

Phase I pilot study of Wilms tumor gene 1 peptide-pulsed dendritic cell vaccination combined with gemcitabine in pancreatic cancer

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Key words

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The prognosis of pancreatic cancer (PC) is poor because most patients have relatively advanced disease at diagnosis. Most have either poor-prognostic locally advanced or metastatic cancer. Gemcitabine monotherapy has been the mainstay of treatment for advanced PC.⁽¹⁾ In Japan, a phase III study was designed to determine whether gemcitabine plus S-1 therapy was superior to gemcitabine alone, but gemcitabine plus S-1 was not found to be more efficacious.⁽²⁾ However, a recent study reported that gemcitabine plus nab-paclitaxel significantly improved the overall survival and the response rate compared to gemcitabine alone.⁽³⁾ Although a large number of randomized trials have been conducted in relation to PC chemotherapy, very few have demonstrated the benefits of combination therapy over gemcitabine alone. Thus,

there is an urgent need to devise a new strategy for PC treatment.

Dendritic cells (DC) are efficient antigen-presenting cells responsible for T-cell activation. With the identification of human tumor antigens, antigen-pulsed autologous DC generated *ex vivo* by culturing monocytes with cytokine combinations have been used for therapeutic cancer vaccination.⁽⁴⁾ A previous study showed that autologous DC vaccines loaded with granulocyte-macrophage colony-stimulating factor (GM-CSF)-used prostatic acid phosphatase prolonged overall survival in patients with prostate cancer, and this vaccine has been approved by the United States Food and Drug Administration.⁽⁵⁾

The selection of tumor antigens for use in DC vaccines is an important consideration. In a pilot project conducted to prioritize

75 known cancer antigens for this purpose, Wilms tumor gene 1 (*WT1*) antigen was listed as the most suitable.⁽⁶⁾ *WT1* was originally defined as a tumor-suppressor gene that encodes a zinc finger DNA-binding protein⁽⁷⁾ that is involved in tumorigenesis through regulation of transcription of growth factor genes (platelet-derived growth factor A chain,⁽⁸⁾ colony-stimulating factor-1⁽⁹⁾ and insulin-like growth factor II⁽¹⁰⁾) and other genes. Additional reports demonstrate that *WT1* is expressed in hematological malignancies and solid tumors, including pancreatic ductal adenocarcinoma⁽¹¹⁾ and can confer oncogenic functions.⁽¹²⁾ A *WT1* peptide vaccine has previously been applied in various solid tumors.^(13,14) In particular, the HLA-A*2402-restricted modified 9-mer *WT1* peptide (CYTWNQMNL) has been reported to elicit tumor-recognizing cytotoxic T lymphocytes more effectively than the natural 9-mer peptide.⁽¹⁵⁾ Furthermore, the clinical efficacy of treatment with the modified 9-mer *WT1* peptide vaccine in combination with gemcitabine seems to be better than that of gemcitabine alone, especially in terms of survival.⁽¹⁶⁾

Gemcitabine has been reported to restore immunocompetence via various mechanisms, including selective deletion of myeloid derived suppressor cells (MDSC) that inhibit antitumor immunity.^(17–19) A previous study also found that gemcitabine induces the proliferation of CD14⁺ monocytes and CD11c⁺ DC, findings that could support combination therapy with gemcitabine and specific immunotherapy.⁽²⁰⁾ In addition, it has also been reported to enhance *WT1* expression in PC cell lines *in vitro* and to sensitize with *WT1*-specific T-cell-mediated anti-tumor immune response.⁽²¹⁾ Therefore, a combination of *WT1* peptide-pulsed DC vaccination and gemcitabine could enhance anti-tumor effects.

Compared to the survival rates observed in a previous study using chemotherapy alone, a study reported that DC vaccines might prolong the survival of advanced PC patients for whom first-line chemotherapy has failed.⁽²²⁾ However, this comparison may have not been accurate: a patient selection bias, such as exclusion of patients with rapidly progressive PC or a very poor prognosis, may have existed. Furthermore, some patients may have experienced immunomodulation because of previous cancer treatment. Therefore, we conducted the present phase I pilot study of *WT1* peptide-pulsed DC vaccination combined with gemcitabine (DCGEM) as the first-line therapy in chemonaïve PC patients with locally advancement or metastasis. In addition to evaluating the feasibility and safety of this therapy and possible anti-tumor effects, we also evaluated various

immunological parameters that may be correlated with the induction of immune responses and anti-tumor effects.

Patients and Methods

Study design. This trial was a phase I pilot study performed at the Keio University (Tokyo, Japan) and Tokyo Midtown Clinic (Tokyo, Japan). The primary endpoint was adverse events graded according to Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. The secondary endpoints were immune induction to the *WT1* peptide, response rate, and overall survival. The clinical response was evaluated on the basis of the Response Evaluation Criteria in Solid Tumors (RECIST) (version 1.1).⁽²³⁾ The planned sample size was 10. Overall survival duration was from the date of obtaining informed consent to the date of death. This study was approved by the institutional review board of Keio University, and informed consent was obtained from all patients. The trial was registered with the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (<http://www.umin.ac.jp/ctr/> number: UMIN-000004855).

Eligibility. The eligibility criteria were as follows: (i) histological or cytological diagnosis of PC; (ii) HLA-A*2402; (iii) a score of 0 or 1 on the Eastern Cooperative Oncology Group performance scale; (iv) no immediate allergy to the *WT1* peptide; (v) lesion that can be evaluated using RECIST; (vi) no previous treatment; and (vii) adequate hematologic, hepatic, renal and cardiac function.

Pre-treatment assessment and follow-up studies. At the baseline, all patients underwent complete history examination, a physical examination, computed tomography or/and magnetic resonance imaging, and laboratory tests before treatment was initiated. Clinicopathological parameters were expressed according to the TNM classification of the International Union against Cancer. Radiological imaging was repeated prior to each cycle and at 4 weeks after the third cycle of treatment.

Dendritic cell vaccination combined with gemcitabine treatment protocol. A fixed dose of 10^7 *WT1* peptide-pulsed DC was injected intradermally in close proximity to the axillary or inguinal lymph nodes on days 8 and 22. A dose of 1000 mg/m² gemcitabine was administered every 4 weeks by intravenous drip infusion for 30 min on days 1, 8 and 15 (Fig. 1). A total of three cycles of DC vaccination was repeated in

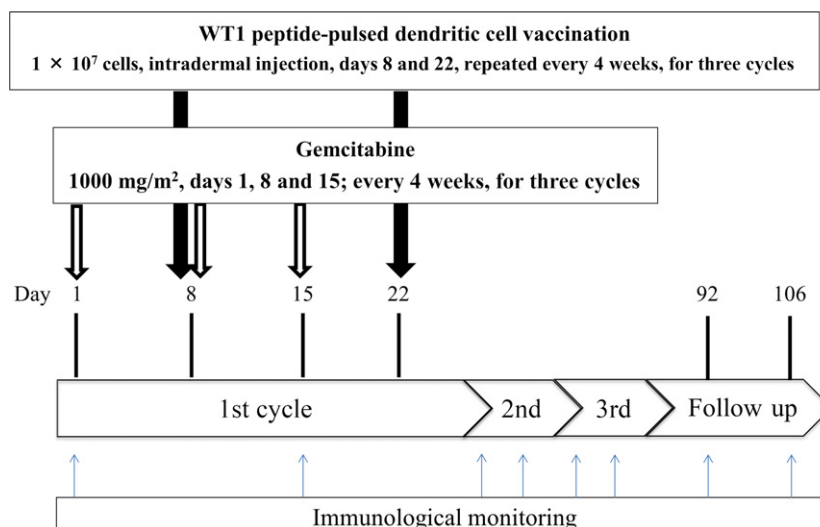


Fig. 1. Dendritic cell (DC) vaccination combined with gemcitabine protocol. *WT1* peptide-pulsed DC (10^7 cells/injection) were injected intradermally on days 8 and 22 every 4 weeks. Gemcitabine (1000 mg/m²) was administered on days 1, 8 and 15. A total of three cycles of DC vaccination were repeated. Immunological monitoring was performed six times on days 1 and 15 of each cycle and two times at 4 weeks after the third cycle.

patients who did not have progressive disease. After completion or termination of the protocol, post-protocol DC vaccination continued with patients' consent.

Dendritic cell vaccine preparation. Procedures for the preparation and quality control of the DC vaccine have been reported previously.⁽²²⁾ The phenotypes CD11c⁺, CD14⁻, CD40⁺, CD80⁺, CD83⁺, CD86⁺, CCR7⁺, HLA-DR⁺ and HLA-ABC⁺ were considered to define mature DC in conformance with the quality criteria for DC vaccines.⁽²⁴⁾

Immunological monitoring. For immunological monitoring, peripheral venous blood was collected from patients six times on days 1 and 15 of each cycle before gemcitabine injection and twice for 4 weeks after the third cycle (Fig. 1). The immune response to the WT1 peptide was analyzed using the delayed-type hypersensitivity (DTH) skin test, interferon (IFN)- γ enzyme-linked immunospot (ELISPOT) and HLA tetramer staining assay.

Delayed-type hypersensitivity test. For immunological monitoring, the DTH skin test against the WT1 peptide was conducted. The diameters of the erythema and induration were measured 48 h after injection of the peptide on day 1 of each cycle and at 4 weeks after the third cycle. An erythema diameter >5 mm was considered a positive result.

Generation of peptide cocktail cultured peripheral blood mononuclear cells *in vitro* by mixed lymphocyte peptide culture. Cryopreserved peripheral blood mononuclear cells (PBMC) from patients were subjected to mixed lymphocyte peptide culture. After thawing and resting, PBMC were stimulated with 10 μ g/mL modified-type WT1 peptide (CYTWNQMNL) (Merck Bioscience AG, Läufelfingen, Switzerland) and 16 μ g/mL CE control peptide pool HLA-A24 (8 peptides; Biosynthesis, TX, USA) in AIM-V CTS Medium (Gibco Life Technologies, New York, USA) supplemented with 10% of human AB serum (MP Biomedicals, Ohio, USA), 20 U/mL of interleukin-2 (IL-2) (Shionogi, Osaka, Japan) and 10 ng/mL of interleukin-7 (IL-7) (Peprotech, NJ, USA). After they were cultured for 9 days, the cells were individually analyzed by the HLA tetramer assay using flow cytometry and the WT1-specific IFN- γ ELISPOT assay.

Wilms tumor gene 1 peptide/HLA-A*2402 tetramer assay. WT1-specific CD8⁺ T-cells in peripheral blood were assessed depending on HLA tetramers as described previously.⁽²⁵⁾ The results were defined as positive when CD3-positive, CD8-positive and WT1/HLA-A24 tetramer-positive cell populations were detected among the cultured cells and no CD8-positive

and HIVenv/HLA-A24 tetramer-positive cells were detected in the negative controls.

Wilms tumor gene 1-specific interferon- γ enzyme-linked immunospot assay. The IFN- γ ELISPOT assay was performed as described previously.⁽²⁶⁾ PBMC were defined to be specifically sensitized when the number of spots indicating IFN- γ release in response to the WT1 peptide was at least two times that in response to HIVenv peptide-pulsed stimulator cells in the ELISPOT assay.

Surface marker analysis for cell phenotyping. Peripheral blood mononuclear cell samples were incubated with fluorescent-conjugated monoclonal antibodies for 45 min at 4°C in the dark. After they were washed with FACS buffer (2% FBS in phosphate-buffered saline), the cells were fixed with stabilizing fixative (BD Biosciences, CA, USA) and examined on a flow cytometer (Gallios; Beckman Coulter, CA, USA). Data were analyzed using the Kaluza software (Beckman Coulter).

Statistical analyses. Statistical analyses were performed using the SPSS Version 21 software (IBM Corporation, Armonk, USA). The immune response was analyzed using the *t*-test. Differences were considered statistically significant at *P*-values <0.05.

Results

Patient characteristics. The clinical characteristics of the patients are shown in Table 1. From January 2011 to December 2012, 24 patients underwent HLA typing. Eleven HLA-A*2402-positive patients were consecutively enrolled, of which 1 patient went off-study before the treatment protocol was initiated because of acute obstructive cholangitis and tumor hemorrhage. The remaining 10 patients (4 with locally advanced and 6 with metastatic PC) had a median age of 58 years (range, 41–69 years). Five patients (50%) completed the protocol, while 5 (50%) terminated the protocol because of rapid disease progression or a severe adverse event; namely, interstitial pneumonia, related to gemcitabine treatment. The relative dose intensity of gemcitabine was 87%. Eight patients received post-protocol DC vaccination after completion or termination of protocol treatment. The median frequency of DC vaccine administration was 8.5 times (range, 3–12 times). S-1 and gemcitabine combination therapy or S-1 monotherapy was administered as post-protocol chemotherapy in 6 of the 10 patients.

Adverse events. All adverse events that occurred within the protocol treatment period are shown in Table 2. There were no adverse skin reactions at the site of vaccination. Two

Table 1. Patient characteristics

Case	Age (years)	Gender	Clinical stage	Site of metastasis	PS	Protocol DC (times)	Post-protocol DC (times)	Total DC (times)	Post-protocol chemotherapy
1	64	M	IV	Peritoneal dissemination	0	6	6	12	S-1
2	67	F	III		0	6	6	12	GEM
3	52	F	IV	Liver	0	3	5	8	GEM+S-1
4	41	F	III		0	6	3	9	None
5	47	F	IV	Liver, spleen, LN	0	2	2	4	S-1
6	50	M	IV	Liver, LN	1	3	0	3	None
7	69	F	III		0	6	0	6	S-1
8	69	M	III		0	4	2	6	None
9	65	M	IV	LN	0	6	5	11	GEM+S-1
10	50	F	IV	Liver	0	4	6	10	None

DC, dendritic cell; GEM, gemcitabine; LN, lymph node; PS, performance status.

Table 2. Adverse events

	Grade				
	1	2	3	4	5
Hematotoxicity					
Neutropenia		1	1	1	
Anemia		2	2	1	
Thrombopenia	1	2	2		
Non-hematotoxicity					
Respiratory disorders					
Hypoxia (Interstitial pneumonia)				1	
Gastrointestinal disorders					
Nausea	1		1		
Vomiting	1	1			
Abdominal distension		1			
Constipation	2	1			
Small intestinal obstruction				1	
Diarrhea	1				
Ascites		2	1		
Hiccups		1			
Anal fistula			1		
Nervous system disorders					
Headache	1				
Dizziness		2			
Dysgeusia	1				
Cerebral infarction (Trousseau syndrome)					1
General disorders					
Fever	1				
Fatigue			1		
Malaise		2			
Pain	1	2			
Edema limbs	2	1			
Metabolism and nutrition disorders					
Anorexia	1	2	1		
Hypoalbuminemia	1		1		
Glucose intolerance			1		
Musculoskeletal disorders					
Myalgia	1				
Skin and subcutaneous tissue disorders					
Urticaria	2				
Cellulitis		1			
Reproductive system disorders					
Irregular menstruation	1				
Investigations					
ALT increase			2		
AST increase		1	1		
Weight loss		3			

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

patients had grade 4 hematotoxicity, neutropenia and anemia. One afebrile patient who developed neutropenia was treated with G-CSF, and the other who developed anemia received a blood transfusion. One patient (case 8 in Tables 1, 3 and 4) had a therapy-induced non-hematological adverse event, namely, hypoxia due to gemcitabine-induced interstitial pneumonia at 4 weeks from the last administration of DC vaccination, and this patient terminated the treatment protocol. Although we conducted a drug-induced lymphocyte transformation test (DLTT) for gemcitabine for investigating the cause of interstitial pneumonia, the patient had a negative DLTT result. After termination of the protocol treatment, that patient received an additional two doses of the DC

Table 3. Results of WT1-specific immune response

Case	DTH	Tetramer (% of Tetramer-positive cells /CD8 ⁺ cells)		ELISPOT (Spots/2.5 × 10 ⁴ PBMC)	
		Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
1	+	0.58	5.57	20	106
2	-	0.16	0.22	110	63
3	-	0.82	1.02	143	184
4	+	0.21	1.17	3	447
5	-	0.55	15.29	27	310
6	-	0.28	2.27	44	248
7	-	0.02	0.61	7	18
8	-	0.27	18.67	0	82
9	+	0.29	20.73	4	502
10	-	0.58	1.76	131	0

DTH, delayed-type hypersensitivity; ELISPOT, enzyme-linked immunospot; PBMC, peripheral blood mononuclear cell.

vaccination without gemcitabine, according to the patient's request. However, interstitial pneumonia did not recur after re-exposure to the DC vaccination. The other patients showed small intestinal obstruction due to peritonitis carcinomatosis and cerebral infarction due to Trousseau syndrome associated with disease progression. Overall, DC vaccination along with gemcitabine did not appear to intensify the hematological adverse effects of gemcitabine; however, careful attention was necessary to monitor the development of therapy-induced interstitial pneumonia.

Immunological monitoring. In terms of induction of WT1 specific T-cell responses, DCGEM elicited a WT1-specific response in 6 of the 10 patients as detected by the HLA/WT1-tetramer assay (Table 3). The number of tetramer-positive WT1-specific T-cells significantly increased after DC vaccination ($P = 0.036$; Fig. 2). Furthermore, in the ELISPOT assay, the WT1-specific T-cell response was found to be enhanced in 7 of the 10 patients (Tables 3 and 4). Cases 1, 4 and 9 showed a significant increase in the response in both the IFN- γ -ELISPOT and HLA-tetramer assays after DC vaccination ($P < 0.05$) (Fig. 3 and Table 3). In these 3 patients, the skin DTH test also showed positivity. In contrast, only 3 of the 7 DTH-negative patients had positive results in both the ELISPOT and the HLA-tetramer assays. Interestingly, as compared to patients without liver metastases, the WT1-specific T-cell responses tended not to be enhanced in patients with liver metastases. Additionally, the DTH skin test was negative in all cases with liver metastases. Thus, it appears that DC vaccination can elicit a WT1-specific T-cell response in combination with gemcitabine as the first-line treatment in chemo-naïve PC patients without liver metastases.

Clinical outcomes. Neither complete response nor partial response was observed for an objective response rate of 0% (Table 4). The disease control rate and median overall survival were 60% and 243 days, respectively. The survival rate after treatment for patients with stable disease was significantly better than that of patients with progressive disease ($P = 0.016$; Fig. 4). While DCGEM could control the development of cancer progression in all patients with locally advanced disease or non-liver metastasis, by contrast, DCGEM did not provide any clinical benefit to patients with liver metastases. Furthermore, disease control was associated

Table 4. Immunological monitoring data and clinical outcomes

Case	Pretreatment laboratory data										Minimum count of lymphocytes during protocol therapy (/μL)	Tetramer	ELISPOT	DTH	Clinical outcomes	Overall survival (days)
	Lymphocytes (/μL)	NLR	CRP (mg/dL)	IL-6 (pg/mL)	IL-8 (pg/mL)	IL-8 (pg/mL)	Granulocytic MDSC (CD15 ⁺ /CD11b ⁺)/CD15 ⁺	MDSC (CD15 ⁺ DR ⁻ CD11b ⁺)/CD15 ⁺								
1	1630	3.4	1.09	2.23	12.53	18.18	812	+	+	+	SD	250				
2	1502	1.9	0.87	19.86	7.38	25.00	270	-	-	-	SD	484				
3	581	7.7	0.07	0.86	7.08	14.29	507	-	-	-	PD	181				
4	1290	2.2	0.01	0.39	6.13	4.55	763	+	+	+	SD	215				
5	1349	4.9	2.2	3.21	28.63	20.73	1282	+	+	+	PD	58				
6	1513	5.8	8.47	17.58	52.26	21.74	412	+	+	+	PD	64				
7	1128	3.0	0.15	28.1	3.63	26.67	702	-	-	-	SD	243				
8	1246	4.0	0.06	3.00	14.47	23.88	354	-	-	-	SD	277				
9	1769	2.7	0.12	1.05	5.89	16.13	1643	+	+	+	SD	530				
10	1635	6.7	9.32	1.81	7.14	31.51	702	-	-	-	PD	244				

CRP, C-reactive protein; DTH, delayed-type hypersensitivity; ELISPOT, enzyme-linked immunospot; IL, interleukin; MDSC, myeloid derived suppressor cells; NLR, neutrophil/lymphocyte ratio; PD, progressive disease; SD, stable disease.

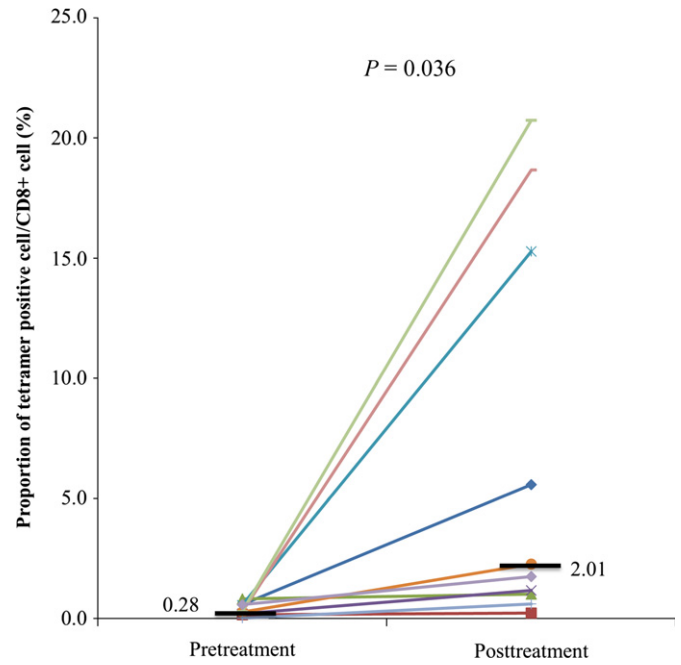


Fig. 2. Proportion of WT1-specific T-cells before and after vaccination in the tetramer assay. Data on the proportion of WT1-specific cytotoxic T lymphocytes expressed as a percentage of CD8⁺ cells. The black horizontal bar shows the median. Differences between values before and after vaccination achieved statistical significance ($P = 0.036$).

with a low neutrophil/lymphocyte ratio (NLR) in the peripheral blood before treatment (Fig. 5). Additionally, patients with liver metastases had high levels of various inflammatory markers and cytokines, such as NLR ($P = 0.001$), C-reactive protein (CRP) ($P = 0.035$) and IL-8 ($P = 0.117$), in comparison with patients with locally advanced disease or non-liver metastasis. In particular, 2 patients (cases 5 and 6) who had multiple liver metastases with high NLR, CRP levels and IL-8 levels before treatment died in <3 months, even though they showed a WT1-specific T-cell response in the HLA-tetramer and IFN- γ -ELISPOT assays. Thus, because of rapid disease progression, DCGEM appears to play a limited role in PC patients with liver metastases and poor immunological parameters.

To identify factors predictive of the immune response to DC vaccination, we evaluated the various immune cell subsets in pretreatment peripheral blood by flow cytometry-based comprehensive leukocyte immunophenotyping. As shown in Figure 6, DTH positivity was significantly correlated with a low percentage of granulocytic MDSC (CD15⁺/HLA-DR⁻/CD11b⁺) ($P = 0.017$) and was associated with a low NLR. However, no difference in the percentages of Th1 (CD4⁺CXCR3⁺CCR6⁻), Th2 (CD4⁺CXCR3⁻CCR6⁻) and Treg (CD4⁺CD25⁺Foxp3⁺CD127^{low}) cells was observed between DTH-positive and DTH-negative patients. These results suggest that DCGEM may be more likely to be effective in PC patients with a low percentage of granulocytic MDSC and low NLR before treatment.

Among the DTH-positive patients, case 9 survived more than 500 days despite multiple metastases to the supraclavicular and para-aortic lymph nodes. The sizes of the metastatic lymph nodes and primary tumor were slightly reduced by DCGEM therapy, but lymph node regrowth and new bone metastasis developed 6 months after DCGEM. Since then,

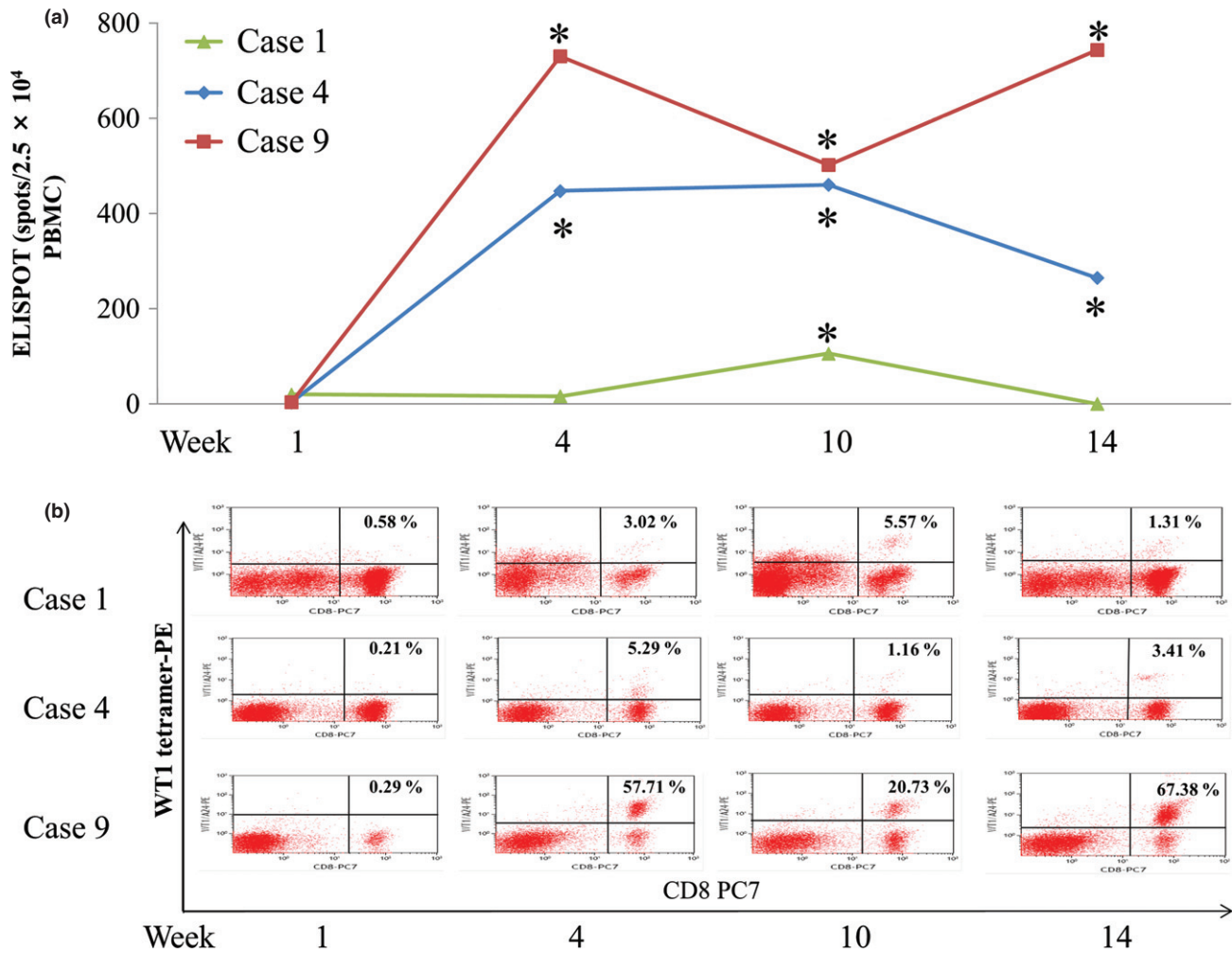


Fig. 3. Enzyme-linked immunospot (ELISPOT) and tetramer assays before and after vaccination. Cases 1, 4 and 9 showed a significant increase in the response in the IFN- γ ELISPOT after vaccination compared to pretreatment (a) (* $P < 0.05$). The percentage of Wilms tumor 1 tetramer+/CD8⁺ lymphocytes also increased after vaccination (b). PBMC, peripheral blood mononuclear cell.

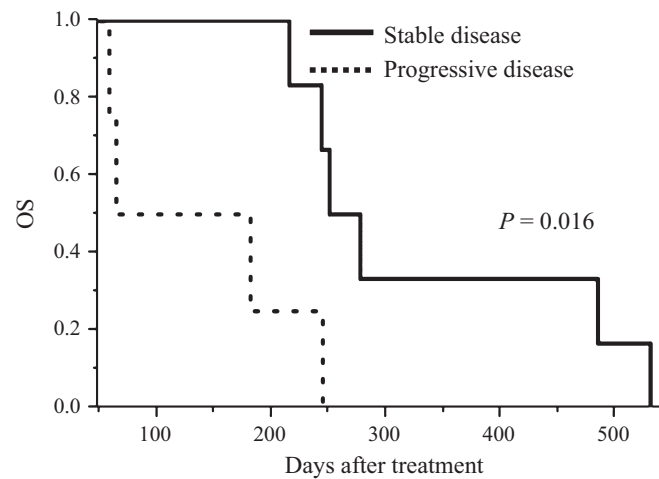


Fig. 4. Overall survival (OS) in patients with pancreatic cancer. Disease control correlated with better survival. ($P = 0.016$; Wilcoxon test).

these metastases have progressed slowly, and the patient has survived more than 11 months after this disease progression. Thus, DCGEM may contribute to prolongation of the survival

of DTH-positive PC patients with favorable immunological parameters before treatment, such as a low percentage of granulocytic MDSC and low NLR.

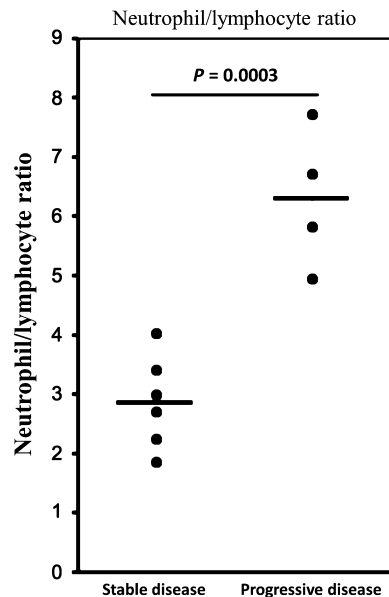


Fig. 5. Correlation of clinical outcome and pretreatment neutrophil/lymphocyte ratio in the peripheral blood. Disease control was correlated with a low neutrophil/lymphocyte ratio ($P < 0.0003$).

Discussion

Safety of dendritic cell vaccination combined with gemcitabine therapy. In the present study, myelosuppression caused by DCGEM was temporary and tolerable and was not accompanied by febrile neutropenia, as is the case gemcitabine alone.^(27,28) In previous studies, relatively high rates of gemcitabine-associated severe lung injury were observed when this drug was combined with other drugs, like bleomycin, docetaxel, or paclitaxel or radiation therapy, which is known to cause lung injury.⁽²⁹⁾ In contrast, pulmonary toxicity, including interstitial pneumonia, has not been reported in a large number of clinical trials for DC vaccination and WT1 peptide vaccination.^(13,30–32) In our study, 1 patient did experience therapy-induced interstitial pneumonia 4 weeks after the last administration of DC vaccination. The real cause of interstitial pneumonia was unclear in this patient. However, we made a clinical diagnosis of gemcitabine-induced interstitial pneumonia in this case, despite a negative DLTT result, because compelling data as to the sensitivity and specificity of the DLTT for drug-induced interstitial pneumonia is currently lacking.^(33,34) In addition, interstitial pneumonia did not recur after re-exposure to the DC vaccine. Overall, DCGEM was as well-tolerated as gemcitabine monotherapy; however, scrupulous attention was necessary to potentiate the adverse effects of gemcitabine's pulmonary toxicity.

First-line use of dendritic cell vaccination in chemo-naïve pancreatic cancer patients compared to gemcitabine monotherapy. The efficacy of DCGEM as first-line therapy was evaluated in chemo-naïve patients because previous retrospective studies on DC-based therapy had various biases that could lead to misinterpretation of the anti-tumor activity of the DC vaccine when compared to gemcitabine monotherapy.

In Japan, with regard to the combination of gemcitabine and cytotoxic agents, a randomized phase III study demonstrated that gemcitabine plus S-1 failed to improve survival as compared with gemcitabine monotherapy.⁽²⁾ By contrast, gemcitabine plus nab-paclitaxel significantly improved overall survival, while rates of peripheral neuropathy and myelosuppression increased.⁽³⁾ In comparison with each combination therapy, the

rate of disease control was 60% with DCGEM, 72% with gemcitabine plus S-1 and 48% with gemcitabine plus nab-paclitaxel. The overall survival was 8.1 months with DCGEM and 8.5 months with gemcitabine plus nab-paclitaxel. Gemcitabine in combination with a cytotoxic agent tends to have a more potent effect. However, this combination also increases both hematological and non-hematological adverse effects. Therefore, it may be not indicated for patients with poor performance statuses. In contrast, the important advantage of the DC vaccine is that it is associated with fewer adverse effects than gemcitabine in combination with a cytotoxic agent. Although our study was planned as a phase I pilot study, the disease control rate and overall survival rate observed with DCGEM treatment was promising, and we observed fewer adverse effects compared with gemcitabine in combination with S-1 or nab-paclitaxel. However, a randomized controlled study is necessary to evaluate the superiority of DCGEM to gemcitabine monotherapy.

Factors predicting immune induction by and therapeutic effect of dendritic cell vaccination. Selection of the appropriate patients is very important for the development of effective immunotherapy. For this purpose, we evaluated various immunological biomarkers in our patients before treatment. Disease control was significantly associated with a low NLR in the pretreatment peripheral blood. The pretreatment NLR has been reported to be an inflammatory predictor of cancer progression and prognosis.⁽³⁵⁾ In particular, disease control was observed in all 3 patients who had positive results in the DTH test for WT1. Thus, DTH may be one of the reactions reflecting the status of patients' *in vivo* anti-tumor immune responses. Other studies also demonstrated a correlation between DTH positivity and a positive clinical outcome in PC,⁽¹⁶⁾ hepatocellular carcinoma,⁽³⁶⁾ melanoma^(37,38) and prostate cancer.⁽³⁹⁾ Patients in the present study with DTH positivity also tested positive in both the IFN- γ -ELISPOT and HLA-WT1-tetramer assays.

Furthermore, DTH was found to be correlated with a low percentage of granulocytic MDSC in pretreatment peripheral blood. Rodriguez *et al.* show that granulocytic MDSC, which suppress anti-tumor immunity through production of immunosuppressive factors such as arginase and vascular endothelial cell growth factor, are associated with a poor prognosis in cancer patients.⁽⁴⁰⁾ Therefore, a low percentage of granulocytic MDSC and low NLR before treatment may be favorable markers for DCGEM efficacy in advanced PC patients.

Patients with DTH positivity in the present study also had relatively low levels of other inflammatory markers, such as CRP, IL6 and IL8. In contrast, 2 patients who died only <3 months after DC vaccination had high levels of these inflammatory markers. These inflammatory parameters have been reported to be predictive markers for cancer prognosis.^(41–44) Several studies revealed that active immunization protocols, including DC vaccination, were suspected to show delayed clinical effects on survival, which were not observed until 4–8 months after the initiation of treatment.^(5,45) Therefore, PC patients with high levels of inflammatory markers may not be appropriate candidates for the DC vaccine. Collectively, the results indicate that it may be worth continuing the evaluation of DCGEM for patients with low levels of inflammatory markers, particularly granulocytic MDSC and NLR, before treatment.

In summary, the present study found that DCGEM therapy was feasible, tolerable and effective as a first-line therapy for inducing anti-tumor T-cell responses in patients with advanced PC without liver metastases. However, WT1 peptide-pulsed DC vaccine has a limited role in the treatment of PC with liver metastases and high levels of inflammatory markers. Therefore,

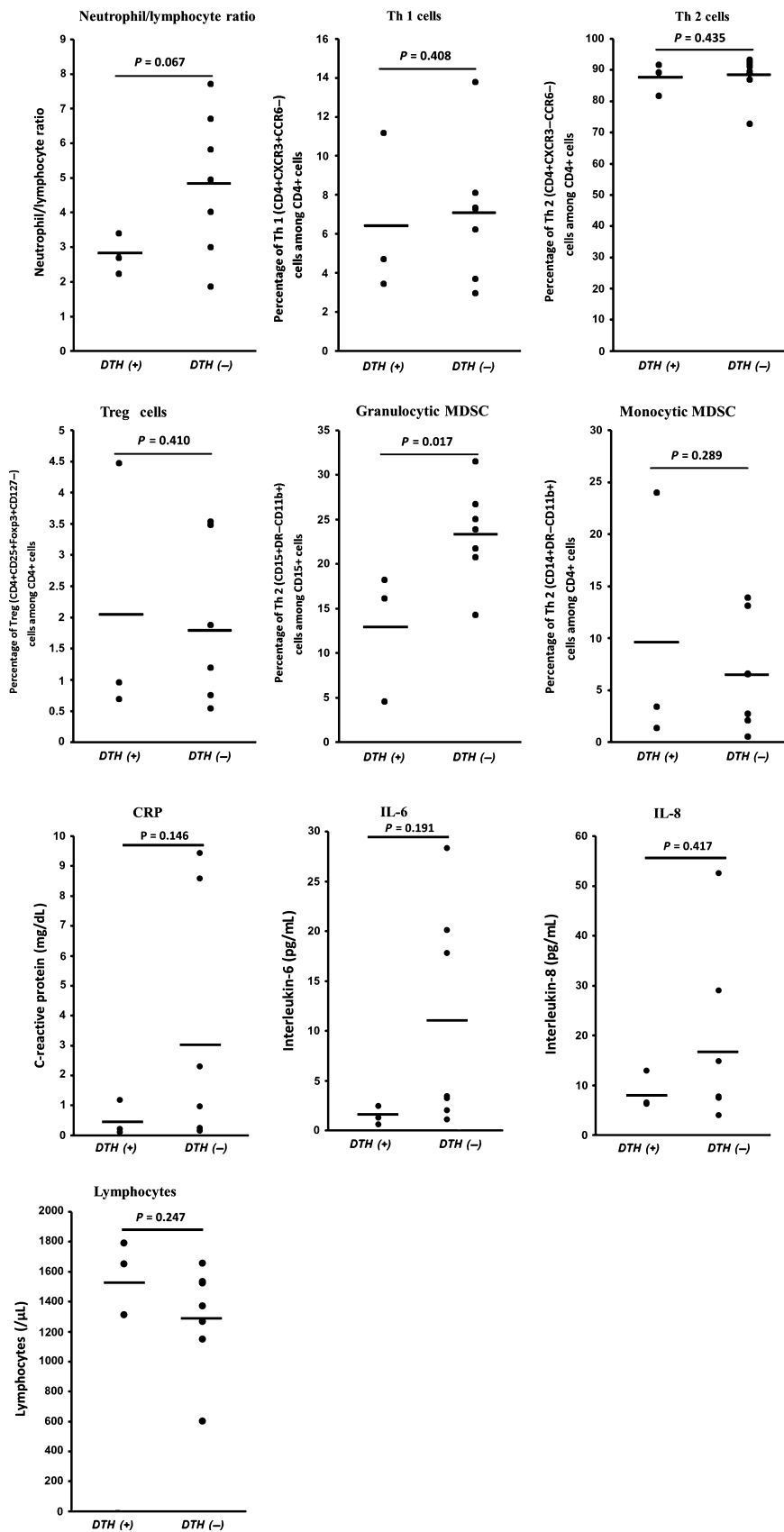


Fig. 6. Pretreatment frequency of various circulating lymphocyte phenotypes in the peripheral blood. The delayed-type hypersensitivity (DTH) response was correlated to a low neutrophil/lymphocyte ratio and significantly correlated to a low percentage of granulocytic myeloid derived suppressor cells (MDSC) (CD15⁺/HLA-DR⁻/CD11b⁺). Monocytic MDSC were identified to be CD14⁺/HLA-DR⁻/CD11b⁺. CRP, C-reactive protein. IL-6, interleukin-6; IL-8, interleukin-8.

we should consider the possibility that our regimen of DCGEM was inappropriate for initial therapy in patients with liver metastases and high levels of inflammatory markers. The results of this phase I pilot study may form the basis of further evaluation of the anti-tumor activity of DCGEM in DTH-positive patients with locally advanced or non-liver metastatic PC and relatively favorable levels of immune parameters.

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References

- Burris HA 3rd, Moore MJ, Andersen J *et al.* Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403–13.
- Ueno H, Ioka T, Ikeda M *et al.* Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study. *J Clin Oncol* 2013; **31**: 1640–8.
- Von Hoff DD, Ervin T, Arena FP *et al.* Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* 2013; **369**: 1691–703.
- Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. *Nat Rev Cancer* 2012; **12**: 265–77.
- Kantoff PW, Higano CS, Shore ND *et al.* Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 2010; **363**: 411–22.
- Cheever MA, Allison JP, Ferris AS *et al.* The prioritization of cancer antigens: a national cancer institute pilot project for the acceleration of translational research. *Clin Cancer Res* 2009; **15**: 5323–37.
- Call KM, Glaser T, Ito CY *et al.* Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* 1990; **60**: 509–20.
- Gashler AL, Bonthron DT, Madden SL *et al.* Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms tumor suppressor WT1. *Proc Natl Acad Sci U S A* 1992; **89**: 10984–8.
- Harrington MA, Konicek B, Song A *et al.* Inhibition of colony-stimulating factor-1 promoter activity by the product of the Wilms' tumor locus. *J Biol Chem* 1993; **268**: 21271–5.
- Drummond IA, Madden SL, Rohwer-Nutter P *et al.* Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. *Science* 1992; **257**: 674–8.
- Oji Y, Nakamori S, Fujikawa M *et al.* Overexpression of the Wilms' tumor gene WT1 in pancreatic ductal adenocarcinoma. *Cancer Sci* 2004; **95**: 583–7.
- Sugiyama H. Cancer immunotherapy targeting Wilms' tumor gene WT1 product. *Expert Rev Vaccines* 2005; **4**: 503–12.
- Miyatake T, Ueda Y, Morimoto A *et al.* WT1 peptide immunotherapy for gynecologic malignancies resistant to conventional therapies: a phase II trial. *J Cancer Res Clin Oncol* 2013; **139**: 457–63.
- Ohno S, Okuyama R, Aruga A *et al.* Phase I trial of Wilms' Tumor 1 (WT1) peptide vaccine with GM-CSF or CpG in patients with solid malignancy. *Anticancer Res* 2012; **32**: 2263–9.
- Tsuboi A, Oka Y, Udaka K *et al.* Enhanced induction of human WT1-specific cytotoxic T lymphocytes with a 9-mer WT1 peptide modified at HLA-A*2402-binding residues. *Cancer Immunol Immunother* 2002; **51**: 614–20.
- Nishida S, Koido S, Takeda Y *et al.* Wilms tumor gene (WT1) peptide-based cancer vaccine combined with gemcitabine for patients with advanced pancreatic cancer. *J Immunother* 2014; **37**: 105–14.
- Suzuki E, Kapoor V, Jassar AS *et al.* Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res* 2005; **11**: 6713–21.
- Sinha P, Clements VK, Bunt SK *et al.* Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J Immunol* 2007; **179**: 977–83.
- Le HK, Graham L, Cha E *et al.* Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice. *Int Immunopharmacol* 2009; **9**: 900–9.
- Soeda A, Morita-Hoshi Y, Makiyama H *et al.* Regular dose of gemcitabine induces an increase in CD14+ monocytes and CD11c+ dendritic cells in patients with advanced pancreatic cancer. *Jpn J Clin Oncol* 2009; **39**: 797–806.
- Takahara A, Koido S, Ito M *et al.* Gemcitabine enhances Wilms' tumor gene WT1 expression and sensitizes human pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune response. *Cancer Immunol Immunother* 2011; **60**: 1289–97.
- Kimura Y, Tsukada J, Tomoda T *et al.* Clinical and immunologic evaluation of dendritic cell-based immunotherapy in combination with gemcitabine and /or S-1 in patients with advanced pancreatic carcinoma. *Pancreas* 2012; **41**: 195–205.
- Eisenhauer EA, Therasse P, Bogaerts J *et al.* New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer* 2009; **45**: 228–47.
- Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. *Nat Med* 2004; **10**: 475–80.
- Ohnishi M, Sakurai T, Heike Y *et al.* Evaluation of cytomegalovirus-specific T-cell reconstitution in patients after various allogeneic haematopoietic stem cell transplantation using interferon-gamma-enzyme-linked immunosorbent and human leucocyte antigen tetramer assays with an immunodominant T-cell epitope. *Br J Haematol* 2005; **131**: 472–9.
- Kuwana M, Okazaki Y, Kaburaki J, Ikeda Y. Detection of circulating B cells secreting platelet-specific autoantibody is useful in the diagnosis of autoimmune thrombocytopenia. *Am J Med* 2003; **114**: 322–5.
- Aapro MS, Martin C, Hatty S. Gemcitabine—a safety review. *Anticancer Drugs* 1998; **9**: 191–201.
- Green MR. Gemcitabine safety overview. *Semin Oncol* 1996; **23**: 32–5.
- Belknap SM, Kuzel TM, Yarnold PR *et al.* Clinical features and correlates of gemcitabine-associated lung injury: findings from the RADAR project. *Cancer* 2006; **106**: 2051–7.
- Kobayashi M, Sakabe T, Abe H *et al.* Dendritic cell-based immunotherapy targeting synthesized peptides for advanced biliary tract cancer. *J Gastrointest Surg* 2013; **17**: 1609–17.
- Shirakata T, Oka Y, Nishida S *et al.* WT1 peptide therapy for a patient with chemotherapy-resistant salivary gland cancer. *Anticancer Res* 2012; **32**: 1081–5.
- Kaida M, Morita-Hoshi Y, Soeda A *et al.* Phase I trial of Wilms tumor 1 (WT1) peptide vaccine and gemcitabine combination therapy in patients with advanced pancreatic or biliary tract cancer. *J Immunother* 2011; **34**: 92–9.
- Matsuno O, Okubo T, Hiroshige S *et al.* Drug-induced lymphocyte stimulation test is not useful for the diagnosis of drug-induced pneumonia. *Tohoku J Exp Med* 2007; **212**: 49–53.
- Matsuno O. Drug-induced interstitial lung disease: mechanisms and best diagnostic approaches. *Respir Res* 2012; **13**: 39.
- Stotz M, Gerger A, Eisner F *et al.* Increased neutrophil-lymphocyte ratio is a poor prognostic factor in patients with primary operable and inoperable pancreatic cancer. *Br J Cancer* 2013; **109**: 416–21.
- Shimizu K, Kotera Y, Aruga A *et al.* Postoperative dendritic cell vaccine plus activated T-cell transfer improves the survival of patients with invasive hepatocellular carcinoma. *Hum Vaccin Immunother* 2014; **10**: 970–6.
- Hersey P, Menzies S, Halliday G *et al.* Phase I/II study of treatment with dendritic cell vaccines in patients with disseminated melanoma. *Cancer Immunol Immunother* 2004; **53**: 125–34.
- de Vries IJ, Bernsen MR, Lesterhuis WJ *et al.* Immunomonitoring tumor-specific T cells in delayed-type hypersensitivity skin biopsies after dendritic cell vaccination correlates with clinical outcome. *J Clin Oncol* 2005; **23**: 5779–87.

- 39 Reyes D, Salazar L, Espinoza E *et al.* Tumour cell lysate-loaded dendritic cell vaccine induces biochemical and memory immune response in castration-resistant prostate cancer patients. *Br J Cancer* 2013; **109**: 1488–97.
- 40 Rodriguez PC, Ernstoff MS, Hernandez C *et al.* Arginase I-producing myeloid-derived suppressor cells in renal cell carcinoma are a subpopulation of activated granulocytes. *Cancer Res* 2009; **69**: 1553–60.
- 41 Pine JK, Fusai KG, Young R *et al.* Serum C-reactive protein concentration and the prognosis of ductal adenocarcinoma of the head of pancreas. *Eur J Surg Oncol* 2009; **35**: 605–10.
- 42 Chen Y, Shi M, Yu GZ *et al.* Interleukin-8, a promising predictor for prognosis of pancreatic cancer. *World J Gastroenterol* 2012; **18**: 1123–9.
- 43 Dima SO, Tanase C, Albulescu R *et al.* An exploratory study of inflammatory cytokines as prognostic biomarkers in patients with ductal pancreatic adenocarcinoma. *Pancreas* 2012; **41**: 1001–7.
- 44 Mitsunaga S, Ikeda M, Shimizu S *et al.* Serum levels of IL-6 and IL-1beta can predict the efficacy of gemcitabine in patients with advanced pancreatic cancer. *Br J Cancer* 2013; **108**: 2063–9.
- 45 Hodi FS, O'Day SJ, McDermott DF *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363**: 711–23.