/DQ/DR. $CD4^+$ T cells specific for MEIOB-#4, -#7, -#19, or -#54 produced IFN-γ in response to peptide-pulsed #4 with HLA-DPB1*05:01- or HLA-DRB1*04-, #7 with HLA-DQB1*03:01-, HLA-DQB1*03:03-, HLA-DQB1*05:01-, HLA-DRB1*14:03-, or HLA-DRB1*08:03-, #19 with HLR-DPB1*05:01- or HLA-DRB1*08:03-, or #54 with HLA-DRB1*09:01-, HLA-DRB1*04-, or HLA-DRB1*0803-expressing EBV-B cell lines (Fig. 4A). These HLA-type restrictions for

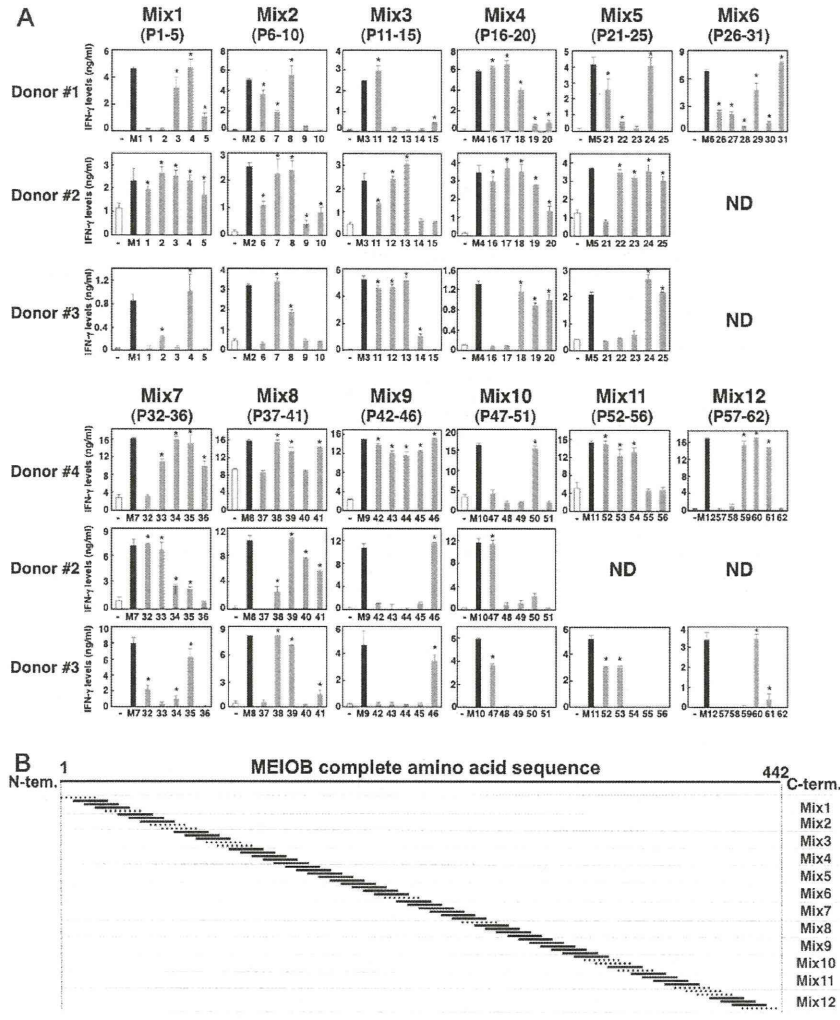
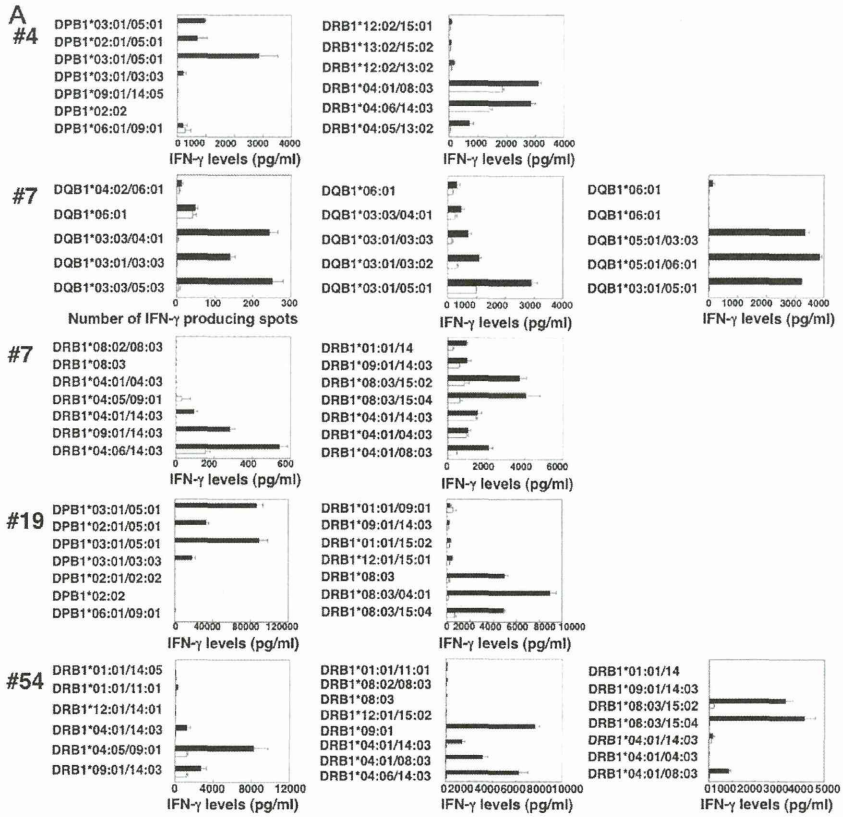


Fig. 3. Induction of antigen-specific CD4$^+$ T cells by MEIOB-overlapping peptides. (A) MEIOB antigen-specific CD4$^+$ T cells were induced by using an overlapping peptide pool (MIX1–MIX12) from purified CD4$^+$ T cells of 4 healthy donors. The vehicle of the peptide was used as a negative control (−). IFN-γ production levels were assessed by ELISA. The mean ± SD of the representative data are indicated in the figure. (B) MEIOB peptides containing helper epitopes are summarized in the figure. Solid lines represent peptides inducing MEIOB-specific helper T cells, and dashed lines mean that T cells were not established in the present experiment. The values represent the mean ± SD of duplicated samples and are representative data of 3 independent experiments. *$P < 0.05$, *versus* values of the negative control (−). ND in the figure means "not detected.".

helper epitopes of peptides #4, #7, #19, and #54 are summarized in Fig. 4B.

3.4. *MEIOB antigen-specific T cells react with endogenously processed antigen in human lung cancer cells*

We assessed whether MEIOB-specific T cells established from PBMCs could recognize MEIOB antigen epitopes that are naturally presented on human cancer cells. We found here that the IFN-γ-treated lung cancer cell lines LC-MS (HLA-DPB1*05:01, HLA-DQB1*04:01, HLA-DRB1*04:05) and A549 (HLA-DPB1*03:01/06:01, HLA-DQB1*02:02/03:01, HLA-DRB1*07:01/11:04) up-regulated the expression levels of HLA class II remarkably more than non-treated cells did, whereas DAC treatment did not affect the HLA expression (Fig. 5A). Moreover, we confirmed that MEIOB gene expression was obviously enhanced in A549 cells after treatment with DAC, IFN-γ, or both, whereas in LC-MS cells, the spontaneous MEIOB gene expression was only slightly enhanced (Fig. 5B). Then, we evaluated the protein expression levels by western blot analysis. As a result, MEIOB protein was detected in the constitutively mRNA expressed LC-MS cells. Moreover, we confirmed that expression levels of MEIOB protein were enhanced by DAC and IFN-γ treatment in A549 cells which less expressed mRNA of MEIOB in the steady state (Fig. 5C). In these experiments, the morphology (Fig. 5D) and viability of the LC-MS and A549 cells did not alter after treatment with DAC and/or IFN-γ (data not shown). We also evaluated whether MEIOB antigen-specific T cells (HLA-DPB1*04:02/05:01, HLA-DQB1*03:03/05:01, HLA-DRB1*01:01/09:01), induced from PBMCs by stimulating with the MEIOB-overlapping peptide, could recognize HLA-DPB1*0501-matched LC-MS and HLA-mismatched A549 cells after treatment with IFN-γ alone, DAC alone, or IFN-γ and DAC combined, or with no treatment. As shown in Fig. 5E, the IFN-γ production by MEIOB-specific T cells co-cultured with either IFN-γ-treated or DAC/IFN-γ-treated LC-MS cells was more significantly enhanced than that co-cultured with non- or DAC-treated cells. The IFN-γ production was remarkably blocked in the presence of anti-HLA class II mAbs but not anti-HLA class I mAbs (Fig. 5E). In addition, we confirmed that the MEIOB-specific T cells do not respond to HLA-mismatched A549 cells, even if the cells



B

ID#	Amino-acid sequence	HLA-class II
#4	NLKVIGIVIGKTDVKGFPDR	DPB1*05:01, DRB1*04
#7	NIGSERYTFSFTIRDSPAHF	DQB1*03:01, DQB1*03:03, DQB1*05:01, DRB1*08:03, DRB1*14:03
#19	CSSYEVDTKLLSLIHLPVKE	DPB1*05:01, DRB1*08:03
#54	SVFLSFHVLIDLTDHTGTLH	DR53(DR4/DR7/DR9), DRB1*08:03

Fig. 4. Induction of MEIOB-specific CD4$^+$ T cells by the overlapping peptides, and HLA restriction for the helper epitopes of the established CD4$^+$ T cells. (A) MEIOB-specific CD4$^+$ T cells were induced from purified CD4$^+$ T cells by using MEIOB-#4, -#7, -#19, or -#54 peptides. HLA restriction was analyzed by using allogeneic EBV-B cell lines pulsed with irrelevant (open bar) or cognate (filled bar) peptides. The IFN-γ production levels were assessed by ELISA or ELISPOT. The mean ± SD of the representative results are indicated in the figure. (B) Summary of the MEIOB amino-acid sequence-containing helper epitopes and the restriction of the corresponding HLA class II.

are treated with DAC plus IFN-γ (Fig. 5E). These results suggested that the established MEIOB-specific CD4$^+$ T cells would at least react to an endogenously presented MEIOB antigen on HLA class II-matched cancer cells.

4. Discussion

In the present work, we first identified that a meiosis-specific protein, MEIOB, was a novel CT antigen and that its gene and protein expression levels were enhanced by DAC treatment of human cancer cells (Figs. 1, 2 and 5). MEIOB gene expression was observed in several cancer cell lines and a primary cancer cells but not in normal tissues or PBMCs (Fig. 1B and data not shown). Therefore, our strategy using DAC-treated demethylated cancer cells would be a useful tool for finding novel CT antigens applicable to cancer vaccine therapy in humans.

A previous clinical study had demonstrated that DAC treatment induced NY-ESO-1 gene expression in 36% of patients with thoracic malignancies [25]. In mouse xenograft models, intraperitoneal injection of DAC enhanced the gene expression levels of various CT antigens in the human xenografts [26–28]. We demonstrated here that DAC treatment remarkably enhanced the gene expression levels of other CT antigens, such as MAGE-A4, XAGE, and BAGE, in addition to MEIOB in various cancer cells (Fig. 1A), suggesting that the DAC-treated cancer cells would increase their immunogenicity. Generally, the restricted gene expression of the CT antigen is regulated mainly by the levels of DNA methylation of the promoter region. Therefore, it may be that the DAC treatment of cancer cells induces CT antigen gene expression by the demethylation [24].

In the present experiments, we confirmed that MEIOB-specific CD4$^+$ T cells, induced by synthetic peptides, responded to IFN-γ-treated target cells in a HLA class II-dependent manner (Fig. 5E). These data suggest that such CD4$^+$ T cells would at least recognize the helper epitopes of naturally processed antigens presented on the HLA class II molecules of cancer cells. In the present study, we confirmed that IFN-γ treatment enhanced HLA class II expression levels in cancer cells (Fig. 5A). Thus, we confirmed that the expression of MEIOB in target cancer cells, the HLA class II expression on

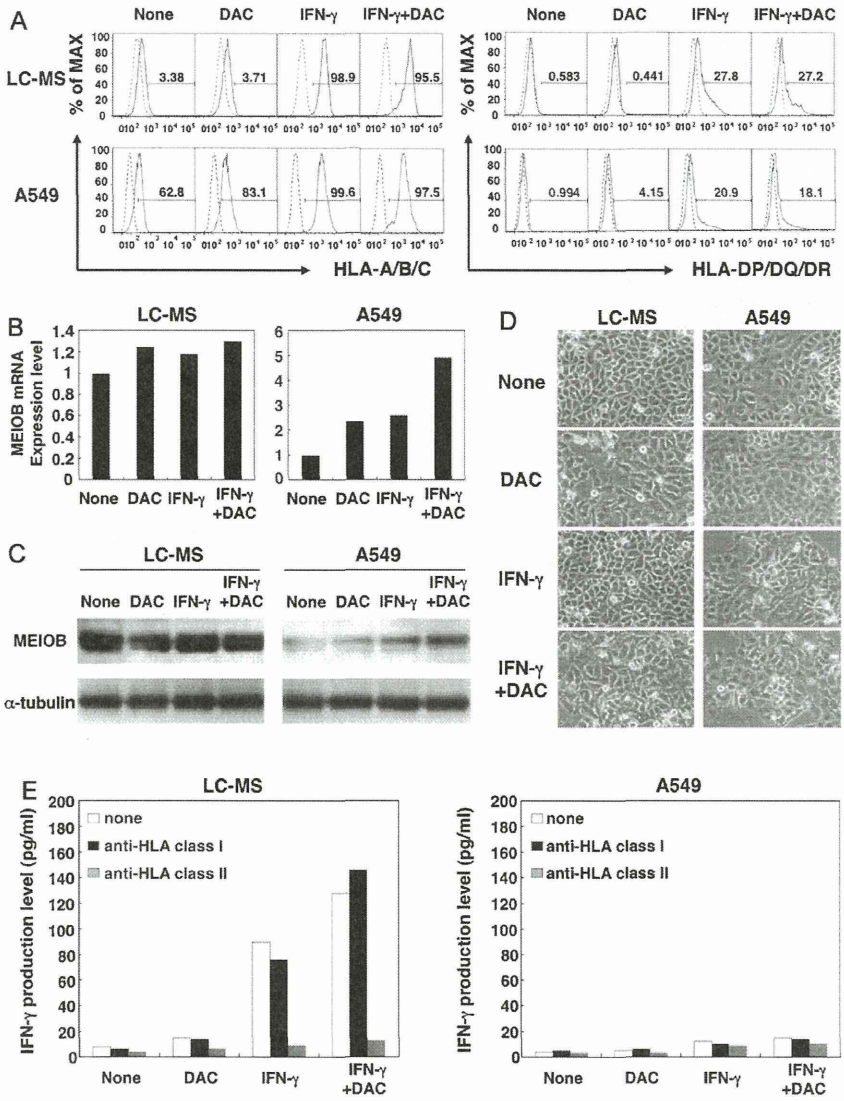


Fig. 5. MEIOB-specific CD4⁺ T cell recognition of the endogenous antigen presented on lung cancer cells. (A) HLA class I and II expression in DAC- and/or IFN-γ-treated cancer cells, as analyzed by flow cytometry. Three independent experiments were performed, and the representative profiles are indicated in the figure. (B) Gene expression of MEIOB in cancer cells treated with DAC and/or IFN-γ, as analyzed by quantitative-PCR. (C) MEIOB expression levels of None-, DAC-, IFN-γ-, DAC and IFN-γ-treated cancer cells were evaluated by Western blot analysis. Three independent experiments were performed, and the representative profiles are indicated in the figure. (D) Representative photographs (×400) of cancer cells treated with DAC and/or IFN-γ. (E) T cells established by MEIOB-overlapping peptides from PBMCs were co-cultured with the HLA-DPB1*05:01-matched LC-MS or HLA-mismatched A549 cells treated with DAC and/or IFN-γ in the presence or absence of anti-HLA class I or II mAbs for 96 h. The IFN-γ production levels were then assessed by ELISA. Representative results are indicated in the figure.

the target cells, and the matching of HLA would be required for the responses of MEIOB-specific T cells against target cancer cells. Taken together, these findings suggest that MEIOB-overlapping peptides would be a promising tool for developing a novel vaccine therapy for patients with MEIOB-expressing cancers.

Recently, we developed the H/K-HELP that artificially combined both helper and killer epitopes of the tumor antigen to induce tumor antigen-specific T cells [19]. Cancer vaccine immunotherapy with the H/K-HELP of the MAGE-A4 antigen has been carried out for advanced cancer patients with MAGE-A4-expressing tumors in a phase I clinical study. We found that antigen-specific immune responses, such as IFN-γ production by T cells and antibody formation, were induced by the vaccination at an early date in the patients [19]. In this study, we found that expression levels of MEIOB protein were enhanced by DAC and IFN-γ treatment in A549 cells (Fig 5C). Since IFN-γ treatment enhanced MHC class I and class II expression levels in A549 cells (Fig. 5A), the combination of DAC and IFN-γ would be a promising strategy for augmentation of cancer antigen-specific CD4⁺ T cell responses against the target tumors. Generally, it has been reported that anti-tumor immunity was severely suppressed by generation of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in tumor-bearing hosts [29]. There might be several issues to improve less half-life of IFN-γ in patients and possibility of systemic side effect caused by the overdose administration. We have been demonstrated that introduction of Th1-dependent anti-tumor immunity into tumor-bearing hosts overcomes the strong immunosuppressive tumor escape mechanisms and exhibited a complete eradication of tumors in various mouse therapy models [11,14]. Therefore, we expected that induction of cancer-specific Th1 cells at the local site by MEIOB

peptide vaccine therapy combined with DAC treatment would be much more effective compared with only administration of IFN-γ or the combination with DAC.

In the present experiments, we found that MEIOB contained many helper epitopes (Figs 3 and 4, Supplementary Fig. S2). Moreover, the MEIOB antigen has many promiscuous peptide regions, which might make it possible to overcome the HLA restriction and bind the cancer peptide to multiple HLA phenotypes of cancer patients. These findings suggest that the MEIOB-H/K-HELP would effectively induce antigen-specific T cells and contribute to the development of an innovative cancer vaccine therapy. Thus, our finding that DAC treatment augments the induction of CT antigens, including a MEIOB antigen, indicates that DAC treatment can be used as a novel strategy to enhance the therapeutic efficacy of cancer vaccine therapy.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2014.01.004.

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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 a cycle of treatment, a vaccine comprising 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: These results suggest that a 7-peptide vaccine used with UFT/LV is safe and to be recommended for further trials for 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 (1). Several of these proteins are tumor-associated antigens (TAAs) as they are highly expressed in CRC, with limited expression in normal tissues.

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Key Words: Clinical trial, 7-peptide vaccine, oral chemotherapy, metastatic colorectal cancer, refractory to standard chemotherapy.

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 (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 antitumor action in clinical settings (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

Table I. *Peptides used in this trial.*

	Protein						
	RNF43	TOMM34	FOXM1	MELK	HJURP	VEGFR1	VEGFR2
Total amino acids	783	309	801	651	748	1338	1356
Functional activity	Cell proliferation	Cell proliferation	Cell proliferation	Cell proliferation	Cell proliferation	Angiogenesis	Angiogenesis
Expression in CRC	83%	92%	60%	73%	85%	Up-regulated for tumor angiogenesis	Up-regulated for tumor angiogenesis
Normally expressed in	-	Testis	Testis	Testis	Testis		
HLA-A2402-restricted peptide sequence	NSQPVWLCL	KLRQEVKQNL	IYTWIEDHF	EYCPGGNLF	KWLISPVKI	SYGVLLWEI	RFVPDGNRI
Reference no.	4	5	6	7	8	9	10

CRC: colorectal cancer; HLA-A2402: human leukocyte antigen-A2402; RNF43: ring finger protein 43; TOMM34: translocase of the outer mitochondrial membrane 34; FOXM1: forkhead box M1; MELK: maternal embryonic leucine zipper kinase; HJURP: holliday junction-recognizing protein; VEGFR: vascular endothelial growth factor receptor.

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/mm^3, hemoglobin ≥10 g/dl and platelet count ≥75,000/mm^3), 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 anticancer 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/m^2/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.

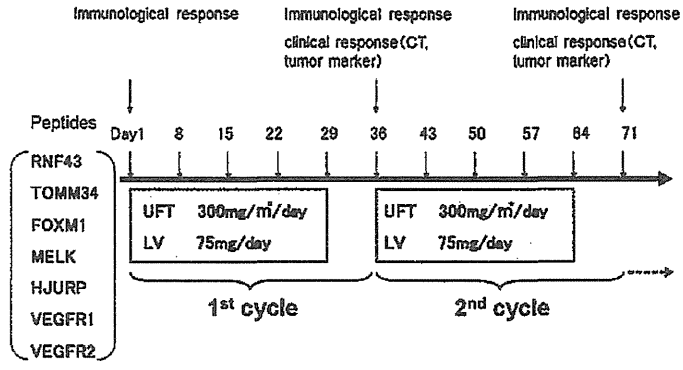

Figure 1. *Clinical protocol. The 7-peptide vaccine mixed with adjuvant were injected weekly, and oral chemotherapy, UFT/LV, was given daily for 4 weeks followed by 1 week of rest. The cycle was repeated twice. The immunological and clinical responses were evaluated at the end of every 5 weeks. After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

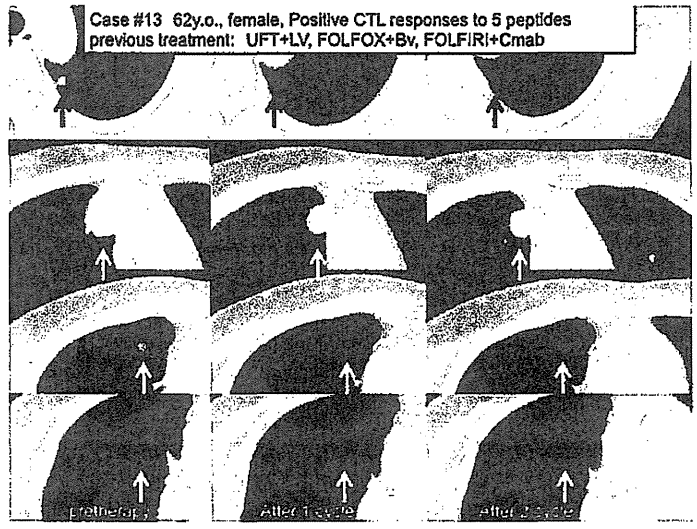

Figure 2. *Imaging of a typical partial response (PR). All 4 lesions of lung metastases (black and white arrows) reduced in size after two cycles of peptide vaccination.*

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 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 (2×10^4 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

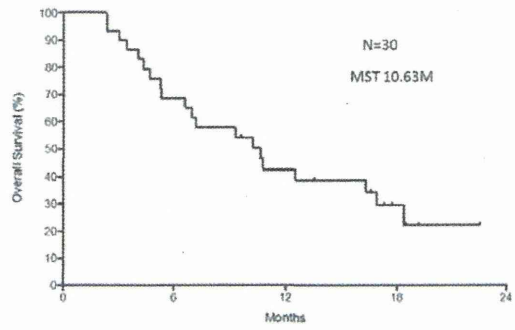

Figure 3. *Overall survival (OS) of all 30 patients. The Kaplan-Meier survival curve shows a median OS of ~~10.8~~ 10.63 months.*

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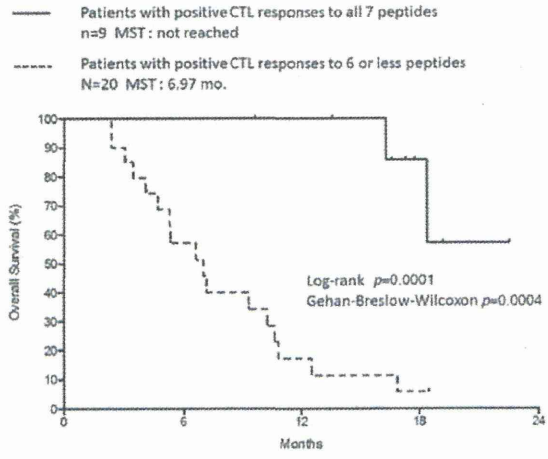

Figure 4. *Correlation of CTL responses to the number of peptides with overall survival (OS). Nine patients with positive CTL responses to all 7 peptides are long-term survivors in this study as compared with patients who had detectable CTL responses to only 6 peptides or less.*

(3 mg/ml) overnight, then applied to IFN-γ ELISPOT assay (2.5×10^3 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

Patient 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 seizure 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 of these 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 as compared with patients who had detectable CTL responses to only six peptides or fewer (Figure 4).

Table II. *Patient characteristics.*

Pt	Gender	Age (years)	Sites of metastases	PS	Previous treatment
1	F	61	Lung, peritoneum	1	FOLFOX+Bv, FOLFIRI+Bv
2	F	62	Lung, bone	1	SOX+Bv
3	F	76	LN	0	SOX+Bv, S-1
4	M	58	LN, lung, bone	1	XELOX+Bv, CPT-11+Cmab
5	F	66	LN, liver	1	FOLFOX+Bv, FOLFIRI+Pmab, S-1
6	M	65	Lung, pelvis	1	UFT+LV, S-1, FOLFIRI+Bv, RT
7	F	58	Lung, bone	1	UFT+LV, CPT-11+Cmab, FOLFOX+Bv, Pmab
8	M	61	LN, lung, liver	1	UFT+LV, FOLFOX, S-1, Cmab
9	F	49	LN	1	FOLFOX, FOLFIRI+Bv
10	M	66	LN, peritoneum	0	FOLFOX+Bv
11	F	62	Lung, pelvis	0	XELOX+Bv, FOLFIRI+Bv
12	M	58	Lung, liver	0	XELOX+Bv, SOX+Bv, IRIS+Bv
13	F	62	Lung, liver	0	UFT+LV, FOLFOX+Bv, FOLFIRI+Cmab
14	M	72	Lung, liver, brain	1	UFT+LV, FOLFOX, FOLFIRI+Bv
15	F	61	Lung, LN, peritoneum	1	UFT+LV, FOLFOX+Bv, FOLFIRI+Cmab
16	F	69	Liver	1	FOLFIRI+Bv
17	F	75	LN, lung	1	XELOX+Bv, FOLFIRI+Bv
18	F	57	Liver	0	FOLFOX, XELOX, FOLFIRI+Bv
19	F	64	LN, lung, liver	0	UFT+LV, FOLFOX, S-1
20	F	53	Liver, peritoneum	0	FOLFOX+Cmab, FOLFIRI+Bv, S-1
21	M	46	Lung, liver	0	XELOX+Bv, IRIS
22	M	64	Liver	0	XELOX, CPT-11+Cmab
23	F	60	LN, lung	0	FOLFOX+Bv, UFT+LV
24	F	61	Bone	0	FOLFOX+Bv, FOLFIRI+Bv
25	F	78	LN, lung, liver	0	FOLFOX, FOLFIRI
26	M	56	Peritoneum	2	FOLFOX, FOLFIRI+Pmab
27	M	63	Liver, pleura	2	XELOX+Bv, IRIS, Xeloda+Bv
28	F	64	LN, lung	0	5-FU+LV, XELOX+Bv, FOLFIRI+Bv
29	M	58	Peritoneum	0	FOLFOX+Bv
30	M	50	LN, lung	1	XELOX+Bv, FOLFIRI+Bv, Pmab

Pt, Patients; M, male; F, female; PS, performance status; LN: lymph nodes; FOLFOX: 5-fluorouracil, leucovorin and oxaliplatin; FOLFIRI: 5-fluorouracil, leucovorin and irinotecan; Bv: bevacizumab; SOX: S-1 and oxaliplatin; XELOX: xeloda and oxaliplatin; Cmab: cetuximab; Pmab: panitumumab; UFT+LV: tegafur-uracil and leucovorin; IRIS: irinotecan and S-1.

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 8 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 with the patients who showed response to only one peptide or none. However, no remarkable clinical responses, PR or CR were observed in that study (2).

A key difference between the former trial and the present one are the breadth of the T-cell responses and the number of different antigens used in the vaccination which appear to 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 of if certain subsets of

Table III. *Adverse events.*

Toxicity	Grade			
	Total	1	2	3
Fatigue	10 (33%)	10 (33%)	0	0
Nausea	3 (10%)	3 (10%)	0	0
Anorexia	5 (17%)	5 (17%)	0	0
Cough	2 (7%)	2 (7%)	0	0
Seizure	1 (3%)	0	0	1 (3%)
Thromboembolic event	1 (3%)	0	1 (3%)	0
Anemia	4 (13%)	4 (13%)	0	0
Blood bilirubin increase	4 (13%)	3 (10%)	0	1 (3%)
Hypercalcemia	1 (3%)	0	0	1 (3%)
Anaphylaxis	1 (3%)	0	0	1 (3%)
Injection-site reaction	25 (83%)	25 (83%)	0	0