months in the gemcitabine alone group were 8.8 months and 35.4%, respectively. These results seemed a little better than those in our study. One reason for this may be the difference in the proportion of the patients with the locally advanced pancreatic cancer, in which survival data were apparently much better than those in metastatic ones. In our study, this proportion was 18.8%, which was lower than that in GEST study (23.8%). The other reason may be PS at baseline, which was also one of the important prognostic factors. The proportion of the patients with ECOG-PS 0, 1, and 2 at baseline in our study were 46.9%, 31.3%, and 21.9%, respectively, whereas those in GEST study were 65.3%, 34.7%, and 0.0%, respectively. It is apparent that our patients are predicted to worse prognosis than those in GEST study. Despite lower proportion of locally advanced stage and worse PS, however, the survival data gained from the patients with DTH-positivity seemed to be better than those in GEST study. These results suggested additional or synergistic effects of WT1 vaccine. Although the number of patients in our present study was too small to reach any

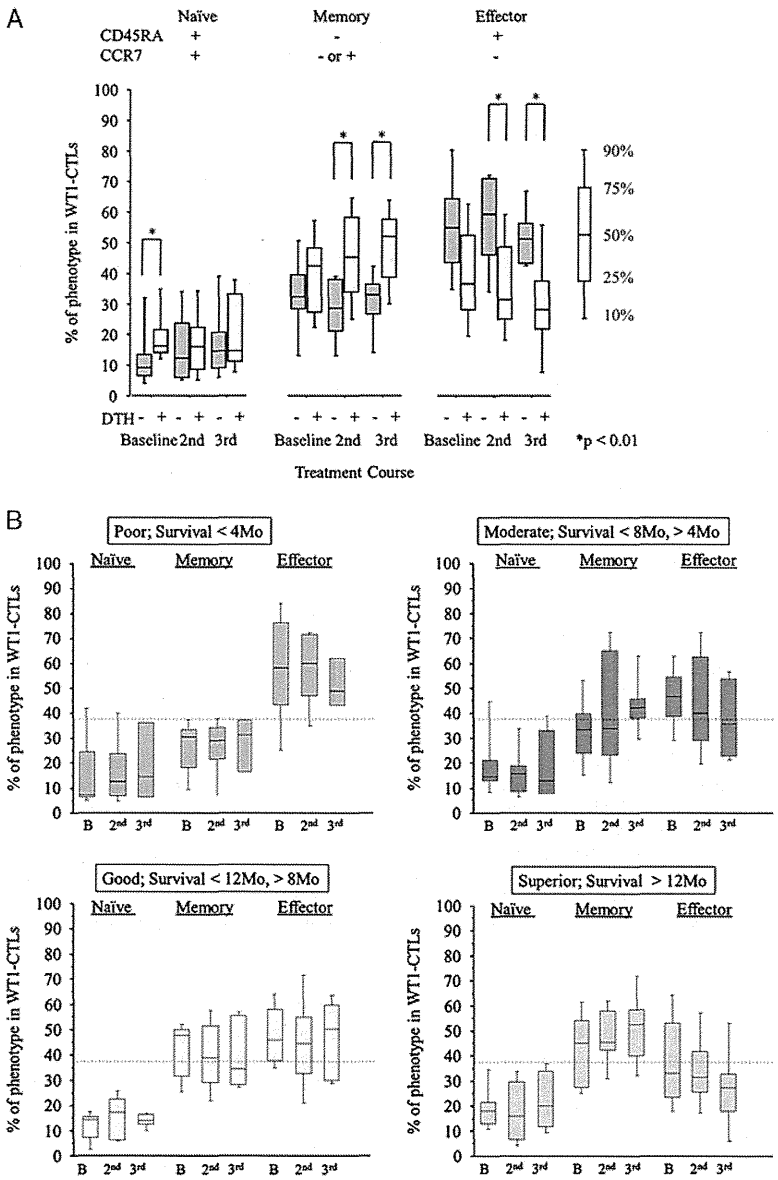

FIGURE 3. Analysis of WT1-specfic immune response. A, Immunologic monitoring of the phenotype analysis of WT1 tetramer$^+$ CD3$^+$CD8$^+$ T lymphocytes (WT1-CTLs) in DTH-positive (light gray columns) and DTH-negative patients (dark gray columns). B, Immunologic monitoring of the phenotype analysis of WT1 tetramer$^+$CD3$^+$CD8$^+$ T lymphocytes (WT1-CTLs) in the patients of 4 groups classified according to overall survival time. The broken line represents the median percentage of memory-phenotype WT1-CTLs at baseline for all patients. WT1 tetramer = PE-conjugated $WT1_{235}$ tetramer [HLA-A*24:02-restricted natural 9-mer WT1 peptide (CMTWNQMNL)], naive (CD45RA$^+$CCR7$^+$), memory (CD45RA-CCR7$^+$ or CD45RA-CCR7$^-$), and effector (CD45RA$^+$CCR7$^-$). 2nd indicates day 1 in the second course; 3rd, day 1 in the third course; B, baseline; DTH, delayed-type hypersensitivity.

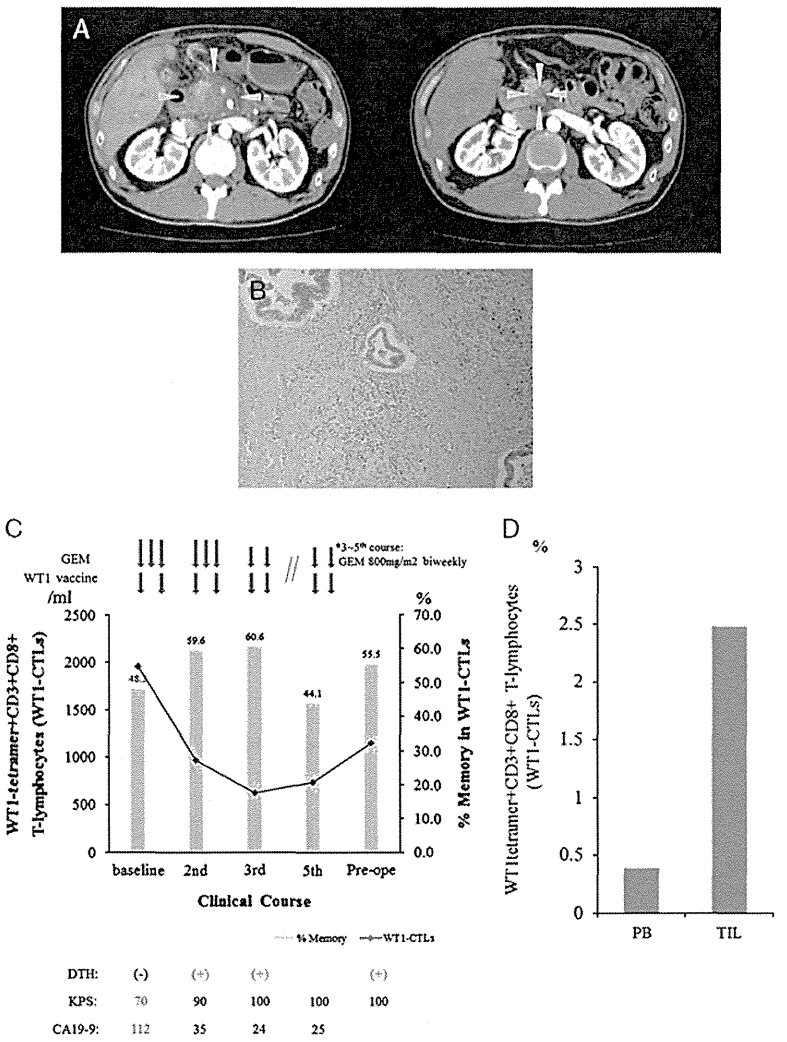

FIGURE 4. Clinical course and immunologic monitoring of 1 patient. A, Abdominal computed tomography (CT) scan before and after treatment. Left: CT scan at baseline showed a large hypodense lesion in the head of the pancreas, which had also invaded the supramesenteric artery and portal vein. Right: 5 months after treatment (before operation), a follow-up CT scan showed >80% regression of the primary lesion. Gray arrows shows primary lesion of pancreas. B, Microscopic findings of the resected specimen (hematoxylin-eosin stain). C, Clinical course and immunologic monitoring. The black line represents the absolute number of WT1 tetramer$^+$CD3$^+$CD8$^+$ T lymphocytes (WT1-CTLs), and the gray column represents the percentage of memory-phenotype WT1-CTLs. D, Percentages of WT1-CTLs in the peripheral blood (PB) and tumor-infiltrating lymphocytes (TIL). CA19-9 indicates carbohydrate antigen 19-9; CTLs, cytotoxic T lymphocytes; DTH, delayed-type hypersensitivity; GEM, gemcitabine; KPS, Karnofsky performance status.

definitive conclusions about clinical efficacy, these findings have been sufficiently encouraging to prompt us to conduct a further clinical study to determine the potency of this combination therapy. No combination chemotherapy, with the exception of FOLFIRINOX,[34] has resulted in a significant improvement in survival of patients with pancreatic cancer although some combination therapies are thought to be more effective for several cancers than single-agent treatments.[1] The use of FOLFIRINOX, however, may have to be limited to patients with good performance status as this regimen has much higher toxicity that sometimes can impair QOL.[34,35] In contrast, as toxicities associated with cancer vaccines are generally mild and acceptable, combination therapies using chemotherapy and cancer vaccine can be expected to exert their clinical benefits without worsening of QOL, which is often impaired by combination chemotherapies using several kinds of cytotoxic agents.

Immunologic monitoring is an important step in the development of evidence-based immunotherapy. Our data provided 2 useful prognostic markers of better clinical outcomes for the combination therapy used in our study. One is DTH to WT1 peptide and the other the frequency of memory-phenotype WT1-CTLs in PB although we did not find the correlation between clinical effects, including survival, and the frequency or absolute numbers of nonphenotypically divided WT1-specific CTLs statistically (data not shown). DTH-positive patients had a notably better prognosis than DTH-negative patients, and the OS curve for DTH-positive

patients showed a late separation beyond the median. As DTH has long been used for evaluation of antigen memory for bacterial, viral, and cancer antigens,[36] the occurrence of DTH to WT1 peptide may reflect the development and persistence of memory-phenotype WT1-CTLs. This can be inferred from our observation that DTH-positive patients showed a significantly higher frequency of memory-phenotype WT1-CTLs than did DTH-negative patients after WT1 vaccination. Furthermore, patients who survived 12 months or longer (superior responders) seemed to have the highest frequency of memory-phenotype WT1-CTLs in their PB although the number of patients in each subgroup was too small to make a statistically valid comparison. It was reported that long-term survivors who had been treated with mutant K-ras vaccine against pancreatic cancer showed the persistence of vaccinated peptide-recognizing T cells (long-term T-cell memory response) for many years after the last vaccination.[37] This report and our results suggest that the development and persistence of TAA-specific CTLs with memory-phenotype resulting from treatment with cancer vaccine contributed to the longer survival. Further investigations are needed to validate these findings in the larger-scale clinical trial.

Despite its potent cytotoxicity, gemcitabine reportedly has immune-modulating functions, such as increase in antigen cross-presentation,[38] and inhibition of B-cells,[39] myeloid-derived suppressive cells,[40] and regulatory T cells,[41] resulting in enhancement of the antigen-specific CTL function. Recently, we reported that gemcitabine enhanced the WT1 expression on human pancreatic cancer cells thus sensitizing the cancer cells to WT1-specific CTL.[11] Furthermore, it was reported that lymphopenia-induced memory-phenotype WT1-CTLs from naive-phenotype WT1-CTLs without self-antigen-induced tolerance.[42] Transient mild to moderate lymphopenia induced by gemcitabine and immediate recovery of T cells could thus promote both the differentiation of naive-phenotype WT1-CTLs into memory-phenotype WT1-CTLs and their proliferation in the clinical application of the combination therapy of gemcitabine and WT1 vaccine. In view of these immunostimulatory properties of gemcitabine, this combination therapy can be expected to generate additional or synergistic effects.

In conclusion, the combination of WT1 vaccine with the standard gemcitabine therapy was well tolerated for patients with advanced pancreatic cancer. WT1 vaccine might have additional effects on gemcitabine to improve survival benefit. An increase in memory-phenotype WT1-CTLs could be a useful predictive marker for a favorable clinical outcome. To determine the clinical efficacy of this combination therapy, we have started a phase 2 randomized clinical study (UMIN000005248).

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CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

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Cancer Therapy: Clinical

Clinical Cancer Research

Treatment with Chemotherapy and Dendritic Cells Pulsed with Multiple Wilms' Tumor 1 (WT1)–Specific MHC Class I/II–Restricted Epitopes for Pancreatic Cancer

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Abstract

Purpose: We performed a phase I trial to investigate the safety, clinical responses, and Wilms' tumor 1 (WT1)-specific immune responses following treatment with dendritic cells (DC) pulsed with a mixture of three types of WT1 peptides, including both MHC class I and II–restricted epitopes, in combination with chemotherapy.

Experimental Design: Ten stage IV patients with pancreatic ductal adenocarcinoma (PDA) and 1 patient with intrahepatic cholangiocarcinoma (ICC) who were HLA-positive for A*02:01, A*02:06, A*24:02, DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01 were enrolled. The patients received one course of gemcitabine followed by biweekly intradermal vaccinations with mature DCs pulsed with MHC class I (DC/WT1-I; 2 PDA and 1 ICC), II (DC/WT1-II; 1 PDA), or I/II–restricted WT1 peptides (DC/WT1-I/II; 7 PDA), and gemcitabine.

Results: The combination therapy was well tolerated. WT1-specific IFNγ-producing $CD4^+$ T cells were significantly increased following treatment with DC/WT1-I/II. WT1 peptide-specific delayed-type hypersensitivity (DTH) was detected in 4 of the 7 patients with PDA vaccinated with DC/WT1-I/II and in 0 of the 3 patients with PDA vaccinated with DC/WT1-I or DC/WT1-II. The WT1-specific DTH-positive patients showed significantly improved overall survival (OS) and progression-free survival (PFS) compared with the negative control patients. In particular, all 3 patients with PDA with strong DTH reactions had a median OS of 717 days.

Conclusions: The activation of WT1-specific immune responses by DC/WT1-I/II combined with chemotherapy may be associated with disease stability in advanced pancreatic cancer. *Clin Cancer Res; 20(16); 4228–39. ©2014 AACR.*

Introduction

Dendritic cells (DC) play important roles in the initiation and regulation of tumor-specific immune responses (1). Cancer cells express tumor-associated antigens (TAA) by MHC class I (MHC-I) molecules, and these antigens are recognized by $CD8^+$ cytotoxic T lymphocytes (CTL). Therefore, DCs have been pulsed with various MHC-I peptides to induce antigen-specific immunity (1). Other groups and our laboratory have shown that antigen-specific immune responses can be induced by DCs pulsed with an MHC-I–restricted peptide in patients with pancreatic ductal adenocarcinoma (PDA; refs. 2, 3). Most DC-based vaccines have targeted only $CD8^+$ CTLs; however, the antitumor effects of these vaccines are not as vigorous in clinical trials (2). Increasing evidence has suggested that $CD8^+$ CTLs depend on $CD4^+$ T cells, which provide the $CD8^+$ CTLs with growth

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Dendritic cells (DC) have been extensively used in the development of anticancer vaccines. Most DC-based cancer vaccines have targeted only $CD8^+$ CTLs; however, the antitumor effects of these vaccines are not as vigorous in clinical settings. $CD4^+$ T cells play a direct role beyond assisting in the generation of antitumor immunity. In this phase I study, we investigated the safety and the clinical and immunologic responses of DCs pulsed with a mixture of three types of WT1 peptides, including MHC class I and II–restricted epitopes (DC/WT1-I/II), when used in combination with chemotherapy. Our results showed that the combination therapy induced WT1 peptide–specific delayed-type hypersensitivity in 4 of the 7 patients with pancreatic cancer, and that these responses lasted throughout long-term vaccination and were associated with clinical responses. These findings suggest that targeting $CD4^+$ and $CD8^+$ T cells with DC/WT1-I/II could be a promising therapy for patients with pancreatic cancer.

factors, such as interleukin (IL)2, and can mediate the destruction of the tumor cells (4–8). Therefore, the presentation of antigenic epitopes of both MHC class I and II (MHC-I/II) induces high affinity T cells that react with the MHC-I/II epitopes (9).

With many TAAs, the establishment of criteria for selecting particular TAAs for clinical development is important. The Wilms' tumor gene 1 (*WT1*) is highly expressed in various types of malignancies, including pancreatic cancer (71%–75%; refs. 10, 11), and has been found to be both oncogenic during tumorigenesis (12) and immunogenic (13–15). Therefore, other groups and our laboratory have performed clinical studies investigating the efficacy of immunotherapies targeting WT1 using a MHC-I restricted peptide for patients with PDA (16, 17). Recently, MHC class II (MHC-II) epitopes derived from WT1 have been made available for use in clinical trials (18, 19). We hypothesized that the use of WT1 peptides, including MHC-I/II–restricted epitopes, would result in disease stability in patients with PDA. Here, we report a phase I clinical trial in stage IV patients with PDA that investigated the safety and effects of chemotherapy treatment combined with DCs pulsed with MHC-I/II–restricted WT1 epitopes.

Materials and Methods

Study design

This phase I study was reviewed and approved by the ethics committee of the Jikei Institutional Review Board, Jikei University School of Medicine (Tokyo, Japan), and by the clinical study committee of Jikei University Kashiwa Hospital [No. 21-204 (6082)]. In addition, this study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number, 000004063). All patients provided written informed consent, and the procedures were performed in accordance with the Helsinki Declaration. The primary endpoint was the assessment of the safety and toxicity of the combination therapy based on the Common Terminology Criteria for Adverse Events (CTCAE v.4.0). Toxicity was defined as hematologic and nonhematologic events, including at the vaccine-injection sites, during the first three courses. The secondary endpoint was the assessment of the immunologic response, tumor response, overall survival (OS), and progression-free survival (PFS) from the first treatment.

Patient population

Patients with pathologically or cytologically confirmed, measurable, metastatic pancreatic or biliary tract adenocarcinoma or with recurrent disease were eligible for this noncomparative, open-label, phase I study. All patients were required to have an HLA type of A*02:01, A*02:06, A*24:02, DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01. Approximately 80% of the Japanese population has HLA types A*02:01, A*02:06, or A*24:02. Additional inclusion criteria included patients with ages between 20 and 75 years, Karnofsky performance status (KPS) of 60% to 100%, a minimum 6-month interval from the completion of any previous treatment for recurrent disease, a life expectancy of $\geq$3 months, and adequate organ function. The exclusion criteria were pregnancy, serious infections, severe underlying disease, severe allergic disease, and a judgment of unsuitability by the principal investigator.

Clinical responses

Computed tomography was performed every 4 weeks during the treatment protocol and every 4 to 8 weeks during the additional treatment until the disease progressed, and treatment efficacy was determined according to the Response Evaluation Criteria in Solid Tumors (RECIST). Stable disease (SD) was defined as disease that was stable for more than 8 weeks after the start of the treatment.

DC preparation

DCs were generated from peripheral blood mononuclear cells (PBMC) prepared from leukapheresis products using Ficoll-Plaque Premium (GE Healthcare, Life Science) density gradient solution, as previously described (3). Briefly, plastic-adherent monocytes were cultured in AIM-V medium (Gibco) containing granulocyte macrophage colony-stimulating factor (50 ng/mL, Primmune Corp) and IL4 (50 ng/mL, R&D Systems) for 5 days to generate immature DCs. The immature DCs were then matured by incubation with penicillin-killed and lyophilized preparations of a low virulence strain (Su) of *Streptococcus pyogenes* (OK-432; 10 μg/mL, Chugai Pharmaceutical) and prostaglandin E2 (PGE2; 50 ng/mL, Daiichi Fine Chemical Co, Ltd) for 24 hours. The DCs were cryopreserved until the day of administration. After thawing, the cell viability for each vaccination was confirmed to be more than 90% using Trypan blue exclusion analysis. To determine the phenotype of the DCs, the cells were incubated with the following monoclonal

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antibodies (mAb): fluorescein isothiocynate (FITC)-conjugated anti-human CD14 (61D3, eBioscience), HLA-ABC (W6/32), CD80 (2D10), CD40 (5C3), phycoerythrin (PE)-conjugated anti-human CCR7 (150503, R&D Systems), CD11c (3.9), HLA-DR (L243), CD83 (HB 15e), and CD86 (IT2.2; BioLegend). The cells were analyzed using MACSQuant Analyzers (Miltenyi Biotec Inc.) and the FlowJo analysis software (Tree Star). The endotoxin levels and bioburden of the DCs were tested at the SRL Inc.. A general mycoplasma test was performed using a PCR ELISA Kit (Roche Applied Science).

WT1 peptide–pulsed DCs (DC/WT1)

For each vaccination, DCs were pulsed with the MHC-I, -II, or -I/II–restricted epitopes of WT1, depending on their HLA. Briefly, mature DCs were incubated with WT1 peptides restricted to HLA-A*02:01, A*02:06 (126-134: RMFPNAPYL, NeoMPS Inc.) or A*24:02 (235-243: CYTWNQMNL, NeoMPS Inc.; refs. 16, 17), and/or DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01 (332-347: KRYFKLSHLQMHSRKH, NeoMPS Inc.; refs. 20, 21, and unpublished data) for 30 minutes and washed with saline. The DC/WT1 cells were suspended in 500 μL of saline for injection. The endotoxin levels and bioburden of these peptides were tested and determined to be acceptable based on the GMP grade for the vaccines.

Combination therapy

Gemcitabine was intravenously administered at a dose of 1,000 mg/m^2 on days 1, 8, and 15 of a 28-day cycle. After the first cycle of gemcitabine administration, the patients were treated with a combination of gemcitabine and DC/WT1. The DC/WT1 vaccine (usually 1×10^7 cells/dose) was intradermally administered biweekly at six different sites (bilateral upper arms, lower abdomen, and femoral regions) regardless of the regimen of chemotherapy. However, nearly all vaccines overlapped with standard chemotherapy. The initial treatment protocol was planned as three courses. The patients without early progressive disease at the completion of the treatment protocol could receive additional treatment until the occurrence of disease progression, unacceptable adverse events, or withdrawal of consent.

Delayed-type hypersensitivity test

The delayed-type hypersensitivity (DTH) test was performed before treatment in all patients and after 2, 4, and 6 vaccinations; it was also performed at suitable times during the additional treatment. Briefly, 30 μg of a WT1 peptide (RMFPNAPYL, CYTWNQMNL, or KRYFKLSHLQMHSRKH) in saline or saline alone was intradermally injected in the forearm, and the maximum diameter of erythema and other skin reactions, including induration, were measured after 48 hours. WT1-specific DTH positivity was defined as erythema greater than 2 mm in diameter, which was the minimum size measurable with a ruler. Moreover, we chose the value of 5-mm erythema to discriminate between weak (2–5 mm) and strong (>5 mm) DTH.

PBMC preparation for immunologic monitoring

PBMCs were obtained from the patients before chemotherapy and during vaccination. The PBMCs were prepared using a Ficoll-Plaque Plus (GE Healthcare Bio-Sciences) density gradient solution and were stored at −80°C in Bambanker (Nippon Genetics Co., Ltd.) without serum within 24 hours of blood collection. After thawing, cell viability was confirmed to be greater than 90% using the Trypan blue exclusion assay.

Detection of WT1-specific immune responses

The cryopreserved PBMCs were thawed and cultured with 10 μg/mL WT1 class I and II peptides in the presence of recombinant human (rh) IL2 (10 U/mL; Shionogi) and IL7 (10 ng/mL, Peprotech) for 9 days. The HIV env peptides and matched isotype IgG were used as negative controls. Cells were assessed using the PE-conjugated tetramer for WT1/HLA-A*24:02 (MBL) and FITC-conjugated anti-human CD8 mAb (BioLegend). The PE-conjugated tetramer for human immunodeficiency virus (HIV env)/HLA-A*24:02 (RYLRDQQLL) was used as a negative control, and cytomegalovirus (CMV pp65)/HLA-A*24:02 (QYDPVAALF) was used as a positive control (MBL). The amount of WT1-specific CD8$^+$ T cells (WT1-CTLs) is shown as the percentage of the double-positive population (WT1/HLA-A*24:02 tetramer–positive CD8$^+$) in the total CD8$^+$ T cells.

Detection of WT1/HLA-A*24:02–specific memory cells in CD8$^+$ T cells

To assess the population of WT1/HLA-A*24:02-specific memory cells in CD8$^+$ T cells, cryopreserved PBMCs from six vaccinations with DC/WT1-I/II were thawed and immediately assessed using the following mAbs: 7-amino-actinomycin D (7-AAD; eBioscience), APC-Cy7–conjugated anti-human CD3, PE-conjugated tetramer for WT1/HLA-A*24:02, FITC–conjugated anti-human CD8, PE-Cy7–conjugated anti-human CD45RA (BioLegend), APC-conjugated anti-human CCR7 (BD Biosciences), or matched isotype control IgG (BioLegend). First, CD3$^+$7-AAD$^-$ cells were gated, and then the lymphocytes were re-gated. The percentage of memory (CD45RA$^-$CCR7$^+$ and CD45RA$^-$CCR7$^-$) phenotypes in the entire CD3+CD8$^+$ T-cell population was determined. Then, the number of WT1/HLA-A*24:02 tetramer–positive cells in the total memory cells was determined. Finally, the percentages of WT1/HLA-A*24:02 tetramer–positive memory cells in CD8$^+$ T cells were determined using MACSQuant Analyzers and the FlowJo analysis software.

ELISA

To assess the production of IFNγ or IL10 in the PBMCs, the PBMCs (1×10^6 cells/mL in each well) from 6 vaccinations were cultured with 10 μg/mL WT1 class I and II peptides in the presence of 10 U/mL rh IL2 and 10 ng/mL IL7 for 6 days. The HIV env peptides were used as negative controls. The supernatants from the samples were analyzed for IFNγ or IL10 using an ELISA (BioLegend) according to the manufacturer's instructions.

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Intracellular staining of IFNγ

To assess the function of the WT1-CTLs, PBMCs (1×10^6 cells/mL in each well) were cultured with 10 μg/mL WT1 class I and II peptides in the presence of 10 U/mL rh IL2 and 10 ng/mL IL7 for 9 days. The HIV env peptides and matched isotype IgG were used as negative controls. The cells (1×10^5 cells/50 μL in each well) were restimulated with 10 μg/mL WT1 class I and II peptides for 6 hours using a GolgiPlug kit (BD Pharmingen); the cells were then stained with FITC-conjugated anti-human CD8 mAb, APC-Cy7–conjugated anti-human CD4 mAb (eBioscience), and APC-conjugated anti-human IFN-γ mAb (BioLegend). The IFNγ-producing cells in the $CD4^+$ or $CD8^+$ T cells were analyzed using MACSQuant Analyzers and the FlowJo analysis software.

Proliferation assays

PBMCs (1×10^6 cells/mL in each well) were cultured with 10 μg/mL WT1 class II peptide in the presence of 10 U/mL rh IL-2 and 10 ng/mL IL7 for 9 days. The cells were stained with FITC-conjugated anti-human CD8 mAb and APC-Cy7–conjugated anti-human CD4 mAb, and then the total $CD4^+$ or $CD8^+$ T-cell numbers were determined.

Detection of immunosuppressive cells

To assess the $CD4^+CD25^+$forkhead box p3 $(Foxp3)^+$ regulatory T cells (Tregs) in the $CD4^+$ T cells, the PBMCs were stained with FITC-conjugated anti-human CD4 mAb, APC-conjugated anti-human CD25 mAb, and PE-conjugated anti-human Foxp3 mAb or matched isotype control IgG using the Human Primate Regulatory T-Cell Staining Kit (eBioscience) according to the manufacturer's instructions. The $CD4^+$ T cells were analyzed for CD25 and Foxp3 expression. For the analysis of the myeloid-derived suppressor cells (MDSC), the PBMCs were stained with 7-AAD, PE-conjugated anti-human CD14 mAb, FITC-conjugated anti-human CD11c mAb, APC-conjugated CD33 mAb (BioLegend), or the matched isotype control IgG. The $CD14\text{-}CD11b^+CD33^+$ populations in the live PBMCs were defined as MDSCs. The cells were analyzed using MACSQuant Analyzers and the MACSQuantify Software (Miltenyi Biotec Inc).

Detection of WT1/HLA-A*24:02–specific $PD1^+$ cells in $CD8^+$ T cells

To assess whether the WT1-CTLs were impaired by the DC/WT1-I/II vaccination, the following mAbs were used: APC-Cy7–conjugated anti-human CD3, FITC-conjugated anti-human CD8, PE-conjugated tetramer for WT1/HLA-A*24:02, and APC-conjugated anti-human CD279 [programmed death 1 (PD1); BioLegend]. The HIV env/HLA-A*24:02 peptide and matched isotype IgG were used as negative controls. First, $CD3^+$ cells were gated and then the lymphocytes were regated. The percentage of $PD1^+$ cells in the entire $CD8^+$ T-cell population was determined. Then, the number of WT1/HLA-A*24:02 tetramer–positive cells in the $PD1^+CD8^+$ T-cell population was determined. Finally, the population of WT1/HLA-A*24:02 tetramer–positive $PD\text{-}1^+$ cells in $CD8^+$ T cells was analyzed using MACSQuant Analyzers and the FlowJo analysis software.

Statistical analysis

OS and PFS were calculated from the date of treatment to the date of death or final follow-up and the date of disease progression, respectively. Statistical analyses of the prognostic factors of OS or PFS were performed using the Kaplan–Meier method and were evaluated using the log-rank test. Immunologic parameters in the patients after therapy were evaluated using Student's t test for two independent groups and a one-way analysis of variance for multiple-group comparisons. A P value less than 0.05 was considered statistically significant.

Results

Patient characteristics

Between August 2011 and January 2013, 11 patients were enrolled. The patient characteristics are presented in Table 1. Ten patients had PDA and 1 patient had intrahepatic cholangiocarcinoma (ICC). First, 2 patients with PDA and 1 patient with ICC were treated with DCs pulsed with MHC-I (HLA-A*02:01, 02:06 and/or 24:02)–restricted WT1 peptides (DC/WT1-I). Then, 1 patient with PDA was treated with DCs pulsed with MHC-II (DRB1*04:05, DRB1*08:03, DRB1*15:01, DRB1*15:02, DPB1*05:01, or DPB1*09:01)–restricted WT1 peptides (DC/WT1-II; Supplementary Fig. S1). We assessed the toxicities in these 4 patients. Next, DCs pulsed with MHC-I/II–restricted WT1 peptides (DC/WT1-I/II) were used for the remaining 7 patients with PDA. All patients completed the initial treatment protocol (first three courses; Supplementary Fig. S2).

Toxicity

The toxicities documented within the first three courses are shown in Supplementary Table S1. During these periods, the average total gemcitabine dose was 7,400 mg/m^2, and the total vaccination was 3.8 times. One patient, PDA-06, with multiple liver metastases showed rapid disease progression in the liver and died of a cerebral infarction 133 days after the first treatment. Finally, PDA-06 received 10 gemcitabine (10,000 mg/m^2) and 7 DC/WT1-I/II treatments. A grade 1 pulmonary fibrosis occurred in one patient (PDA-07) after a total of 12 gemcitabine (11,800 mg/m^2) and 6 DC/WT1-I/II treatments. All 11 patients experienced grade 1 skin reactions at the site of vaccination. Grade 1 to 3 leukocytopenia and anemia thought to be caused by gemcitabine were observed in all 11 patients. Grade 1 to 3 lymphopenia and grade 2 to 3 neutropenia were also observed in 10 patients. Other major nonhematologic adverse events included grade 1 to 2 anorexia and nausea, all of which were previously reported as major adverse events associated with gemcitabine. Grade 1 to 3 hepatic transaminase elevation related to disease progression and/or hepatobiliary infection was detected in 5 patients.

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Table 1. Patient characteristics

Patient	Sex	Age, y	UICC stage	Location	Metastases	Prior systemic treatment	HLA genotype						Vaccine	Times	WT1 peptide–specific DTH													
							HLA-A		DRB1		DPB1				A*02:01 Vaccine times					A*24:02 Vaccine times					DRB1/DPB1 Vaccine times				
															0	2	4	6	8	0	2	4	6	8	0	2	4	6	8
PDA-01	M	41	IV	PDA (body–tail)	Bone, LN	No	02:01	—	08:03	12:01	02:02	15:01	DC/WT1-I	19	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PDA-02	M	69	IV	PDA (body–tail)	Lung, peritonitis, LN	No	24:02	26:01	12:01	15:02	05:01	09:01	DC/WT1-I	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PDA-03	M	73	IV	PDA (head)	Peritonitis	No	26:03	33:03	08:02	08:03	02:02	05:01	DC/WT1-II	13	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ICC-01	F	58	IV	ICC	Peritonitis, LN	No	02:01	24:02	04:05	15:02	09:01	—	DC/WT1-I	15	–	+	+	+	+	–	+	++	++	++	–	–	–	–	–
PDA-04	M	70	IV	PDA (body)	Peritonitis	No	02:01	24:02	04:05	15:02	05:01	09:01	DC/WT1-I/II	35	–	+	++	++	++	–	+	++	++	++	–	+	++	++	++
PDA-05	M	68	IV	PDA (body)	Liver, LN	Ope, Cx	24:02	33:03	08:03	13:02	02:02	04:01	DC/WT1-I/II	46	–	–	–	–	–	–	+	++	++	++	–	+	++	++	++
PDA-06	F	49	IV	PDA (head)	Liver, peritonitis, LN	No	02:01	24:02	04:05	09:01	02:02	05:01	DC/WT1-I/II	7	–	–	–	–	N.D.	–	–	–	–	N.D.	–	–	–	–	N.D.
PDA-07	M	35	IV	PDA (body)	Liver, LN	No	02:01	—	09:01	15:01	02:01	05:01	DC/WT1-I/II	6	–	–	–	–	N.D.	–	–	–	–	N.D.	–	–	–	–	N.D.
PDA-08	F	72	IV	PDA (body)	Peritonitis, LN	No	02:06	24:02	08:02	12:01	02:01	05:01	DC/WT1-I/II	14	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
PDA-09	F	69	IV	PDA (body–tail)	LN	No	24:02	33:03	13:02	15:01	04:01	13:01	DC/WT1-I/II	44+	–	–	–	–	–	–	+	++	++	++	–	+	++	++	++
PDA-10	M	39	IV	PDA (head–body)	Peritonitis	Ope	02:10	24:02	15:01	15:02	02:02	09:01	DC/WT1-I/II	20	–	–	–	–	–	–	–	+	+	+	–	–	+	+	+

Abbreviations: Cx, chemotherapy; DTH -, erythema <1 mm; DTH +, erythema 2- 5 mm; DTH ++, erythema > 5 mm; LN, lymph nodes; N.D., not done; Ope, operation.

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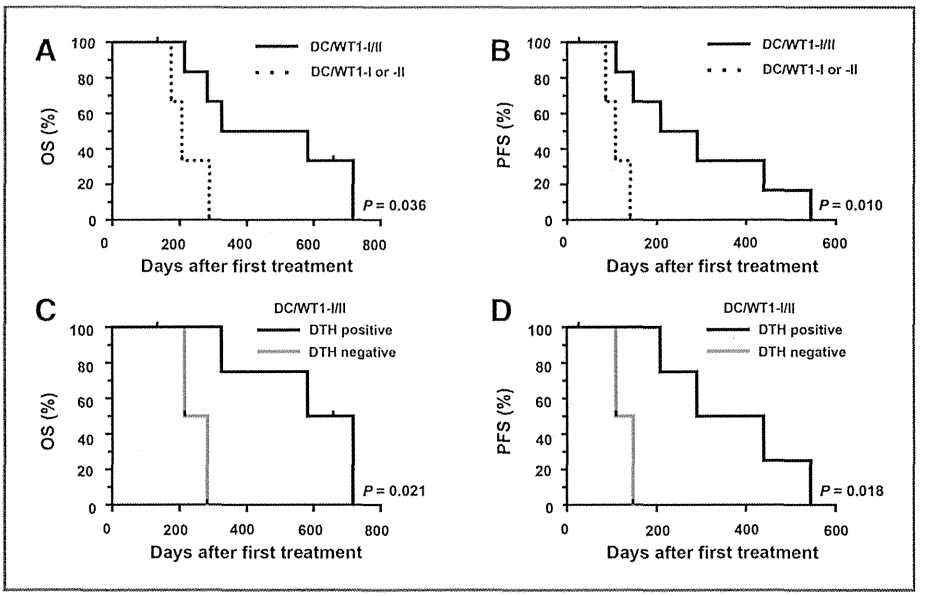

Figure 1. OS and PFS in patients with PDA. A, Kaplan–Meier estimates of OS for patients with PDA who were treated with DCs pulsed with WT1 MHC class I (DC/WT1-I) or -II (DC/WT1-II; $n = 3$) or -I/II peptides (DC/WT1-I/II; $n = 7$). B, PFS in patients with PDA who received DC/WT1-I or -II ($n = 3$) or DC/WT1-I/II ($n = 7$). C, OS in delayed type hypersensitivity (DTH)-positive ($n = 4$) or -negative ($n = 3$) PDA patients who received DC/WT1-I/II. D, PFS in DTH-positive ($n = 4$) or -negative ($n = 3$) patients with PDA who received DC/WT1-I/II.

Clinical responses

None of the 7 patients with PDA vaccinated with DC/WT1-I/II reached a complete or partial response, and 6 of these patients (85.7%) exhibited SD (Supplementary Table S2). However, 1 of the 3 PDA patients (33.3%) vaccinated with DC/WT1-I or -II exhibited SD, and the remaining 2 patients (66.7%) exhibited progressive disease (PD; Supplementary Table S2). The median survival time (MST) and the median PFS of the patients with PDA vaccinated with DC/WT1-I/II were significantly longer than those receiving the DC/WT1-I or -II vaccines ($P = 0.036$ and $P = 0.010$, respectively; Fig. 1A and B).

Assessment of WT1-specific immune responses *in vivo*

No patients with PDA exhibited DTH reactivity against the WT1 peptides during pretreatment. After vaccination with DC/WT1-I/II, 4 of the 7 patients with PDA (57.1%) showed DTH positivity, and all conversions were detected during the treatment protocol (Table 1). However, DTH positivity was not detected in all 3 patients with PDA vaccinated with DC/WT1-I or -II (Table 1). Interestingly, the MST and the median PFS of the DTH-positive PDA patients vaccinated with DC/WT1-I/II were significantly longer than that of the DTH-negative PDA patients ($P = 0.021$ and $P = 0.018$, respectively; Fig. 1C and D). Moreover, 3 patients with PDA who were vaccinated with DC/WT1-I/II were strongly DTH-positive during the entire treatment period and had an MST of 717 days and a median PFS of 440 days (Supplementary Table S2).

Analysis of WT1-specific immune responses *in vitro*

We first analyzed the production of IFNγ or IL10 from the PBMCs of patients vaccinated with DC/WT1-I, -II, or -I/II. After six vaccinations, all vaccines induced extremely high levels of IFNγ upon stimulation with WT1-I/II peptides *in vitro* compared with the levels of IL10 (Fig. 2A). Next, the percentages of the IFNγ–positive $CD4^+$ or $CD8^+$ T-cell populations were examined (Fig. 2B). The maximum levels of IFNγ-producing cells in the $CD4^+$ T cells were significantly increased by vaccinations with DC/WT1-I/II compared with DC/WT1-I or -II (Supplementary Table S3). In addition, IFNγ-positive T cells were at baseline levels before the vaccinations (data not shown). To assess the more critical role of the WT1-specific $CD4^+$ T-cell response that is restricted by MHC class II molecules with the DC/WT1-I/II vaccine, proliferation assays were performed. Figure 2C shows that, upon stimulation with WT1 class II peptide *in vitro*, $CD4^+$ T cells from DC/WT1-I/II-vaccinated patients with PDA showed significant proliferation compared with DC/WT1-I or -II vaccination. Moreover, no significant difference between the proliferation of $CD4^+$ or $CD8^+$ T cells before and after vaccination with DC/WT1-I or -II was observed.

HLA-A*24:02-positive patients were selected to assess the induction of HLA-A*24:02–restricted WT1-CTLs (Fig. 3A), as adequate HLA-A*02:01 and HLA-DRB1/DPB1 tetramers were not available. Eight patients were positive for HLA-A*24:02 among all enrolled patients. In all 8 of the HLA-A*24:02-positive patients, 2 were vaccinated with DC/WT1-I, and 6 were vaccinated with DC/WT1-I/II. Before treatment, the number of WT1-CTLs was extremely low in all 8 patients. However, the WT1-CTLs were induced in all 8 patients during the vaccination period (Fig. 3B). The percentage of WT1-CTLs in the total $CD8^+$ T-cell population derived from the patients vaccinated with DC/WT1-I/II was higher, but not significantly higher, than that in the patients vaccinated with DC/WT1-I after seven or eight vaccinations (Fig. 3C, left). In the DC/WT1-I group, the WT1-CTLs were increased early

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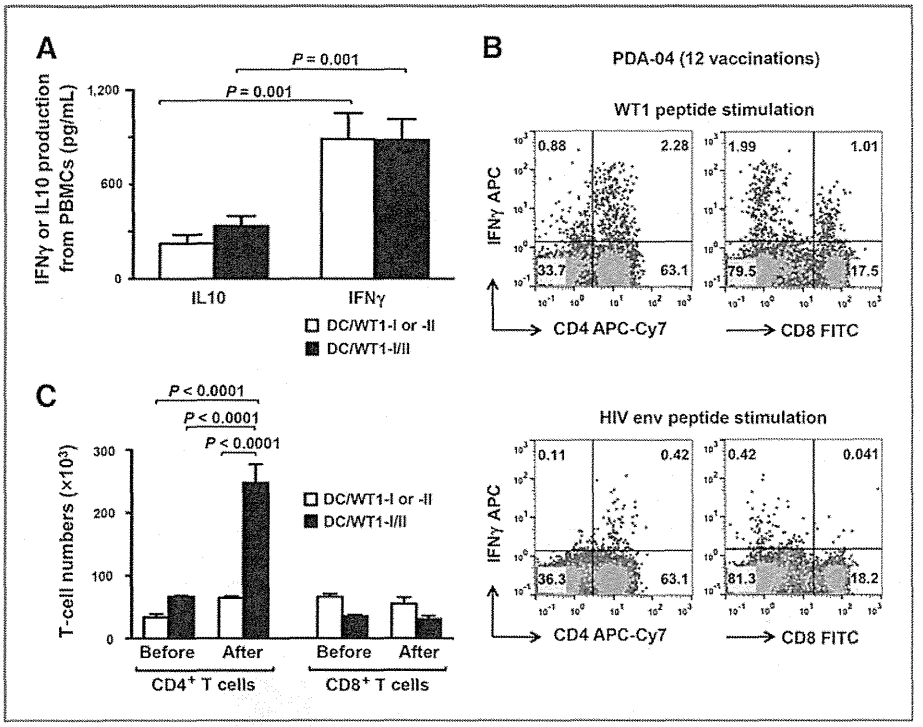

Figure 2. $CD4^+$ T-cell responses after vaccination with DC/WT1-I/II. A, The production of IFNγ or IL10 in peripheral blood mononuclear cells (PBMC) after six vaccinations with DC/WT1-I or -II ($n = 4$) or DC/WT1-I/II ($n = 7$) following stimulation with WT1 class I and II peptides was assessed. B, dot blots of PBMCs (PDA-04) analyzed for the IFNγ-producing cells in the $CD4^+$ and $CD8^+$ T cells upon stimulation with WT1 class I and II peptides *in vitro* are shown. HIV env peptides and matched isotype IgG were used as controls. C, $CD4^+$ and $CD8^+$ T-cell numbers upon stimulation of PBMCs from vaccinated patients [DC/WT1-I or -II ($n = 4$) or DC/WT1-I/II ($n = 7$)] with WT1 class II peptide *in vitro*. The results are expressed as the mean ± SE.

after vaccination, but this increase was not maintained during the entire vaccination period (Fig. 3B left). However, the WT1-CTLs in the circulating $CD8^+$ T-cell population derived from the DTH-positive patients vaccinated with DC/WT1-I/II were maintained during the entire vaccination period (Fig. 3B middle). We also assessed the association between survival and the WT1-specific immune responses in the HLA-A*24:02-positive PDA patients vaccinated with DC/WT1-I/II. As an OS of ≥1 year generally indicates that the treatment has been beneficial (22), the patients vaccinated with DC/WT1-I/II were classified into 2 groups: OS ≥ 1 year (super-responders) and OS < 1 year (nonsuper-responders). Three super-responders, all of who received DC/WT1-I/II, were discovered. We could not detect a difference in the percentage of WT1-CTLs in the total $CD8^+$ T-cell populations between these 2 groups after 7 or 8 vaccinations (Fig. 3C right).

Because assessing the WT1-specific memory $CD8^+$ T cells may be more important for determining a patient's response, the percentages of WT1/HLA-A*24:02 tetramer–positive memory ($CD45RA^-CCR7^+$ and $CD45RA^-CCR7^-$) cells in $CD8^+$ T cells were analyzed (Fig. 4A and B). Interestingly, the association between the percentages of WT1/HLA-A*24:02-specific memory cells in $CD8^+$ T cells and the OS of super-responders was significant after six vaccinations with DC/WT1-I/II ($P = 0.046$; Fig. 4B). Moreover, the combined analysis of the tetramer and functional IFNγ assay confirmed that almost all the HLA-A*24:02–restricted WT1-CTLs produced IFNγ (data not shown).

Immunosuppressive factors

The vaccination of patients with PDA with DC/WT1-I/II did not generate a significantly increased population of $CD25^+Foxp3^+CD4^+$ T cells or $CD14^-CD11b^+CD33^+$ PBMCs compared with the DC/WT1-I or -II vaccines (Supplementary Fig. S3). Moreover, in the patients with PDA vaccinated with DC/WT1-I/II, the super-responders exhibited a decreased percentage of both $CD25^+Foxp3^+CD4^+$ T cells and $CD14^-CD11b^+CD33^+$ PBMCs compared with the non–super-responders but this difference was not significant ($P = 0.052$ and 0.328, respectively; data not shown). Next, we assessed the population of WT1/HLA-A*24:02 tetramer–positive $PD1^+$ cells in the $CD8^+$ T cells in DC/WT1-I/II–vaccinated PDA (Fig. 5A and B). A low percentage of WT1/HLA-A*24:02 tetramer–positive $PD1^+$ cells was observed in the circulating $CD8^+$ T cells before therapy; however, after 10 vaccinations, the population was significantly increased in the non–super-responders compared with the super-responders ($P = 0.018$; Fig. 5B). In contrast, in the super-responders, there was no difference in the populations before and after 10 vaccinations (Fig. 5B).

Discussion

Our phase I study is the first trial to use mature DCs pulsed with a mixture of three types of WT1 peptides restricted by MHC-I/II into one site in combination with chemotherapy.

The safety profile constituted the primary end point. One patient PDA-06 with multiple liver metastases showed rapid disease progression and died of a cerebral infarction. DTH

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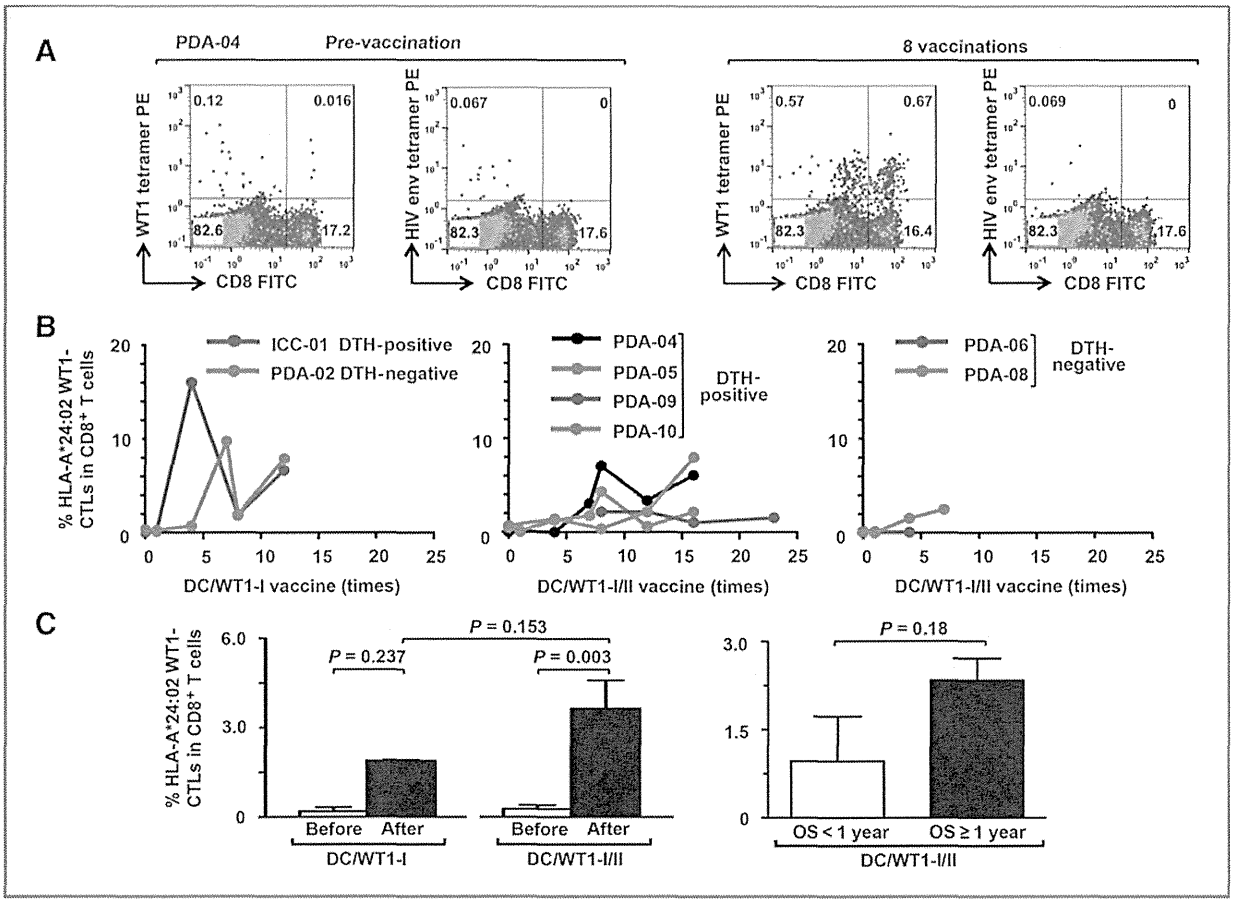

Figure 3. Induction of HLA-A*24:02–restricted WT1-specific CTLs. A, dot plots of HLA-A*24:02–restricted, WT1 tetramer-positive populations in $CD8^+$ T cells derived from PDA-04 are shown before and after eight vaccinations. B, the percentage of HLA-A*24:02–restricted, WT1-specific CTLs (WT1-CTLs) in $CD8^+$ T-cell populations was analyzed after patients were vaccinated with DC/WT1-I (left) or with DC/WT1-I/II [4 DTH-positive (middle) and 2 DTH-negative (right)]. C, the percentages of WT1-CTLs in the $CD8^+$ T-cell populations of patients treated with DC/WT1-I or DC/WT1-I/II were compared with those before vaccination (left panel). After 7 or 8 vaccinations with DC/WT1-I/II, the percentages of WT1-CTLs in the $CD8^+$ T-cell populations in patients with an overall survival (OS) time of ≥1 year (PDA-04, -05, and -09) and <1 year (PDA-06, -08, and -10) were compared (right). The results are expressed as the mean ± SE.

to the WT1-I/II peptides was negative during all vaccination periods. Cerebral infarction, reported here as a severe adverse event, could be caused by the pancreatic cancer itself and/or the administration of gemcitabine, both of which are associated with a high risk of developing thrombotic disease (23). In particular, patients with PDA with metastatic disease are at the highest risk for cancer-associated thromboembolic stroke (24). The supervising Data Safety and Monitoring Board (DSMB) determined that the patient died of stroke induced by a cancer-related hypercoagulable state. Finally, the DSMB determined that the case was not related to the treatment protocol. Moreover, grade 1 pulmonary fibrosis occurred in one patient, PDA-07, after a total of 12 gemcitabine and 6 DC/WT1-I/II treatments. At that time, the drug lymphocyte stimulation test (DLST) for gemcitabine was positive. Moreover, DTH to the WT1-I/II peptides was negative during the entire vaccination period. This adverse event was considered to be multifactorial, and the DSMB determined that it was definitely related to the combination therapy, as the DLST for gemcitabine was positive. The patient continued treatment with S-1, an oral fluoropyrimidine, which is the major chemotherapy regimen for PDA in Japan (25), without additional toxicity. In all 7 enrolled patients, except for skin reactions at the local injection sites, the toxicity profiles of the DC/WT1-I/II vaccine in combination with gemcitabine were consistently similar to those of gemcitabine alone (25).

WT1 peptide–specific DTH reactivity was induced in 4 of the 7 patients with PDA vaccinated with DC/WT1-I/II; however, no patients with PDA vaccinated with DC/WT1-I or -II were DTH-positive. Moreover, in all 4 DTH-positive patients with PDA vaccinated with DC/WT1-I/II, no complete response (CR) or partial response (PR) was observed, but long-term SD was observed and exhibited prolonged survival times. Modern trial experience suggests the response ratio (RR) of gemcitabine is approximately 10% (25). Because cancer vaccines do not work as quickly as cytotoxic agents, the RECIST criteria may not adequately

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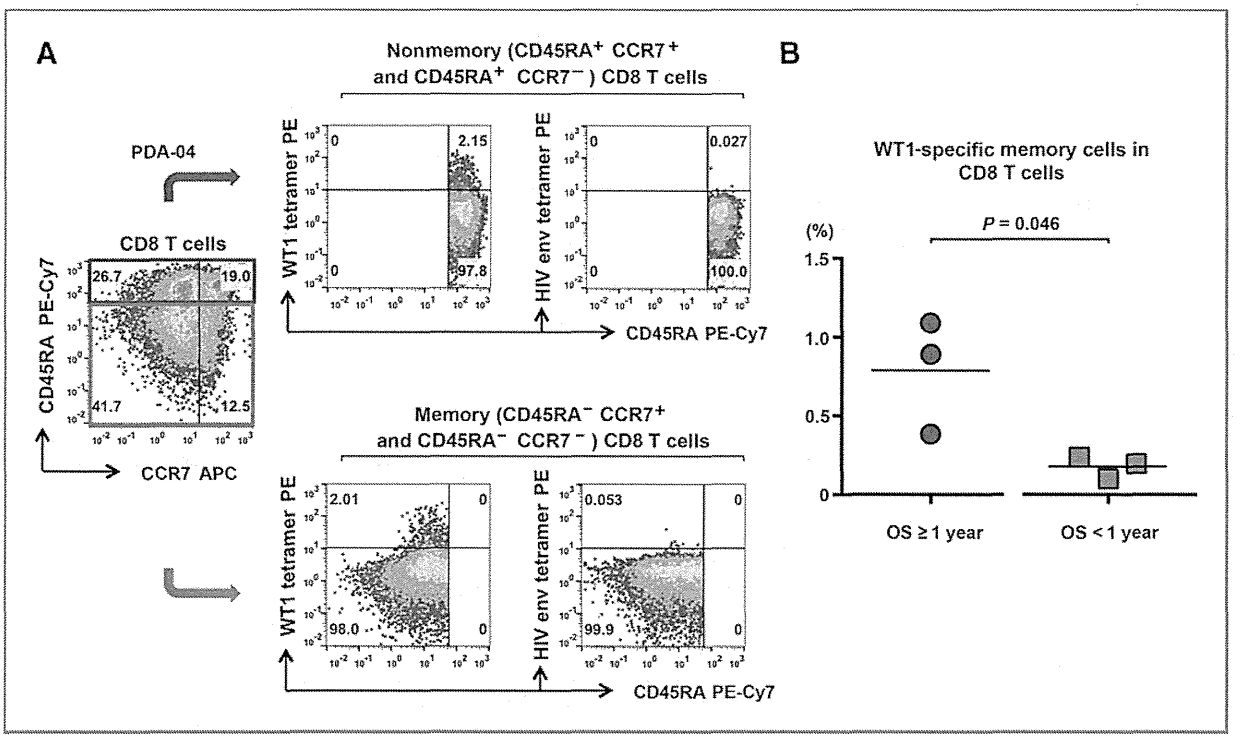

Figure 4. WT1/HLA-A*24:02–specific memory cells in $CD8^+$ T cells. A, dot plots of the memory ($CD45RA^-CCR7^+$ and $CD45RA^-CCR7^-$) and non-memory ($CD45RA^+CCR7^+$ and $CD45RA^+CCR7^-$) phenotypes in $CD3^+CD8^+$ T cells from PDA-04 treated with six vaccinations of DC/WT1-I/II are shown (left). The WT1/HLA-A*24:02$^+$ memory (bottom) and non-memory (top) cells in the $CD3^+CD8^+$ T cells are shown. The HIV env tetramer was used as a control. B, after six vaccinations with DC/WT1-I/II, the percentages of WT1/HLA-A*24:02$^+$ memory cells in the $CD3^+CD8^+$ T cells between patients with an OS time ≥1 year [PDA-04, -05, and -09 (blue) and those with an OS time <1 year (PDA-06, -08, and -10 (red)] were compared.

capture the clinical benefit of cancer vaccines (26). The long-term SD that is shown in this study may be a unique characteristic of cancer vaccines. Interestingly, 3 of the 7 patients vaccinated with DC/WT1-I/II were strongly DTH positive during the entire treatment period. DTH is an inflammatory reaction mainly mediated by $CD4^+$ effector-memory T cells that infiltrate the injection site of the antigen against which the immune system has been primed by the cancer vaccines (27). Our results support previous findings that showed there was a significant correlation between favorable clinical outcomes and the presence of a vaccine-related antigen-specific DTH test (27, 28). These findings suggest that effective cancer vaccines not only stimulate CTL activity but also maintain long-term memory T cells capable of mounting functional antitumor immune responses to secondary antigenic peptide challenges.

Assessing whether WT1-CTLs can be induced by the combination therapy is essential. We used the HLA-A*24:02 tetramer to detect WT1-CTLs because adequate HLA-A*02:01 and HLA-DRB1/DPB1 tetramers were not available. In all 11 enrolled patients, 8 patients were positive for HLA-A*24:02. The patients vaccinated with DC/WT1-I showed more than 10% WT1/HLA-A*24:02-specific tetramer-positive cells in the $CD8^+$ T-cell population shortly after vaccination, suggesting that the DC/WT1-I vaccine induced the expansion of the self-restricted WT1-CTLs that originated from preexisting memory $CD8^+$ T cells (29). Importantly, the CTLs induced by the DC/WT1-I vaccine were not maintained for the entire duration of the treatment protocol. These findings suggest that the WT1-CTLs generated following vaccination with DC/WT1-I may be functionally impaired, resulting in short-lived WT1-specific immune responses (30). In contrast, all 4 DTH-positive patients maintained WT1/HLA-A*24:02–specific tetramer-positive cells during the entire treatment period. The maintenance of the WT1-CTLs may be, at least in part, associated with prolonged survival. Moreover, a significant increase in the number of IFNγ-producing cells in the $CD4^+$ T-cell populations in patients with PDA vaccinated with DC/WT1-I/II was detected upon stimulation with WT1 class II peptide *in vitro*, suggesting that concurrent $CD4^+$ T-cell activation is essential to induce functional CTLs. Furthermore, almost all of the HLA-A*24:02–restricted WT1-CTLs produced IFNγ following challenge with WT1 peptides. These findings support the hypothesis that the coactivation of WT1-specific $CD4^+$ helper T cells augments the induction and proliferation of functional, circulating WT1-CTLs. Indeed, vaccination with DC/WT1-I/II resulted in populations of circulating functional memory CTLs that were specific for WT1 and were long-lived, lasting for the entire treatment period in the super-responders. In pancreatic cancer, long-term survivors who had been vaccinated with a mutant K-ras peptide

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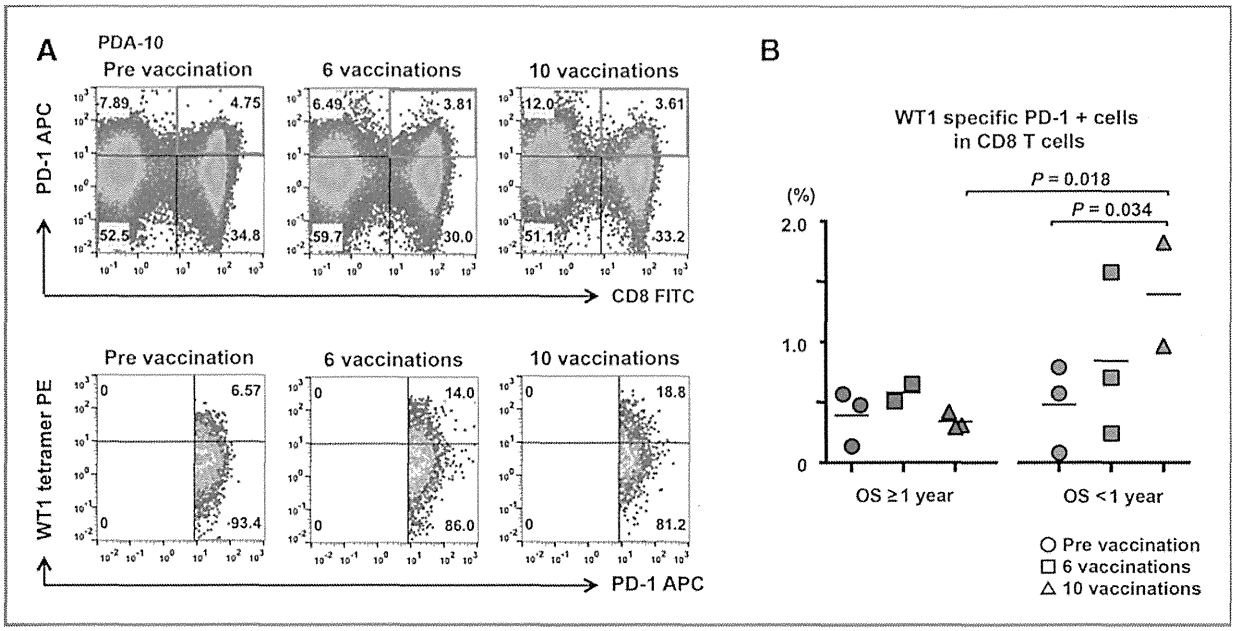

Figure 5. Frequency of WT1/HLA-A*24:02–specific $PD1^+$ cells in $CD8^+$ T cells. A, dot plots of PD1 and CD8 expression in lymphocytes from PDA-10 before and after vaccinations with DC/WT1-I/II are shown (top). Dot plots of the WT1/HLA-A*24:02$^+$ cells in the $PD1^+CD8^+$ population from PDA-10 before and after vaccinations with DC/WT1-I/II are shown (bottom). B, After six or ten vaccinations with DC/WT1-I/II, the percentages of WT1/HLA-A*24:02$^+$ $PD1^+$ cells in CD8 T cells between patients with an OS time ≥1 year [PDA-04, -05, and -09 (blue)] and those with an OS time <1 year [PDA-06, -08, and -10 (red)] were compared. PDA-06 received the DC/WT1-I/II vaccination seven times. In six vaccinations, PBMCs from PDA-09 were not available.

designed to elicit Th responses were reported to exhibit persistent K-ras–specific memory CTLs (31). Importantly, our results also showed that the generation and maintenance of WT1-specific memory CTLs by DC/WT1-I/II were significantly linked to beneficial clinical outcomes. Therefore, vaccination with DCs pulsed with WT1 peptides for both MHC class I and II may be associated with the long-term survival of patients with PDA.

Although WT1-CTLs were generated by vaccination with DC/WT1-I/II and were detected in the circulation of the vaccinated patients in this study, these CTLs may not act against the tumor. In the tumor microenvironment, there are many immunosuppressive cells, including $CD4^+$ $CD25^+Foxp3^+$ Tregs and MDSCs (32). Moreover, the interaction of PD1 in activated CTLs with its ligand PDL1 in the tumor cells plays a major role in immune escape (33). After vaccination with either type of DC/WT1 vaccine, the percentages of $CD25^+Foxp3^+$ cells in the $CD4^+$ T-cell population and the $CD14^-CD11b^+CD33^+$ cells in the PBMCs were not significantly changed. After 10 vaccinations with DC/WT1-I/II, the non–super-responders showed a significantly increased population of WT1/HLA-A*24:02–specific $PD1^+$ cells in $CD8^+$ T cells, compared with the super-responders. The low percentage of the exhausted WT1-CTLs in the super-responders may be, at least in part, associated with longer survival times. However, these results were obtained from peripheral cells. Therefore, we must understand the immune responses observed in the peripheral blood versus the responses at the tumor site. Of the 3 super-responders, we could examine PDL1 expression in 2 patients, PDA-05 and PDA-09, and detected strong PDL1 expression on both pancreatic cancer cells (data not shown). Endogenous inflammatory immune responses induced by IFNγ-producing CTLs have been shown to promote the expression of PDL1 on cancer cells (34). Therefore, the overexpression of PDL1 on tumor cells and the lower frequency of WT1/HLA-A*24:02–specific $PD1^+$ cells in $CD8^+$ T cells that was observed in the super-responders, compared with the non–super-responders, may be associated with long-term survival (34, 35). Indeed, an immune checkpoint blockade by antibodies targeting inhibitory immune receptors, such as PD1 and PDL1, can be used to successfully treat patients with advanced melanoma (33). Monitoring of WT1-CTLs and immunosuppressive cells at the tumor site may be directly associated with clinical responses. However, cells from the tumor site are not readily available for monitoring purposes in patients with PDA. Antigen-specific T cells obtained from DTH sites have been reported to be significantly correlated with favorable clinical outcomes (27). Therefore, the ability to assess the function of the WT1-CTLs and the immunosuppressive cells from the DTH site may serve as useful prognostic markers for survival during treatment with this combination therapy. Future studies are needed to address these issues and to identify the immune biomarkers that are capable of predicting clinical response.

In conclusion, the combined treatment of chemotherapy and DCs pulsed with a mixture of 3 types of WT1 peptides, including both MHC class I and II–restricted epitopes, was well tolerated, induced WT1-CTLs during long-term vaccination, and appeared to provide some clinical benefits to

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DTH-positive patients. Further investigations are needed to validate these findings in a larger-scale clinical trial.

Disclosure of Potential Conflicts of Interest

M. Okamoto holds ownership interest in Tella, Inc. H. Sugiyama is the inventor of patents PCT/JP02/02794 and PCT/JP04/16336 which are held by the International Institute of Cancer Immunotherapy. No potential conflicts of interest were disclosed by the other authors.

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Treatment with Chemotherapy and Dendritic Cells Pulsed with Multiple Wilms' Tumor 1 (WT1)–Specific MHC Class I/II–Restricted Epitopes for Pancreatic Cancer

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Immunogenic Modulation of Cholangiocarcinoma Cells by Chemoimmunotherapy

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Indication: Experimental study

Running title: Immunogenic Modulation of Tumor Cells

Abstract. Background/Aim: Chemoimmunotherapy have been used to treat intrahepatic cholangiocarcinoma (ICC). However, little is known about the phenomena underlying the immunomodulation of ICC cells elicited by chemoimmunotherapy. Materials and Methods: Primary ICC cells were cultured from a patient with ICC who received gemcitabine followed by 5-FU, both combined with dendritic cells pulsed with Wilms' tumor 1 (WT1) peptides. The ICC cells were treated with gemcitabine, 5-fluorouracil (5-FU) or interferon (IFN)-γ *in vitro*. The phenotype of the ICC cells was examined by flow cytometry and quantitative reverse transcription polymerase chain reaction. Results: Stimulation of the ICC cells with gemcitabine resulted in up-regulation of *WT1* mRNA, programmed death receptor ligand-1 (PDL1) and calreticulin. Gemcitabine, 5-FU and IFN-γ induced up-regulation of mucin-1. Moreover, human leukocyte antigen (HLA)-ABC, HLA-DR and PDL1 were extremely up-regulated by IFN-γ. Conclusion: Chemoimmunomodulating agents alter the immunogenicity of ICC cells, resulting in complex clinical efficacy results.