

Peptide-pulsed dendritic cell vaccine in combination with carboplatin and paclitaxel chemotherapy for stage IV melanoma

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In this study, we aimed to evaluate the feasibility and efficacy of peptide-pulsed dendritic cell (DC) vaccine in combination with carboplatin and paclitaxel chemotherapy (DCCP) for patients with stage IV melanoma previously treated with dacarbazine-containing regimen. Six HLA-A24⁺ and 3 HLA-A02⁺ patients were treated with carboplatin (area under the curve 5) and paclitaxel (175 mg/m²) on day 1 and DCs (2 × 10⁷ cells) pulsed with Wilms tumor gene 1 (WT1), gp100, tyrosinase, and either MAGE-A3 (for HLA-A24⁺) or MAGE-A2 (for HLA-A02⁺) peptides on days 8 and 22 in 28-day cycle for up to three cycles. DCCP was well tolerated, and median progression-free survival and median overall survival were 2.3 and 12.0 months, respectively. In four of nine patients, a WT1-specific immune response (WT1-IR) was detected using the interferon- γ enzyme-linked ImmunoSpot assay and WT1/HLA tetramer assay. DCCP was more likely to elicit a WT1-IR in patients who received DCs pulsed with the HLA-A24-restricted peptide (75%) compared with patients who received DCs pulsed with the HLA-A02-restricted peptide (0%, $P = 0.058$).

Introduction

Melanoma is a highly aggressive skin cancer that is particularly immunogenic, evident by its ability to undergo spontaneous regression [1]. The observation that vitiligo, an autoimmune skin disease characterized by patchy depigmentation, occasionally appears simultaneously with tumor regression supports this consideration [2,3]. Vitiligo is caused by the destruction of melanocytes by CD8⁺ T cells and can be induced by immunotherapy such as administration of a cancer vaccine to melanoma patients. CD8⁺ T cells that cause vitiligo are specific for melanocyte/melanoma-shared antigens such as gp100 and can reduce melanoma mass and prevent tumor recurrence [4–6]. Indeed, vitiligo induced by immunotherapy is a favorable prognostic factor [3,7].

To induce melanocyte/melanoma antigen-specific effector and memory T cells similar to the induction of vitiligo, numerous trials of dendritic cell (DC) vaccines have been performed for patients with advanced melanoma [8,9]. However, the efficacy of the DC vaccine is limited,

Furthermore, three (75%) of four patients with a WT1-IR survived longer than 12 months, whereas only one (20%) of five patients without a WT1-IR who received the BRAF inhibitor after DCCP survived longer than 12 months. These results suggest that DCCP may be beneficial for HLA-A24⁺ melanoma patients with a WT1-IR. *Melanoma Res* 00:000–000 Copyright © 2017 Wolters Kluwer Health, Inc. All rights reserved.

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with an objective response rate of 8.5% [10]. Although DC vaccines induce tumor-reactive T cells in the majority of the patients [11], in-vivo tumor reduction is difficult because of immunosuppression mediated by the tumor microenvironment, including exposure to immune checkpoint molecules such as PD-1/PD-L1 [12]. Therefore, combined treatment with a DC vaccine is required to attenuate immunosuppression and increase efficacy [13].

Carboplatin and paclitaxel chemotherapy (CP therapy) is a commonly used second-line chemotherapy for melanoma. In addition to causing direct cytotoxicity, carboplatin downregulates the inhibitory molecule PD-L2 expressed by DCs and melanoma cells to enhance T-cell activation [14]. Furthermore, paclitaxel reduces the number of regulatory T cells (Tregs), decreases production of the cytokines interleukin (IL)-10 and transforming growth factor- β by Treg, and stimulates DC-mediated antigen presentation [15–17]. Therefore,

the addition of CP therapy would likely enhance the efficacy of a DC vaccine.

The selection of tumor antigens is an important component of developing DC vaccines. The National Cancer Institute pilot project was conducted to prioritize 75 cancer antigens and concluded that Wilms tumor gene 1 (WT1), a zinc-finger transcription factor expressed in melanomas, which promotes proliferation, is the most promising cancer antigen [18–20]. Furthermore, melanoma differentiation antigen gp100 and tyrosinase as well as cancer/testis antigen MAGE-A3 are ranked within the top 20 most promising cancer antigens [18]. Moreover, several peptide vaccines comprising MAGE-A proteins induce antivaccine T cells as well as a far larger number of antitumor T cells that recognize other antigens expressed in the melanoma [21,22].

Accordingly, we hypothesized that DCs pulsed with peptides derived from WT1, gp100, tyrosinase, and either MAGE-A3 (for HLA-A24⁺) or MAGE-A2 (for HLA-A02⁺) peptides, which represents a multiple peptide cocktail-pulsed DC vaccine not yet explored, in combination with CP therapy (DCCP), may show higher efficacy compared with available DC vaccines used to treat patients with melanoma. To test this hypothesis, we conducted a phase I/II pilot study aimed to investigate the feasibility and efficacy of DCCP for patients previously treated with dacarbazine-based chemotherapy. Furthermore, we investigated immunological factors that may correlate with the induction of immune responses and prognosis.

Methods

Study design

This trial was a phase I/II pilot study conducted at Keio University School of Medicine (Tokyo, Japan) and Tokyo Midtown Clinic (Tokyo, Japan). The primary endpoint of this study was to explore the feasibility of DCCP. Adverse events were assessed according to the Common Terminology Criteria for Adverse Events, version 4.0. The secondary endpoints were overall survival (OS), progression-free survival (PFS), response rate, and induction of immunity against the WT1, gp100, tyrosinase, MAGE-A3, and MAGE-A2 peptides. The clinical response was evaluated according to the Response Evaluation Criteria in Solid Tumors (version 1.1). Events were measured from the first day of CP chemotherapy. This study was approved by the Institutional Review Board (IRB) of Keio University and fulfilled the principles of Declaration of Helsinki. All patients provided written informed consent. As a peptide-pulsed DC vaccine is not an approved therapy and CP chemotherapy is off-label use in melanoma, we obtained the review and approval of investigational use of DCCP by the IRB of Keio University and Oncology Board of Keio University Hospital. The trial was registered with the University Hospital Medical Information Network Clinical Trials Registry (<http://www.umin.ac.jp/ctr/>, number: UMIN-000006629).

Patients

Patients with histologically confirmed stage IV melanoma previously treated with dacarbazine-containing regimen were eligible. Other inclusion criteria were as follows: (a) 20–75 years of age; (b) HLA-A24⁺ or HLA-A02⁺ phenotype; (c) lesion that can be evaluated using Response Evaluation Criteria in Solid Tumors; (d) more than 4 weeks passed since prior treatment for melanoma; (e) Eastern Cooperative Oncology Group clinical performance status of 0 or 1; (f) adequate hematologic, hepatic, renal, and cardiac function; and (g) life expectancy of more than 4 months. Exclusion criteria were as follows: (i) other active primary malignancies; (ii) history of severe allergy; (iii) severe comorbidity (infections, cardiovascular disease, renal disease, liver disease, or uncontrolled diabetes); (iv) pleural effusion or pericardial fluid requiring treatment, (v) pregnancy or nursing; (vi) desiring to become pregnant; (vii) carrier of hepatitis virus, human T-cell lymphotropic virus type 1, or HIV; (viii) severe psychiatric disease; (ix) history of autoimmune disease; and (x) ongoing treatment with immunosuppressive agents or steroids.

DCCP treatment protocol

Intravenous carboplatin area under the curve 5 plus intravenous paclitaxel (175 mg/m²) was administered on day 1 of a 28-day cycle. Patients received intradermal injections of 2×10^7 DCs pulsed with WT1, gp100, tyrosinase, and either MAGE-A3 (for HLA-A24⁺) or MAGE-A2 (for HLA-A02⁺) peptides close to the axillary or inguinal lymph nodes on days 8 and 22 (Fig. 1). Patients continued treatment for up to three cycles unless they developed progressive disease (PD). After the completion of the protocol, DC vaccination continued with the patient's consent.

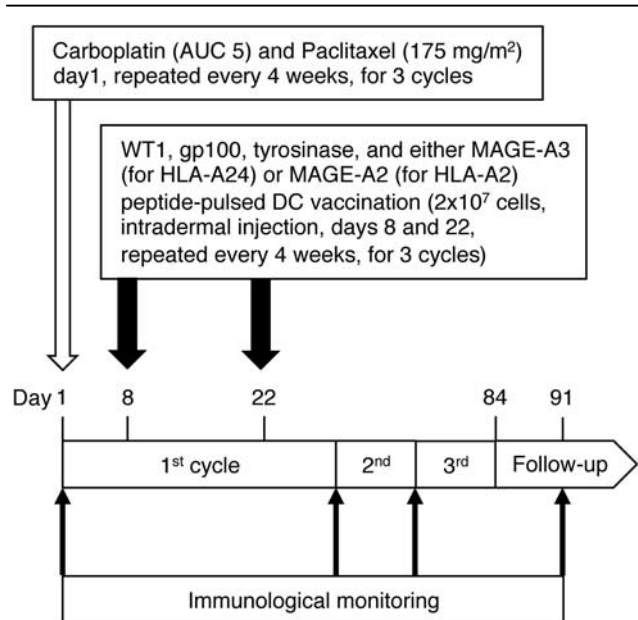
Pretreatment assessment and clinical monitoring

All patients underwent complete history taking, physical examination, blood tests, and computed tomography before treatment commenced. During DCCP treatment, patients underwent physical examinations and blood tests at least every 2 weeks to assess toxicities. In addition, computed tomography was repeated before each cycle and 4 weeks after the third cycle of DCCP to evaluate clinical response.

Generation of peptide-pulsed DC vaccine

DCs were generated from peripheral blood mononuclear cells (PBMCs) prepared from leukapheresis products as previously described [23]. Briefly, after leukapheresis, monocytes were enriched through their adherence to plastic and cultured using AIM-V Medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing granulocyte macrophage colony-stimulating factor (50 ng/ml; Primmune, Kobe, Japan) and IL-4 (50 ng/ml; R&D Systems, Minneapolis, Minnesota, USA) for 5 days to generate immature DCs. On day 6, the immature DCs

Fig. 1



Protocol of DC vaccination combined with carboplatin and paclitaxel chemotherapy. WT1, gp100, tyrosinase, and either MAGE-A3 (for HLA-A24⁺) or MAGE-A2 (for HLA-A02⁺) peptide-pulsed DCs (2×10^7 cells) were injected on days 8 and 22 every 4 weeks. Intravenous carboplatin at area under the curve 5 (AUC 5) and intravenous paclitaxel (175 mg/m^2) were administered on day 1 of a 28-day cycle. Immunological monitoring was performed on day 1 of each cycle before CP therapy and 1 week after the third cycle. CP, CP, carboplatin and paclitaxel; DC, dendritic cell.

were incubated with prostaglandin E2 (50 ng/ml; Daiichi Fine Chemical, Tokyo, Japan) and penicillin-killed and lyophilized preparations of a low-virulence strain (Su) of *Streptococcus pyogenes* (OK-432, 10 $\mu\text{g/ml}$; Chugai Pharmaceutical, Tokyo, Japan) for 24 h to generate mature DCs. The mature DCs were subjected to quality control according to a published method [24] or cryopreserved until the day of administration. The phenotypes CD11c⁺, CD14⁻, CD40⁺, CD80⁺, CD83⁺, CD86⁺, CCR7⁺, HLA-DR⁺, and HLA-ABC⁺ were defined as those of mature DCs, according to the quality criteria for DC vaccines. For each vaccination, cryopreserved DCs were thawed and pulsed with a mixture of WT1 peptide (CYTWNQMNL; PolyPeptide, San Diego, California, USA), gp100 peptide (VWKTWGQYW; AnyGen, Samtae, South Korea), tyrosinase peptide (AFLPWHRLF; PolyPeptide), and MAGE-A3 peptide (IMPKAGLLI; PolyPeptide) that were restricted to HLA-A24 or with a mixture of WT1 peptide (RMFPNAPYL; PolyPeptide), gp100 peptide (IMDQVPFSV; PolyPeptide), tyrosinase peptide (YMDGTMSQV; AnyGen), and MAGE-A2 peptide (YLQLVFGIEV; PolyPeptide) that were restricted to HLA-A02 for 60 min and washed twice before their use. All peptides used for generating peptide-pulsed DC vaccine were products synthesized under GMP conditions.

Immunological monitoring and PBMC preparation

PBMCs were collected and cryopreserved from patients on day 1 of each cycle before CP therapy and 1 week after the third cycle (day 91) for immunological monitoring. The immune responses to the WT1, gp100, tyrosinase, MAGE-A3, and MAGE-A2 peptides were analyzed using the interferon (IFN)- γ ELISPOT assay, HLA tetramer assay, and delayed-type hypersensitivity (DTH) skin test.

Cryopreserved PBMCs were thawed and pulsed with 10 $\mu\text{g/ml}$ of WT1, gp100, tyrosinase, and MAGE-A3 peptides restricted to HLA-A24 or with WT1, gp100, tyrosinase, and MAGE-A2 peptides restricted to HLA-A02 in AIM-V CTS Medium (Thermo Fisher Scientific) supplemented with 10% human AB serum (MP Biomedicals, Solon, Ohio, USA), 20 U/ml IL-2 (Shionogi, Osaka, Japan), and 10 ng/ml IL-7 (Peprotech, Rocky Hill, New Jersey, USA). After 9 days, the cells were analyzed using the IFN- γ ELISPOT assay and HLA tetramer assay.

WT1-specific, gp100-specific, tyrosinase-specific, MAGE-A3-specific, and MAGE-A2-specific IFN- γ ELISPOT assays

The IFN- γ ELISPOT assay was performed as previously described [25]. PBMCs were defined to be specifically sensitized by peptides when the number of spots in the ELISPOT assay indicating IFN- γ release in response to the WT1, gp100, tyrosinase, MAGE-A3, or MAGE-A2 peptide was at least 15 spots and of at least 1.5-times the response to HIVenv (for HLA-A24⁺) or HIVgag (for HLA-A02⁺) peptide-pulsed PBMCs.

HLA tetramer assay of PBMCs cultured with peptide cocktails

WT1-specific, gp100-specific, tyrosinase-specific, MAGE-A3-specific, and MAGE-A2-specific CD8⁺ T cells in patients' PBMCs were assessed using HLA tetramers (T-Select MHC Tetramer; Medical & Biological Laboratories, Nagoya, Japan), as previously described [26]. The results were defined as positive when WT1-, gp100-, tyrosinase-, MAGE-A3- or MAGE-A2-tetramer-positive CD3⁺CD8⁺ T cells were detected in clusters and not in a diffused population as well as no detectable HIVenv/HLA-A24 or HIVgag/HLA-A02 tetramer-positive CD3⁺CD8⁺ T cells.

DTH test

The DTH skin test was performed as previously described [27]. The diameters of the erythemas and indurations were measured 48 h after the injection of the WT1, gp100, tyrosinase, MAGE-A3, or MAGE-A2 peptide on day 1 of each cycle and at 4 weeks after the third cycle. An erythema diameter greater than 5 mm was defined as a positive reaction.

Flow cytometric analysis of cell phenotypes

PBMC samples were incubated with fluorescent dye-conjugated monoclonal antibodies for 45 min at 4°C in

the dark. After they were washed with FACS buffer (2% fetal bovine serum in PBS), the cells were fixed with stabilizing fixative (BD Biosciences, San Jose, California, USA) and analyzed using a flow cytometer (Gallios; Beckman Coulter, Pasadena, California, USA). Data were analyzed using Kaluza software (Beckman Coulter).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, California, USA). The survival curve was generated using the Kaplan–Meier method. Univariate analysis was conducted using the log-rank test. The immune response was analyzed using the *t*-test. Probability (*P*) values less than 0.05 were two sided and considered statistically significant.

Results

Patient characteristics

From October 2011 to October 2013, nine patients were enrolled. Patient clinical characteristics are listed in Table 1. The median age was 57 years (range: 43–68 years). Among the nine patients, the primary lesions of three patients were mucosal melanomas. Three of the nine patients had lung metastasis (stage IV-M1b) and the other six patients had metastasis to visceral organs other than the lung (stage IV-M1c). Six patients were positive for HLA-A24, and their HLA-A⁺ genotypes were HLA-A2402. All nine patients were positive for HLA-A02 and those three (33%) patients who were not HLA-A2402 received DCs pulsed with HLA-A02-restricted peptides. Five patients completed the protocol, and four (44%) patients terminated the protocol because of disease progression during the second cycle. One patient was administered postprotocol DC vaccination. The median frequency of DC vaccine administration was six times (range: 3–10 times). Three patients received CP therapy and four patients received postprotocol dacarbazine-based chemotherapy. One patient received vemurafenib, a BRAF inhibitor, and one patient received mogamulizumab, a humanized anti-CCR4 monoclonal antibody, as post-protocol therapy.

Adverse events

Adverse events that occurred within the protocol treatment period are summarized in Table 2. There were no adverse skin reactions at the site of DC vaccination. DCCP was well tolerated and adverse events that showed grade 3 or higher toxicity were related to hematotoxicity associated with CP therapy. One patient who developed grade 4 neutropenia was treated with granulocyte-colony stimulating factor and recovered promptly. No patient received blood or platelet transfusion. The non-hematological toxicities arthralgia and myalgia were treated with NSAIDs for several days during each cycle. Overall, treatment with the DC vaccine did not appear to exacerbate the adverse effects of CP therapy.

Clinical outcomes

One of nine (11%) patients achieved a partial response (PR), four (44%) had stable disease (SD), and the disease control rate was 55%. The median PFS and OS were 2.3 and 12.0 months, respectively (Fig. 2a and b). Furthermore, the median PFS and OS of patients treated with DCs pulsed with HLA-A24-restricted peptides were 2.9 and 14.1 months, respectively. The OS of patients with PR or SD was significantly better compared with those of patients with PD (*P*=0.0027, Fig. 2c). Among six patients treated with DCs pulsed with HLA-A24-restricted peptides, one patient exhibited PR, three patients exhibited SD, and four patients survived longer than 12 months. However, of the three patients treated with DCs pulsed with HLA-A02-restricted peptides, one patient who had been treated with vemurafenib, a BRAF inhibitor, after DCCP, exhibited SD and survived longer than 12 months (Table 2). Furthermore, there were no significant differences in the values of various inflammatory markers and cytokines such as the neutrophil/lymphocyte ratio, lactate dehydrogenase, C-reactive protein, IL-6, IL-8, and tumor necrosis factor- α between patients who achieved PR or SD and those who exhibited PD (data not shown).

Table 1 Patient characteristics and clinical outcomes

Case number	Age/sex	Primary sites	Metastatic sites	LDH	AJCC M category	Genotypes of HLA-A*	DC (times)	Postprotocol therapy	Types of response	PFS (months)	OS (months)	
1	54/female	Rectum	Distant LN	> ULN	M1c	2402	–	6	CP, DAC-Tam	PR	2.9	16.8
2	47/male	Esophagus	Retroperitoneum, distant LN	≤ULN	M1c	2402	0206	6	CP	SD	10.2	24.4
3	67/female	Lung	Peritoneum	≤ULN	M1c	2402	2601	6	DAC-Tam, mogamulizumab	SD	5.2	12.0
4	43/male	Shoulder	Liver	≤ULN	M1c	2402	–	6	CP, DAC-Tam	SD	2.8	14.1
5	68/male	Chest	Liver, lung	≤ULN	M1b	2402	2601	4	None	PD	1.2	4.4
6	57/female	Toe	Liver	> ULN	M1c	2402	0206	3	None	PD	1.0	2.9
7	67/male	Chest	Lung, distant LN, distant skin	≤ULN	M1b	0201	3101	10	DC, vemurafenib	SD	2.3	21.6
8	66/female	Abdomen	Liver, lung	≤ULN	M1c	0207	2602	4	DTIC	PD	1.2	4.6
9	44/male	Shoulder	Lung, distant LN	≤ULN	M1b	0201	0206	4	None	PD	1.4	2.2

AJCC, American Joint Committee on Cancer; CP, carboplatin and paclitaxel; DAC-Tam, chemotherapy combined with dacarbazine, nimustine hydrochloride, cisplatin, and tamoxifen; DC, dendritic cell vaccine; DTIC, dacarbazine; LDH, lactate dehydrogenase; LN, lymph node; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; ULN, upper limit normal.

Table 2 Adverse events

	N=9 [n (%)]		
	Grade 1/2	Grade 3	Grade 4
Neutrophil count decreased	2 (22)	5 (56)	1 (11)
Anemia	8 (89)	1 (11)	-
White blood cell decreased	6 (67)	2 (22)	-
Platelet count decreased	5 (56)	1 (11)	-
Arthralgia/myalgia	5 (56)	-	-
Alopecia	5 (56)	-	-
Fatigue	4 (44)	-	-
Peripheral sensory neuropathy	3 (33)	-	-
Diarrhea	3 (33)	-	-

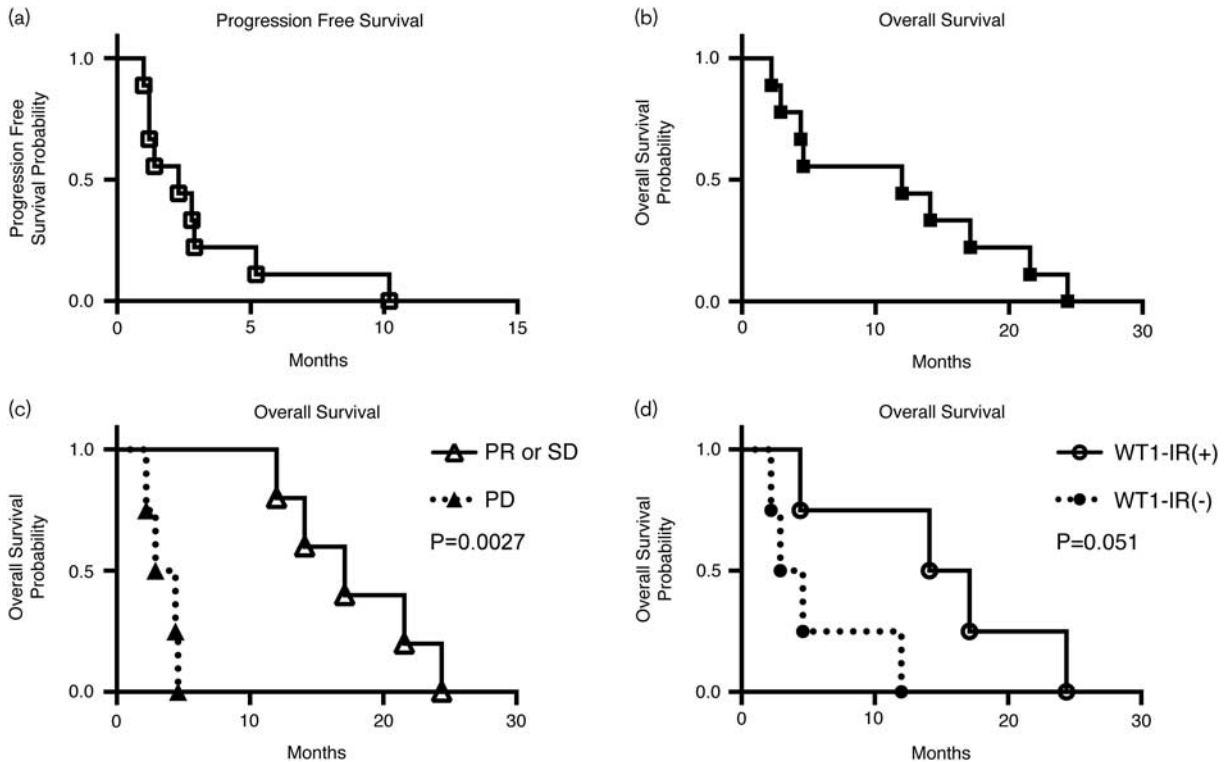
Immunological monitoring

In terms of induction of WT1-specific, gp100-specific, tyrosinase-specific, MAGE-A3-specific, and MAGE-A2-specific T-cell responses, DCCP elicited a WT1-specific immune response (WT1-IR) in four patients (cases 1, 2, 4 and 5), which was detected using the IFN- γ ELISPOT and HLA tetramer assay (Table 3). In addition, case 1 showed a gp100-specific immune response in the IFN- γ ELISPOT assay, and case 9, who received the DC vaccine pulsed with HLA-A02-restricted peptides, showed a gp100-specific immune response in the IFN- γ ELISPOT and HLA tetramer assay. Interestingly, DCCP was more likely to elicit a WT1-IR

in patients treated with DCs pulsed with HLA-A24-restricted peptides (66%, 4/6 patients) compared with patients treated with DCs pulsed with HLA-A02-restricted peptides (0/3 patients) ($P=0.058$). Furthermore, the number of WT1 tetramer-positive CD8⁺ T cells significantly increased after DC vaccination of patients treated with DCs pulsed with HLA-A24-restricted peptides ($P=0.047$), in contrast to those treated with DCs pulsed with HLA-A02-restricted peptides (Fig. 3a). Moreover, the analysis of OS, excluding the patient who showed significant tumor regression induced using BRAF inhibitor, showed possible longer survival in patients with a WT1-IR compared with those without WT1-IR ($P=0.051$, Fig. 2d). Given that patients with a WT1-IR were treated with DCs pulsed with HLA-A24-restricted peptides, these results suggest that DCCP may be beneficial for patients with HLA-A24⁺ melanomas that mount a WT1-IR.

As regards the DTH test, no patient treated with DCs pulsed with HLA-A02-restricted peptides exhibited DTH positivity to any of the peptides. Among the six patients treated with DCs pulsed with HLA-A24-restricted peptides, three patients reacted with gp100, tyrosinase, and MAGE-A3 at baseline (cases 2, 4, and 5), and one patient developed responses to gp100, tyrosinase, and MAGE-A3 peptides (case 1) after immunization (Table 3). Interestingly, no

Fig. 2



Kaplan-Meier analysis for (a) progression-free survival and (b) overall survival of melanoma patients treated with DCCP. Comparison of OS (c) in patients who achieved PR or SD ($N=5$) and PD ($N=4$), (d) in patients with a WT1-IR ($N=4$) and without a WT1-IR ($N=4$). A patient treated with vemurafenib (BRAF inhibitor) was excluded from the analysis. DCCP, dendritic cell vaccination combined with carboplatin and paclitaxel chemotherapy; PD, progressive disease; PR, partial response; SD, stable disease.

Table 3 Clinical outcomes and immunological monitoring data

Case number	HLA-A of DC vaccine	Types of response	OS (m)	HLA tetramer assay				IFN-γ ELISPOT assay				DTH test			
				Post-treatment				Post-treatment				Pretreatment/post-treatment			
				gp100	TYR	MAGE-A3/A2	WT1	gp100	TYR	MAGE-A3/A2	WT1	gp100	TYR	MAGE-A3/A2	WT1
1	A24	PR	16.8	-	-	-	+	+	-	+	+	-/+	-/+	-/+	-/-
2	A24	SD	24.4	-	-	-	+	-	-	-	+	+/-	+/+	+/-	-/-
3	A24	SD	12.0	-	-	-	-	-	-	-	-	-/-	-/-	-/-	-/-
4	A24	SD	14.1	-	-	-	+	-	-	-	+	+/+	+/+	+/+	-/-
5	A24	PD	4.4	-	-	-	+	-	-	-	+	+/-	+/+	+/-	-/-
6	A24	PD	2.9	-	-	-	-	-	-	-	-	-/-	-/-	-/-	-/-
7	A02	SD	21.6	-	-	-	-	-	-	-	-	-/-	-/-	-/-	-/-
8	A02	PD	4.6	-	-	-	-	-	-	-	-	-/-	-/-	-/-	-/-
9	A02	PD	2.2	+	-	-	-	+	-	-	-	-/-	-/-	-/-	-/-

DC, dendritic cell vaccine; DTH, delayed-type hypersensitivity; IFN, interferon; PD, progressive disease; PR, partial response; SD, stable disease; TYR, tyrosinase; +, positive; -, negative; +/-, positive at pretreatment DTH test and turned to negative in post-treatment DTH test.

patient exhibited DTH reactivity to WT1 peptides, whereas WT1-specific T-cell responses were detected in four patients in IFN-γ ELISPOT assay and HLA tetramer assay. This discrepancy suggests that these four patients were in immunosuppressed state and were unable to mount a strong immune response sufficiently robust to develop a DTH skin reaction *in vivo*, although there were WT1-specific T cells in blood that could induce WT1-specific T-cell responses *ex vivo*.

Prognostic factors

To identify factors predictive of the immune response to DC vaccination, we evaluated the various subsets of immune cells in pretreatment peripheral blood. Because there was no WT1-specific immune response among the HLA-A02+ patients, we investigated the immune response among patients who were HLA-A24 positive and found that a WT1-IR significantly correlated with a low percentage of Th17 (CD3+CD4+CXCR3-CCR6+) cells (P=0.036, Fig. 3b). In contrast, there was no significant difference in the percentages of naïve, effector, central memory CD8+ T cells, that of naïve, effector, central memory CD4 T cells, that of Th1, Th2, Treg, monocytic myeloid-derived suppressor cells (MDSCs), and granulocytic MDSCs between WT1-IR-positive and WT1-IR-negative patients (data not shown). These results suggest that DCCP is more likely to be effective in patients with HLA-A24+ melanoma with a low percentage of Th17 cells before treatment.

Discussion

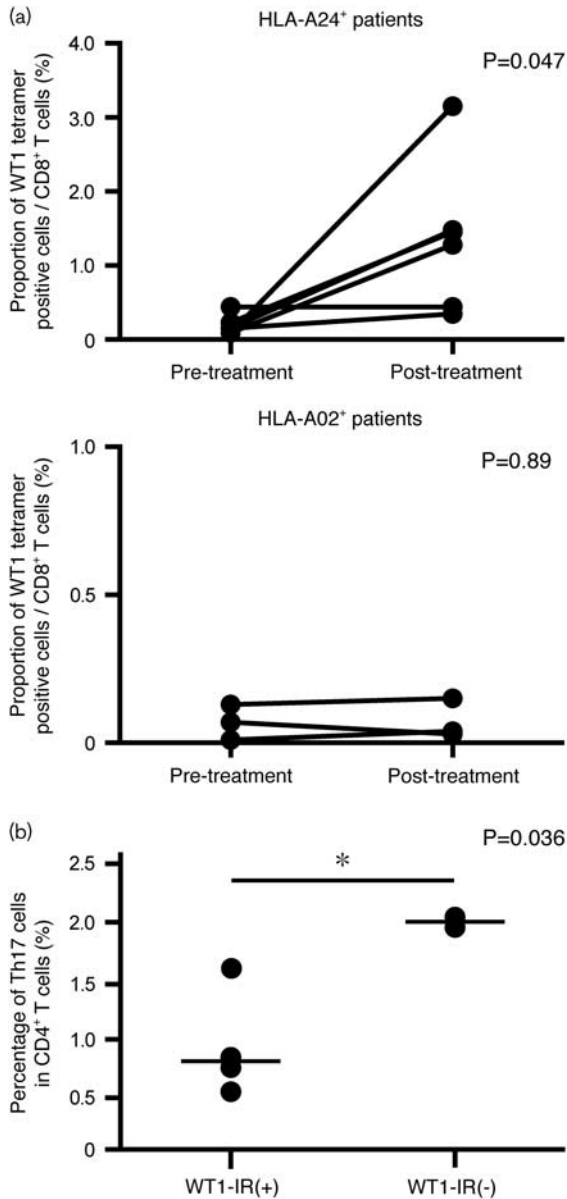
In this study, we evaluated the feasibility and efficacy of DCCP for treating patients with stage IV melanoma patients previously treated with dacarbazine-containing regimen. The most common adverse events were hematologic abnormalities, and their frequencies were comparable to those described in other studies of CP therapy [28–30]. Nonhematologic adverse events were mild (grade 1 or 2). Overall, the toxicity profile of DCCP was clinically acceptable.

Until the recent application of BRAF inhibitors, MEK inhibitors, and immune checkpoint blockers such as antibodies against CTLA-4 and PD-1, stage IV melanoma had an extremely poor prognosis because of its resistance to chemotherapy. Dacarbazine chemotherapy had been the standard first-line therapy; however, because of the low objective response rate of ~12%, which is often transient, CP therapy had been commonly used as a second-line regimen [31]. Given that the objective response rate and median PFS and OS of patients treated with CP are 5–22% and 2.3–4.1 and 5.2–9.7 months, respectively [28–30], DCCP seems to improve OS compared with CP therapy, but not response rate or PFS. These results suggest that the benefit of the combination of peptide-pulsed DCs with CP therapy is characterized by a delayed clinical effect, which is typically shown by various immunotherapeutic drugs [32–34]. Indeed, the metastatic lesions of all patients who survived longer than 12 months with a WT1-IR slowly progressed after they were evaluated as PD.

In the present study, none of the patients treated with DCs pulsed with HLA-A02-restricted peptides elicited a WT1-IR. DCCP was more likely to elicit a WT1-IR in patients treated with DCs pulsed with HLA-A24-restricted WT1 peptide compared with those treated with DCs pulsed with HLA-A02-restricted WT1 peptide (P=0.058). Furthermore, the median OS of patients with HLA-A24+ melanoma with a WT1-IR was 15.0 months, whereas the median OS of other patients (patients with HLA-A24+ melanoma without a WT1-IR and patients with HLA-A02+ melanoma) was 4.6 months. These results suggest that DCCP may be beneficial for HLA-A24+ melanoma patients with the induction of WT1-IR.

In humans and mice, the ability of the gp100 peptide to bind MHC class I molecules increases and enhances the induction of melanoma-reactive CD8+ T cells when amino acid residue substitutions are introduced into the wild-type gp100 peptide [35,36]. Similarly, the modified 9-mer HLA-A24-restricted WT1 peptide, a peptide with

Fig. 3



(a) Frequency of WT1-specific CD8⁺ T cells in peripheral blood of HLA-A24⁺ patients ($N=6$, upper panel) and HLA-A02⁺ patients ($N=3$, lower panel) before and after DCCP. (b) Pretreatment frequency of Th17 cells in peripheral blood of HLA-A24⁺ melanoma patients ($N=6$). Th17 cells were identified to be CD3⁺CD4⁺CXCR3⁻CCR6⁺. * $P<0.05$. DCCP, dendritic cell vaccination combined with carboplatin and paclitaxel chemotherapy.

a single amino acid residue substitution of the wild-type WT1 peptide, significantly increases the binding affinity to the HLA-A24 molecule and elicits WT1-specific CD8⁺ T cells more effectively compared with the wild-type WT1 peptide [37]. Here, we used a modified 9-mer HLA-A24-restricted WT1 peptide for treating HLA-A24⁺ patients, whereas we used the wild-type WT1 peptide for HLA-A02⁺ patients. Thus, the binding

affinity of the peptide for the HLA molecule appears to affect the induction of a WT1-IR and improved OS.

Numerous studies report that the DTH response is induced after DC vaccination and that DTH positivity correlates with improved OS [38]. In contrast, there are reports that DTH outcomes do not correlate with clinical response [39]. Here, only one patient developed DTH reactivity after immunization and the DTH outcome did not correlate with clinical response. However, four patients reacted with the WT1 peptide in both ex-vivo assays, IFN- γ ELISPOT assay and HLA tetramer assay, and these patients tended to have a more favorable prognosis compared with others, although none of these four patients reacted in the DTH assay to the WT1 peptide.

In a recent study, the WT1 peptide-pulsed DC vaccination combined with gemcitabine to treat pancreatic cancer was positive in the DTH assays of three of 10 patients, and all patients with liver metastasis were DTH-negative, although some were positive in the IFN- γ ELISPOT assay and HLA tetramer assay [27]. Similarly, here we found that all patients with liver metastasis had no DTH reactivity to WT1, and, among the four patients who had a WT1-specific T-cell response in IFN- γ ELISPOT assay and HLA tetramer assay, two had liver metastasis. Furthermore, immunosuppressive agents such as steroids can decrease DTH reactivity and, less frequently, cause anergy, which leads to the absence of a detectable DTH skin reaction [40]. Here we used steroids for longer times (3 days each cycle) to prevent emesis induced by CP therapy compared with a study of WT1 peptide-pulsed DC vaccination combined with gemcitabine administered to patients with pancreatic cancer (1 day each cycle) [27]. Therefore, another possible explanation for the lack of DTH positivity to the WT1 peptide in the current study is the prolonged use of steroids. Indeed, one (case 2) of four patients with a WT1-IR lost DTH reactivity to gp100 and MAGE-A3 peptides after the initiation of DCCP, although the patient did not have liver metastasis and achieved the longest OS. These results indicate the importance of patient selection and consideration of the period of using steroids to evaluate whether the DTH test serves as a useful indicator of prognosis.

We evaluated various immunological biomarkers in our patients before treatment, and we found that, among patients who received DCs pulsed with HLA-A24-restricted peptides, WT1-IR significantly correlated with a low percentage of Th17 cells. IL-17A is a key cytokine produced by Th17 cells, and the delayed growth of subcutaneously transplanted B16 melanoma cells in mice deficient in IL-17A reveal that the Th17 cell response can promote tumor growth [41]. However, when B16-F10 melanoma cells are intravenously injected, metastasis of melanoma to the lung increases in IL-17A-deficient mice, suggesting that Th17 suppresses the

progression of melanoma [42]. Furthermore, in a survivin-peptide vaccination trial for patients with previously treated stage IV melanoma, high frequencies of Th17 cells before vaccination were more likely to develop survivin-specific T-cell reactivity after vaccination, which is opposite to our results [43]. Thus, Th17 cells may play different roles depending on tumor stage and the antigenicity of a peptide. The relevance of Th17 cells for melanoma is controversial and warrants further investigation.

Conclusion

DCCP was well tolerated in patients with stage IV melanoma previously treated with dacarbazine-containing regimen and may be beneficial for HLA-A24⁺ patients with a WT1-IR. The induction of a WT1-IR seemed to be associated with the effect of a modified WT1 peptide with higher affinity for HLA-A24 and induces more WT1-specific CD8⁺ T cells compared with the wild-type WT1 peptide. Moreover, it has been reported recently that patients with metastatic melanoma who responds to a CTLA-4 or PD-1 inhibitor, which are immune checkpoint inhibitors and are recommended for first-line and second-line treatment of stage IV melanoma [44], had significantly higher numbers of CD8⁺ T cells in the tumor after two to three courses of treatment compared with those of nonresponders [45]. The HLA-A24-restricted peptide-pulsed DC vaccine of DCCP induced WT1-specific CD8⁺ T cells in four (67%) of six cases. Therefore, we believe that our results provide justification for the continued investigations of combination therapy comprising a DC vaccine and immune checkpoint inhibitors in patients with HLA-A24⁺ melanoma.

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Conflicts of interest

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