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RESEARCH PAPER

Baseline immunity predicts prognosis of pancreatic cancer patients treated with WT1 and/or MUC1 peptide-loaded dendritic cell vaccination and a standard chemotherapy

Shuichi Ota[a], Mamiko Miyashita[b], Yuka Yamagishi[c], and Masahiro Ogasawara[a,b]

[a]Department of Internal Medicine, Sapporo Hokuyu Hospital, Sapporo, Japan; [b]Institute for Artificial Organ, Transplantation and Cell Therapy, Sapporo Hokuyu Hospital, Sapporo, Japan; [c]Cell Processing Center, Sapporo Hokuyu Hospital, Sapporo, Japan

ABSTRACT

The prognosis of patients with advanced pancreatic cancer is poor despite the recent introduction of immune checkpoint inhibitors. Therefore, the development of new therapeutic approaches is urgently required. In the present phase I/II study, we have evaluated the safety, the efficacy and the prognostic factors of Wilms' tumor 1 (WT1) and/or mucin 1 (MUC1) peptide-loaded dendritic cell (DC) vaccination in combination with a chemotherapy employing gemcitabine plus nab-paclitaxel or a combination chemotherapy regimen consisting of oxaliplatin, irinotecan, fluorouracil and leucovorin (FOLFIRINOX) in patients with advanced or relapsed pancreatic ductal adenocarcinoma (PDAC). Forty-eight eligible patients were enrolled and received the vaccinations approximately every 2–4 weeks at least seven times. No severe adverse events related to the vaccinations were observed. Median progression free survival and overall survival were 8.1 months and 15.1 months, respectively. DC vaccinations augmented tumor specific immunity which might be related to clinical outcome. The multivariate analyses demonstrated that WT1 or MUC1-specific interferonγ enzyme-linked immunospot number prior to DC vaccination was an independent prognostic factor related to overall survival. These results indicate that DC-based immunotherapy combined with a conventional chemotherapy is safe and has clinical benefits for patients in advanced stage of PDAC. The precise evaluation of the baseline antitumor specific immunity is critical to predict clinical outcome.

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Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death with an estimated 60,430 new cases and 48,220 deaths in the United States in 2020[1]. The numbers of new cases and deaths worldwide are approximately 458,900 and 432,200 according to GLOBOCAN 2018 estimates.[2] The prognosis of patients with advanced pancreatic ductal adenocarcinoma (PDAC) is extremely poor with 5-year survival rate less than 10%.[3] PFS and OS in patients with metastatic and inoperable disease are reported to be approximately from 3.7 to 11.7 months and from 6.1 to 15.9 months, respectively from the initiation of the standard chemotherapy.[4] Surgical resection is the only curative treatment of PDAC. However, the majority of patients are already in unresectable advanced stage at the time of diagnosis and need multimodal therapy.[3] Although multiagent chemotherapy has been shown to prolong survival by several months, clinical benefit remains unsatisfactory.[3] While immune checkpoint inhibitors have demonstrated remarkable efficacy in several solid tumors, clinical trials targeting immune checkpoint molecules have failed in PDAC.[5,6] Therefore, the development of new therapeutic approaches is urgently needed.

Dendritic cells (DCs) are potent antigen presenting cells capable of presenting tumor-associated antigens (TAAs) to T lymphocytes.[7] PDAC cells express various TAAs which elicit T cell responses against them.[8] These include Wilms' tumor 1 (WT1), mucin 1 (MUC1), human telomerase reverse transcriptase, p53, mutated K-RAS, survivin, carcinoembryonic antigen, HER-2/neu, and α-enolase. Both WT1 and MUC1 are considered to be the most suitable tumor-associated antigens for immunotherapy based on the fact that these antigens are highly immunogenic and over-expressed or over-expressed in an incompletely glycosylated form in various cancers including pancreatic cancer, while the expression in normal tissues is limited.[9,10] The expression of WT1 in normal tissue is demonstrated in mesothelium, glomerular podocytes and mesangial cells of the kidney, hematopoietic progenitor cells, Sertoli cells of the testis, stromal cells, surface epithelium, and granulosa cells of the ovary and myometrium and endometrial stromal cells of the uterus.[11,12] No expression of WT1 in normal pancreatic tissue is reported.[9] MUC1 is reported to be overexpressed in an incompletely glycosylated form in various human cancers.[13] However, the expression of MUC1 is not observed in specimens from normal pancreas, chronic pancreatitis, or ductal hyperplasia of the pancreas.[14] Clinical trials using these peptides or peptide-loaded DC vaccination in patients with advanced PDAC have demonstrated immune responses against tumor antigens with limited clinical efficacy.[15–20]

Gemcitabine (GEM) plus nab-paclitaxel (nab-PTX) or a combination chemotherapy regimen consisting of oxaliplatin, irinotecan, fluorouracil, and leucovorin (FOLFIRINOX) is recommended as the first line chemotherapy for patients with advanced or relapsed PDAC.[3] GEM plus nab-PTX and

CONTACT Masahiro Ogasawara ogasawara@hokuyu-aoth.org Department of Internal Medicine, Sapporo Hokuyu Hospital, 6-6-5-1 Higashi-Sapporo, Shiroishiku, Sapporo 003-006, Japan

FOLFIRINOX improved clinical outcome compared with GEM or S-1 (tegafur gimeracil oteracil).[21,22] However, median overall survival is less than a year.

Immune-regulatory functions have been reported in several chemotherapeutic agents; GEM induces an upregulation of TAAs expression on tumor cells and the proliferation of monocytes and DCs.[23,24] 5-FU increases the frequency of tumor-infiltrating T cells and favors myeloid-derived suppressor cells (MDSCs) differentiation.[25,26] Paclitaxel favors tumor infiltration by natural killer (NK) cells and cytotoxic T cells (CTLs).[27] Oxaliplatin increases the CTL/regulatory T cells (Tregs) ratio and depletes MDSCs.[28] Based on these facts, we hypothesized that the combined DC vaccination with conventional chemotherapies might improve clinical outcome. In fact, an add-on effect of DC or peptide vaccination to a chemotherapy including GEM and/or S-1 in inoperable pancreatic cancer was suggested by us and others.[15–19] However, clinical benefits of the combination of DC vaccination and GEM plus nab-PTX or FOLFIRINOX remain to be elucidated.

In the present phase I/II study, we have evaluated the safety, the clinical and immunological responses and analyzed the prognostic factors related to survival in patients with metastatic and unresectable or relapsed PDAC who received WT1 and/or MUC1 peptide-loaded DC vaccination in combination with a toll-like receptor (TLR) 4 agonist, OK-432, and a chemotherapy employing GEM plus nab-PTX or FOLFIRINOX regimen.

Methods

Patients and eligibility criteria

Sixty-two patients with metastatic and unresectable or relapsed PDAC were referred from local community hospitals for DC vaccination. Fourteen patients were excluded due to incompatibility to the inclusion criteria and 48 patients were enrolled in the present study from May 2017 to December 2019. The inclusion criteria were as follows: (1) pathologically diagnosed PDAC with metastatic and unresectable or recurrent disease; (2) WT1 or MUC1 expression was confirmed by an immunohistochemistry; (3) PS 0–2; (4) an expected prognosis of over 4 months; (5) white blood cell (WBC) count more than 2500 cells/mm^3; (6) hemoglobin more than 9.0 g/dL; (7) platelet count more than 90,000 cells/mm^3; and (8) no serious dysfunction of vital organs.

This study was approved by the Institutional Review Board (IRB) at Sapporo Hokuyu Hospital and performed in accordance with the Declaration of Helsinki. All patients signed informed consent forms before enrolling in this study. This study is registered in University Hospital Medical Information Network (UMIN) in Japan. The registration number is UMIN 000027279. The registration in clinicaltrials.gov was not requested by the IRB at Sapporo Hokuyu Hospital, so we registered in the UNIM in Japan.

Preparation of DC

DCs were prepared from PBMCs obtained by leukapheresis as described previously.[29–31] DCs were loaded with HLA-A2-, HLA-A24-, or HLA-A26-restricted 9-mer WT1 peptides and/or MUC-1 peptide and cryopreserved until the day of administration. The phenotype characteristic to mature DC (CD14$^{-/low}$/HLA-DR$^+$/HLA-ABC$^+$/CD80$^+$/CD83$^+$/CD86$^+$/CD40$^+$) was confirmed by flow cytometry (FACS) analysis. Negativity of mycoplasma (polymerase chain reaction [PCR] method) and endotoxins (Endospecy™; Seikagaku Co.) in cell suspension were confirmed before cryopreservation. Three HLA-restricted 9-mer WT1 peptides were used depending on the HLA-A allele the patients had. Amino acid sequences of WT1 peptides are as follows: HLA-A2 peptide, RMFPNAPYL; HLA-A24 peptide, CYTWNQMNL; HLA-A26, VTFDGTPSY. WT1 peptides were obtained from NeoMPS Inc. and AnyGen Co. Ltd. MUC1 peptide (TRPAPGSTAPPAHGVTSAPDTRP APGSTAP) was used for patients whose tumor was positive for MUC1 regardless of HLA type. MUC1 peptide was obtained from AnyGen Co. Ltd.

Patient treatment

The vaccination regimen has been described previously.[29–31] Briefly, WT1 and/or MUC1 peptide-loaded mature DCs (approximately 1×10^7) were injected intradermally at four positions at each side of axilla approximately every 2–4 weeks if WBC count was above 2000 cells/μl. Patients without progressive disease upon the completion of the standard 7th vaccination received additional vaccinations until the occurrence of disease progression or the administration of the last tube. The median number of vaccinations was 9 times (range: 5–29 times). OK-432 (0.5–2.0 KE), a penicillin-killed and lyophilized preparation of Streptococcus pyogenes (Chugai Pharmaceutical Co. Ltd.), was administered subcutaneously at each side of axilla in the proximity of vaccine sites to activate DC functions.

Patients were treated with either GEM plus nab-PTX or FOLFIRINOX regimen concurrently depending on the referring physician's decision. In the GEM plus nab-PTX regimen, nab-PTX 125 mg/m^2 was administered on day 1 and GEM 1000 mg/m^2 was administered on day 1, 8, and 15 and repeated every 4 weeks. In FOLFIRINOX regimen, oxaliplatin 85 mg/m^2, l-leucovorin 200 mg/m^2, irinotecan 180 mg/m^2 were administered on day 1. 5-FU 400 mg/m^2 was administered i.v. bolus followed by 2400 mg/m^2 continuous i.v. infusion over 46 hours. 5-FU i.v was discontinued when the dose reduction was required due to a serious toxicity. This regimen was repeated every 2 weeks. Dose reduction by up to 40% was permitted depending on the degree of adverse events. To prevent cytotoxicity of chemotherapeutic agents, DC were administered on day 3 and day 17 in the GEM plus nab-PTX regimen and day 5 in in the FOLFIRINOX regimen.

Evaluation of toxicity and clinical responses

Patients were examined for signs of adverse events during the treatment. The US National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 was used to classify the grades. Physical examinations and laboratory tests were conducted at each vaccination. A computed tomography (CT) was performed within a month prior to and post DC vaccination and repeated

approximately every 1–3 months until the disease progression. The clinical response was evaluated on the basis of the Response Evaluation Criteria in Solid Tumors (RECIST) (version 1.1). Follow-up information was obtained from the referring physicians.

ELIspot assay

Cryopreserved PBMCs ($1–2 \times 10^6$), obtained from the patients before and after the vaccination, were cultured with WT1 or MUC1 peptide (10 μg/ml each) and interleukin-2 (50 U/ml, Peprotech Inc.) for 2 weeks. After harvesting, washing, and evaluating the viability of cells, serially threefold diluted cells ($3x10^5$–$1x10^3$) were incubated with WT1, MUC1 or HIV peptide-pulsed antigen presenting cells (APCs; K562 human erythroleukemia cells transduced with HLA-A*02:01 or HLA-A*2402 gene) in duplicate in a separate well of a nitrocellulose plate pre-coated with anti-human IFNγ moAb. Irrelevant HIV peptide-pulsed APCs were used as a negative control to evaluate nonspecific response. The viability of harvested cells, examined by a trypan blue dye exclusion method, was in the range of 80–95%. The presence of IFNγ-secreting WT1 or MUC1-specific T cells were examined by an IFNγ ELIspot assay kit (Invitrogen/ThermoFisher Scientific, Inc.) following the procedure recommended by the manufacturer. The number of IFNγ spots were measured by an image analyzer and software (Cellular Technology Ltd.). The number of WT1 or MUC1 peptide-specific spots was calculated by subtracting the mean spot number of duplicated cells stimulated with HIV peptide-pulsed APCs from that of duplicated cells stimulated with WT1 or MUC1 peptide-pulsed APCs.

FACS analysis

The expression of surface molecules on DCs or PBMCs was examined by FACS using various fluorescent dye-conjugated monoclonal antibodies (moAbs) as described previously;[29–31] fluorescein isothiocyanate (FITC)-conjugated moAbs against cluster of differentiation (CD) 4, CD8 or T cell receptor (TCR) γδ (Becton Dickinson Japan Co.), Phycoerythrin (PE)-conjugated moAbs against CD11b, CD56,CD62L or CD127 (Becton Dickinson Japan Co.). Peridinin chlorophyll protein (PerCP)-conjugated moAbs against CD3, CD4 or HLA-DR and PE-conjugated moAbs against CD33 or HLA-ABC (BioLegend Inc.). FITC-conjugated moAbs against CD25, CD45RO, CD80 or CD83, and PE-conjugated moAbs against CD86 (Beckman Coulter Co.). FITC-conjugated anti-CD14 moAb (Miltenyi Biotec Co.). After 15 minutes incubation, cells were washed with FACS buffer, applied to FACSCalibur and analyzed using CellQuest software (Becton Dickinson Japan Co.).

Immunohistochemistry and HLA typing

The expression of WT1 and MUC1 on tumor cells in biopsy samples was examined by immunohistochemistry using rabbit polyclonal anti-WT1 Ab (C-19; Santa Cruz Biotechnology, Inc.) and anti-MUC1 moAb (VU4H5; Santa Cruz Biotechnology, Inc.) as described by Oji et al.[9] HLA typing was carried out by using a HLA DNA typing kit obtained from Wakunaga Pharmaceutical Co. following the procedure recommended by the manufacturer.

Statistical analysis

Statistical significance of the differences was calculated by the Wilcoxon signed-ranks test or the Mann-Whitney *U*-test. $P < .05$ was considered significant. OS and PFS were analyzed by the Kaplan-Meier method and the survival was measured in months from the date of the first vaccine to the date of death or final follow-up and the date of disease progression, respectively. Survival curve comparisons were conducted with the log-rank test. Univariate or multivariate analysis of the factors related to survival was conducted with the log-rank test and the Cox's proportional hazards regression model, respectively. Statistical analyses were performed using Statcel software (OMS Publishing Co.) and EZR (version 1.36; Saitama Medical Center, Jichi Medical University).

Results

Patient characteristics and treatment

Characteristics of patients enrolled in this study are shown in Table 1. Median age of patients was 64 years (range; 41–83). The male to female ratio was 1 to 1.29. Eastern Cooperative Oncology Group performance status (PS) at the first DC vaccination was below 1 except 2 patients in PS 2. Pancreatic tumor location in head or body/ tail was 22 and 26 patients, respectively. Metastatic sites at DC vaccination were local in 8, liver in 16, lung 9, peritoneum in 5 and multiple sites in 10 patients. Median serum CA19.9 and CEA levels were 499.8 U/ml and 7.0 ng/ml, respectively. Previous treatments were chemotherapy employing S-1 and/or GEM, GEM plus nab-PTX or FOLFIRINOX in 21 patients, surgery in 11 patients and radiation in 3 patients. 13 patients were chemo-naïve. Concurrent treatments were GEM plus nab-PTX in 33 patients and FOLFIRINOX in 15 patients. The median treatment cycle of GEM plus nab-PTX and FOLFIRINOX was 7.0 (range; 3.0–15.0) and 9.0 (range; 5.0–21.0), respectively. Postvaccination chemotherapies were S-1 and/or GEM in 19 patients, GEM plus nab-PTX in 25 patients and FOLFIRINOX in 12 patients. The median number of DC vaccinations were 9.0 times (range; 5.0–29.0). Six patients received vaccinations five or six times due to the insufficient amount of DCs generated.

Clinical outcome

Although no patient had complete response, 7 patients had partial response (PR), 20 patients had stable disease (SD), and the remaining 21 patients had progressive disease (PD) following the 7th DC injection. Therefore, an objective response rate and a disease control rate were 14.6% and 56.3%, respectively. Median progression free survival (PFS) and overall survival (OS) was 8.1 months (95% confidence interval, 4.4–10.0 months) and 15.1 months (95% confidence interval, 9.3–18.9 months) from the initiation of DC vaccination,

Table 1. Patient characteristics.

		n	%
Age	Median	64	
	Range	41–83	
Gender	Male	21	44
	Female	27	56
Performance status	0	29	60
	1	17	35
	2	2	4
Pancreatic tumor location	Head	22	46
	Body/Tail	26	54
Metastatic site	Local invasion	8	17
	Liver	16	33
	Lung	9	19
	Peritoneal	5	10
	Multiple	10	21
CA19-9 (U/ml)	Median	499.8	
	Range	0.5–20379.8	
CEA (ng/ml)	Median	7	
	Range	2.0–281.4	
Previous therapy	Chemotherapy	21	44
	Surgery	11	23
	Radiation	3	6
	None	13	27
Concurrent chemotherapy	GnP	33	69
	FOLFIRINOX	15	31
Postvaccination chemotherapy	S-1	10	16
	GEM	6	10
	GEM+S-1	3	4
	GnP	25	41
	FOLFIRINOX	12	20
	BSC	5	8

Abbreviations: CA19-9, carbohydrate antigen 19–9; CEA, carcinoembryonic antigen; S-1, tegafur gimeracil oteracil; GEM, gemcitabine; GnP, GEM+nab-paclitaxel ; FOLFIRINOX, oxaliplatin, irinotecan, fluorouracil, and leucovorin; BSC, best supportive care.

respectively with the median observation period of 13.1 months. One year PFS and OS was 30.6% and 61.9%, respectively (Figure 1(a,b)).

Adverse events

Adverse events (AEs) observed in more than 20% of patients during the treatment period are shown in Table 2. Grade 3 or 4 leukocytopenia, neutropenia, anemia, and thrombocytopenia occurred in 44%, 46%, 13%, and 8% of patients, respectively. Febrile neutropenia occurred in 4 (8%) patients. Seventeen (35%) patients were treated with granulocyte colony stimulating factor to resolve neutropenia. The most frequent nonhematological AEs were fatigue (48%), nausea (46%), alopecia (44%), anorexia (42%), and peripheral sensory neuropathy (42%) followed by pain (38%) and constipation (33%). The most common AEs directory related to DC vaccination were transient low grade erythema and/or induration at the injected sites (100%) and mild fever related to OK-432 (40%).

Immunological monitoring

WT1 or MUC1 specific immune responses were evaluated by an Interferonɤ (IFNɤ) enzyme-linked immunospot (ELIspot) assay following in vitro culture with WT1 or MUC1 peptide. As shown in Figure 2(a), WT1 specific IFNɤ spot number per 1×10^4 peripheral blood mononuclear cells (PBMCs) increased from 9.0 to 83.4 on average following vaccination ($p < .001$).

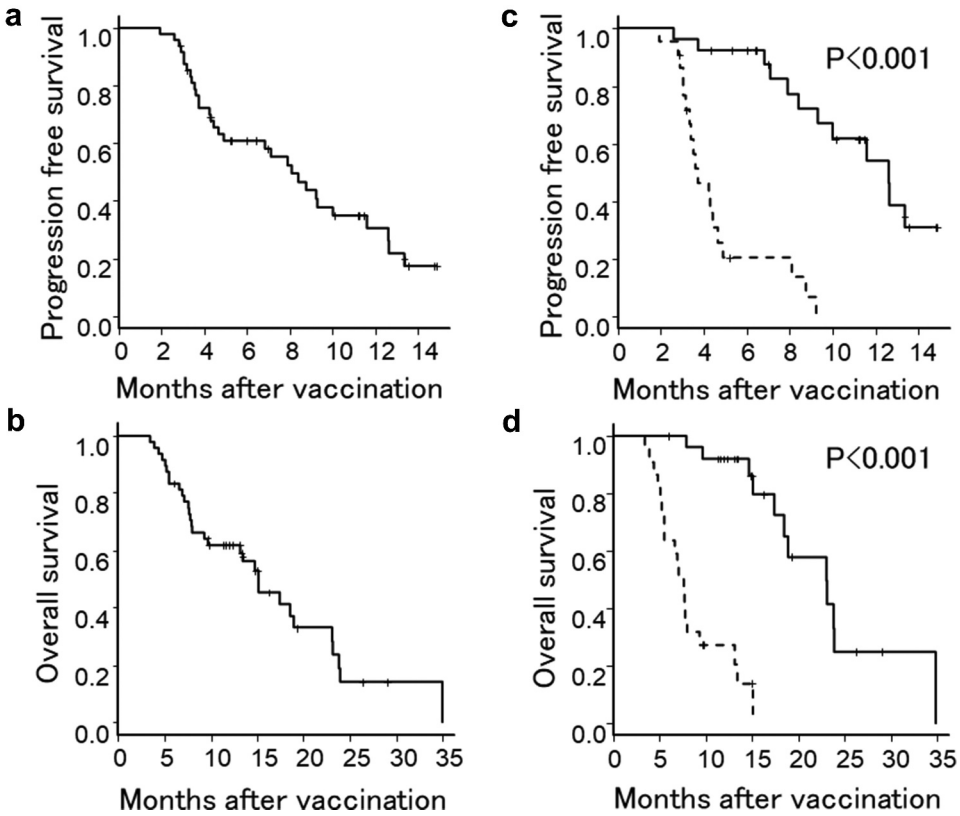

Figure 1. Kaplan-Meier estimates of PFS and OS of patients treated with a combined DC vaccination with a chemotherapy. (a and b) PFS and OS of all patients. (c and d) PFS and OS of patients who showed positive immune responses in either WT1 or MUC1 ELIspot assay (solid line) and patients who showed negative immune responses in both assays (dotted line).

Table 2. Adverse events[†].

	Any grade		Grade 3, 4	
	n	%	n	%
Hematological AEs				
Leukocytopenia	40	83	21	44
Neutropenia	35	73	22	46
Anemia	32	67	6	13
Thrombocytopenia	21	44	4	8
Nonhematological AEs				
Anorexia	20	42	2	4
Diarrhea	14	29	0	0
Constipation	16	33	0	0
Nausea	22	46	0	0
Fatigue	33	48	1	2
Alopecia	21	44	0	0
Fever	19	40	0	0
Injection site reaction	48	100	0	0
Peripheral sensory neuropathy	20	42	0	0
Pain	18	38	0	0
ALT elevation	17	35	1	2
AST elevation	16	33	1	2
Bilirubin elevation	11	23	0	0
ALP elevation	10	21	0	0
Hypoalbuminemia	15	31	0	0

[†]Adverse Events (AEs) listed are those that occurred in more than 20% of patients. The US National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 was used to classify the grades. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

The increment in spot number was significantly higher in the patients who had PR or SD (responding patients) in comparison with PD patients ($p < .001$). Similarly, we observed a significant increase in the MUC1 specific IFNɣ spot number following vaccination ($p < .001$) and significantly greater magnitude of increase in responding patients ($p < .001$, Figure 2 (b)). These results demonstrated that WT1 and MUC1 specific immunity were augmented significantly by WT1 and/or MUC1 peptide-loaded DC vaccination.

The relationship between clinical outcome and immune responses was demonstrated by a survival analysis using a log-rank test. Median PFS and OS were significantly longer in patients who showed positivity in either WT1 or MUC1 ELIspot assay than in patients who were negative in both assays ($p < .001$) (Figure 1(c,d)). These results suggested that augmentation of WT1 or MUC1 specific immunity might contribute to a better clinical outcome.

Prognostic factors related to PFS and OS

The univariate analyses with log-rank tests showed that none of the clinico-laboratory factors such as age, gender, PS, primary tumor localizations, metastatic sites, and previous treatments were significantly correlated with PFS and OS. Similarly, neither concurrent chemotherapy regimens nor difference of peptides were prognostic factors for PFS and OS (Table 3). Instead, neutrophil to lymphocyte ratio (NLR) more than 5 (PFS, $p = .0206$; OS $p = .0164$), prognostic nutritional index (PNI) less than 40 ($p = .0174$; OS, $p = .0109$) and modified Glasgow prognostic score (mGPS) 1 or 2 (PFS, $p = .0169$: OS $p = .0251$) were significantly related to shorter PFS and OS among the clinico-laboratory factors. On the other hand, carcinoembryonic antigen (CEA) above median ($p = .0444$) and platelet to lymphocyte ratio (PLR) more than 150 was significantly related to shorter PFS ($p = .028$) (Table 4). Because there was a variation in the staining level in WT1 and MUC1 in immunohistochemistry, we compared PFS and OS between the patient groups depending on the staining level. We found no significant difference in PFS and OS between the patient groups with moderate or weak staining of WT1 and MUC1 (Table 4).

Among the immune-related factors, we found that Treg number in peripheral blood (PB) below median (PFS, $p = .00306$; OS, $p = .0159$), marked skin reaction (≧30 mm) (PFS, $p = .0107$; OS, $p = .00174$), WT1-specific IFNɣ ELIspot number above median (PFS, $p = .000914$; OS $p < .0001$), and MUC1-specific IFNɣ ELIspot number above median (PFS, $p < .0001$; OS, $p < .0001$) were significantly related to longer PFS and OS (Table 5).

The multivariate analyses with a Cox's proportional hazards regression model among the significant factors in the univariate analyses demonstrated that WT1-specific IFNɣ ELIspot number was an independent prognostic factor related to OS ($p = .038$) and that MUC1-specific IFNɣ ELIspot number was an independent prognostic factor related to both PFS ($p = .012$) and OS ($p = .014$) (Table 6).

Taken together, these results indicated that baseline tumor-specific and nonspecific immunity and the augmentation of tumor-specific immune response following DC vaccination play a pivotal role and influence the clinical outcome.

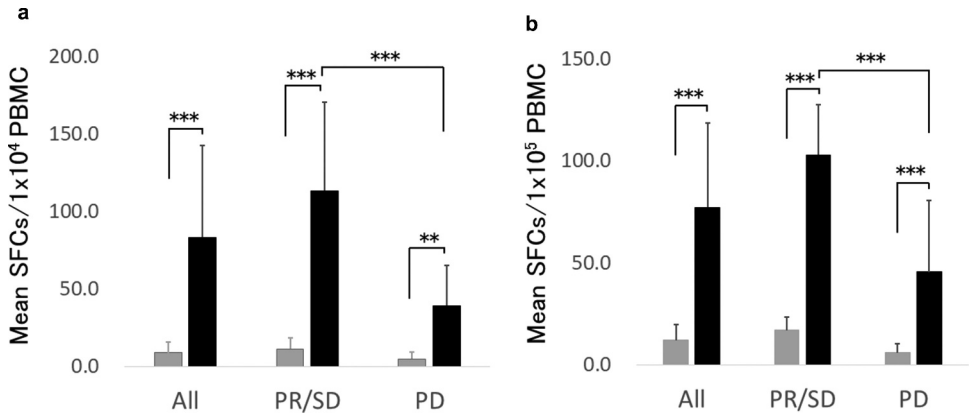

Figure 2. IFNɣ ELIspot assay.

Table 3. Univariate analysis of the association of clinical factors with PFS and OS.

			PFS		OS	
Factor	Group	n	Median survival	p value	Median survival	p-value
Age	≧64	25	8.4 (4.4–12.6)	0.319	15.1 (9.7–23.8)	0.391
	≦64	23	4.9 (3.5–11.6)		13.1 (7.6–18.5)	
Gender	Female	27	8.4 (4.2–13.4)	0.297	17.4 (7.9–23.9)	0.684
	Male	21	7.1 (3.7–10.0)		14.7 (7.6–23.1)	
Performance status	1,2	19	8.4 (3.7–12.6)	0.705	15.1 (7.6–18.9)	0.308
	0	29	7.1 (4.2–12.6)		15.1 (7.8-NA)	
Tumor location	Body/tail	26	7.1 (4.3–9.3)	0.439	15.1 (7.6–23.8)	0.688
	Head	22	8.8 (3.7–13.4)		15.1 (7.9–18.9)	
Local invasion	No	41	8.8 (4.4–12.6)	0.614	15.1 (8.0–18.5)	0.75
	Yes	7	7.9 (3.0–10.0)		18.9 (4.8–23.8)	
Liver metastasis	No	30	8.1 (3.7–10.0)	0.743	14.7 (7.8–18.9)	0.284
	Yes	18	7.1 (3.6-NA)		15.1 (7.7-NA)	
Lung metastasis	No	40	7.9 (4.3–9.3)	0.469	15.1 (8.0–18.9)	0.448
	Yes	8	12.6 (2.8-NA)		23.9 (5.1-NA)	
Peritoneum metastasis	No	43	8.4 (4.7–10)	0.921	15.1 (9.7–23.1)	0.215
	Yes	5	4.3 (3.2-NA)		7.9 (5.5-NA)	
Multiple metastasis	No	38	7.9 (4.3–12.6)	0.532	17.4 (9.3–23.8)	0.0936
	Yes	10	8.1 (1.9–11.6)		13.4 (3.4–15.1)	
Chemotherapy	No	27	8.8 (4.4–12.6)	0.546	15.1 (8.0–23.1)	0.987
	Yes	21	6.8 (3.5–13.4)		15.1 (6.6–23.8)	
Surgery	No	37	9.2 (4.4–12.6)	0.122	14.7 (7.9–23.1)	0.848
	Yes	11	6.8 (2.6–8.1)		15.1 (5.2–23.8)	
Radiation	No	45	8.1 (4.4–11.6)	0.739	15.1 (9.3–23.1)	0.767
	Yes	3	9.3 (3.2-NA)		18.5 (6.6-NA)	
GEM+nab-PTX	No	15	7.1 (3.4–11.6)	0.753	14.7 (5.5-NA)	0.959
	Yes	33	8.4 (4.4–12.6)		15.1 (9.7–23.1)	
FOLFIRINOX	No	33	8.4 (4.4–12.6)	0.753	15.1 (9.7–23.1)	0.959
	Yes	15	7.1 (3.4–11.6)		14.7 (5.5-NA)	
Peptide	MUC1	13	12.6 (3.0-NA)	0.415	15.1 (4.8-NA)	0.511
	WT1+ MUC1	35	7.9 (4.4–9.3)		14.7 (7.9–23.1)	

Parenthesis represents a 95% confidence interval. PFS, progression free survival; OS, overall survival; GEM+nab-PTX, gemcitabine+nab-paclitaxel; FOLFIRINOX, oxaliplatin, irinotecan, fluorouracil and leucovorin. MUC1, Mucin1; WT1, Wilms' tumor 1.

Table 4. Univariate analysis of the association of laboratory factors with PFS and OS.

			PFS		OS	
Factor	Group	n	Median survival	p value	Median survival	p-value
WBC	≧4380/mm^3	24	8.4 (4.2–11.6)	0.479	14.7 (7.6–23.1)	0.621
	<4380/ mm^3	24	8.1 (3.7–13.4)		15.1 (8–23.8)	
Hb	≧11.4 g/dL	24	8.4 (4.2–11.6)	0.99	15.1 (8–18.9)	0.808
	<11.4 g/dL	24	6.8 (3.7–12.6)		13.1 (7.1–23.9)	
Plt	≧199/ mm^3	25	7.9 (4.2–9.3)	0.42	15.1 (7.6–23.8)	0.734
	<199/ mm^3	23	8.8 (3.7–12.6)		17.4 (9.3–23.1)	
Alb	≧3.95 g/dL	24	8.1 (4.3–12.6)	0.596	17.4 (9.3–23.1)	0.447
	<3.95 g/dL	24	8.4 (3.5–12.6)		14.7 (7.1–23.9)	
CRP	≧0.195 mg/dL	24	8.4 (4.4–12.6)	0.899	18.5 (7.1–23.)	0.857
	<0.195 mg/dL	24	7.9 (3.5–13.7)		15.1 (8–23.1)	
CA19.9	≧499.8 U/ml	24	8.4 (4.2–12.6)	0.858	15.1 (7.7–18.9)	0.291
	<499.8 U/m	24	7.9 (3.7–12.6)		15.1 (7.9–23.8)	
CEA	≧7.0 mg/ml	19	4.7 (3.0–9.3)	0.0444*	8.0 (5.1–18.5)	0.203
	<7.0 mg/ml	19	8.4 (4.9-NA)		18.9 (9.3–23.8)	
NLR	≧5	11	4.2 (3.3-NA)	0.0206*	8.0 (6.6–15.1)	0.0164*
	<5	37	9.2 (6.8–12.6)		17.4 (13.1–23.8)	
PNI	≧40	40	9.2 (6.8–12.6)	0.0174*	17.4 (13.4–23.1)	0.0109*
	<40	8	4.6 (1.9-NA)		7.4 (3.4–13.1)	
mGPS	0	36	9.2 (6.8–12.6)	0.0169*	17.4 (13.4–23.1)	0.0251*
	1.2	12	4.4 (3.0–8.8)		7.4 (3.9–13.1)	
PLR	≧150	32	6.8 (3.6–9.2)	0.0228*	13.1 (7.7–18.5)	0.0604
	<150	16	11.6 (4.4-NA)		18.9 (14.7-NA)	
WT1	Weak	33	8.8 (4.3–11.6)	0.62	14.7 (7.9–23.1)	0.968
	Moderate	15	8.4 (3.5-NA)		17.4 (4.8–23.8)	
MUC1	Weak	19	8.1 (3.7–12.6)	0.746	18.5 (7.1–23.1)	0.799
	Moderate	29	7.1 (4.2–13.4)		14.7 (7.9-NA)	

Two groups were divided by the median value. The cutoff values for the NLR, PNI mGPS and PLR are determined on the basis of previous studies.[17,32–34] The classification of the intensity of staining in immunohistochemistry is determined on the basis of the report by Kanai et al.[35] Weak represents faint and barely perceptible staining in PDAC cells under high magnification (x200). Moderate represents moderate complete staining under low magnification (x40). Parenthesis represents a 95% confidence interval. PFS, progression free survival; OS, overall survival; WBC, white blood cell; Hb, hemoglobin; Plt, platelet; Alb, albumin; CRP, C-reactive protein; CA19-9, carbohydrate antigen 19–9; CEA, carcinoembryonic antigen; NLR, neutrophil to lymphocyte ratio; PNI, prognostic nutritional index; mGPS, modified Glasgow prognostic score; PLR, platelet to lymphocyte ratio; WT1, Wilms' tumor 1 staining; MUC1, Mucin 1 staining. * represents statistically significant.

Table 5. Univariate analysis of the association of immune-related factors with PFS and OS.

			PFS		OS	
Factor	Group	n	Median survival	p value	Median survival	p-value
CD3	≧1253/mm^3	24	8.4 (4.3–13.4)	0.549	17.4 (7.8–23.1)	0.837
	<1253/mm^3	24	8.1 (3.5–12.6)		13.4 (7.6–23.1)	
CD4	≧539/mm^3	24	8.4 (4.3–13.4)	0.533	17.4 (9.7–23.8)	0.158
	<539/mm^3	24	8.1 (3.6–11.6)		13.1 (6.9–18.5)	
CD8	≧369/mm^3	24	7.9 (4.2–11.6)	0.436	15.1 (7.1–18.9)	0.553
	<369/mm^3	24	9.0 (3.7–12.6)		13.4 (8.0–23.9)	
Th1	≧109.3/mm^3	24	8.8 (4.2–11.6)	0.944	17.4 (13.4–23.1)	0.235
	<109.3/mm^3	24	6.8 (3.5–12.6)		9.7 (6.9–23.1)	
Th2	≧162.4/mm^3	24	7.9 (4.2–11.6)	0.708	15.1 (8.0–23.1)	0.909
	<162.4/mm^3	24	8.8 (3.7–12.6)		15.1 (6.6–23.9)	
Treg	≧26.2/mm^3	24	4.7 (3.3–9.3)	0.00306*	9.3 (7.1–17.4)	0.0159*
	<26.2/mm^3	24	11.6 (7.1-NA)		18.9 (13.1-NA)	
δT	≧18.2/mm^3	24	7.9 (4.2–12.6)	0.529	17.4 (7.7–23.8)	0.492
	<18.2/mm^3	24	8.8 (3.7–11.6)		13.4 (7.8–18.5)	
NK	≧129.3/mm^3	24	8.4 (4.2–10.0)	0.503	15.1 (7.9–18.9)	0.278
	<129.3/mm^3	24	7.1 (3.7–13.4)		17.4 (6.9-NA)	
NKT	≧16.9/mm^3	24	8.1 (4.3–9.3)	0.493	15.1 (7.9–23.1)	0.686
	<16.9/mm^3	24	8.8 (3.5–13.4)		13.1 (6.9–23.9)	
MDSC	≧6.2/mm^3	24	7.9 (4.3–11.6)	0.342	14.7 (7.8–18.9)	0.363
	<6.2/mm^3	24	9.3 (3.7–13.4)		18.5 (7.7–23.1)	
MUC1 ELspot	≧12.8/10^{5}PBMC	24	12.6 (9.3-NA)	<0.0001*	23.1 (17.4-NA)	<0.0001*
	<12.8/10^{5}PBMC	23	3.7 (3.2–4.9)		7.6 (5.5–9.7)	
WT1 ELIspot	≧9.3/10^{4}PBMC	16	12.6 (6.8-NA)	0.000914*	23.9 (15.1-NA)	<0.0001*
	<9.3/10^{4}PBMC	16	4.3 (3.3–8.8)		7.6 (5.5–13.1)	
Skin reaction	≧30 mm	25	9.3 (7.1-NA)	0.0107*	18.9 (15.1–23.9)	0.00174*
	<30 mm	23	4.7 (3.6–9.2)		7.9 (6.6–15.1)	

Two groups were divided by the median value. Parenthesis represents a 95% confidence interval. The cutoff values for the skin reaction is determined on the basis of previous studies.[17] CD3, CD3$^+$T cell; CD4, CD4$^+$T cell; CD8, CD8$^+$T cell; Th1, type 1 helper T cell; Th2, type 2 helper T cell; Treg, regulatory T cell; γδT, T cell receptor γ and δ chain$^+$ T cell; NK. Natural killer cell; NKT, natural killer T cell; MDSC, myeloid-derived suppressor cell; MUC1, Mucin1; WT1, Wilms' tumor 1; ELIspot, enzyme-linked immunospot. *represents statistically significant.

Table 6. Multivariate analysis.

	PFS		OS	
Factor	Hazard ratio	p value	Hazard ratio	p value
CEA (<7.0 mg/ml vs ≧7.0 mg/ml)	0.29 (0.07–1.29)	0.11		
Treg (≧26.2/mm^3 vs <26.2/mm^3)	1.22 (0.20–7.49)	0.83	1.15 (0.31–4.19)	0.84
mGPS (1.2 vs 0)	0.27 (0.03–2.46)	0.25	3.04 (0.47–19.59)	0.24
NLR (<5 vs ≧5)	0.24 (0.05–1.27)	0.093	0.60 (0.14–2.45)	0.47
PNI (<40 vs ≧40)	3.24 (0.20–51.25)	0.4	0.36 (0.04–2.98)	0.34
MUC1 ELIspot (<12.8 vs≧12,8)	23.68 (1.99–282.00)	0.012*	19.26 (1.81–204.90)	0.014*
WT1 ELIspot (<9.3 vs ≧9.3)	0.51 (0.04–6.10)	0.6	6.11 (1.11–33.79)	0.038*
Skin reaction (<30 mm vs ≧30 mm)	4.91 (0.23–102.80)	0.3	4.43 (0.89–22.13)	0.07

Two groups in the CEA, Treg, MUC1 ELIspot and WT1 ELIspot were divided by the median value. The cutoff values for the mGPS, NLR, PNI and skin reaction are determined on the basis of previous studies.[17,28,29,32,33] Parenthesis represents a 95% confidence interval. PFS, progression free survival; OS, overall survival; CEA, carcinoembryonic antigen; Treg, regulatory T cell; mGPS, modified Glasgow prognostic score; NLR, neutrophil to lymphocyte ratio; PNI, prognostic nutritional index; MUC1, Mucin1; WT1, Wilms' tumor 1; ELIspot, enzyme-linked immunospot. *represents statistically significant.

Discussion

Previous studies of immunotherapy in PDAC demonstrated both immunological responses and clinical efficacy. However, the clinical benefit is limited.[8] In the present study, we aimed to examine the hypothesis that a combination of DC vaccination and the first-line chemotherapy employing GEM plus nab-PTX or FOLFIRINOX might improve clinical outcome in patients with advanced or relapsed PDAC. We also investigated the contribution of both the baseline immunity and the immune responses following DC vaccination to the clinical outcome.

Findings in this study are the followings; (1) We demonstrated a substantial prolongation of median PFS and OS. Median PFS and OS were 8.1 months and 15.1 months, respectively, from the initiation of vaccination, which exceeded the previously reported median PFS and OS. (2) We observed a significant enhancement of the immune responses specific to WT1 or MUC1 following DC vaccination in the majority of patients which was correlated with the prolongation of median PFS and OS. (3) WT1 specific IFNγ ELIspot number prior to vaccination was an independent prognostic factor related to OS and MUC1 specific IFNγ ELIspot number prior to vaccination was an independent prognostic factor related to both PFS and OS.

A phase III trial (MPAC trial) or a phase II/III trial (PRODIGE/ACCORD trial) in patients with metastatic pancreatic cancer demonstrated that median PFS and OS in GEM plus nab-PTX were 5.5 months and 8.5 months, respectively and those in FOLFIRINOX were 6.4 months and 11.1 months, respectively.[21,22] A systemic review of 34 studies including 6915 patients with metastatic or advanced pancreatic cancer who were treated with either GEM plus nab-PTX or FOLFIRINOX in the

first-line setting showed a range of median OS and PFS as follows; median OS of GEM plus nab-PTX and FOLFIRINOX ranged from 6.1 to 14.4 months and from 8.6 to 15.9 months, respectively, and median PFS of GEM plus nab-PTX and FOLFIRINOX ranged from 4.0 to 8.5 months and from 3.7 to 11.7 months, respectively.[4] Based on these data and considering the fact that more than 70% of the patients in this study relapsed or were refractory to the first-line treatment and that there seemed to be no marked differences in the characteristics of patients in this study compared with the previous studies particularly regarding median age and PS, the clinical outcome of this study might be favorable, suggesting an add-on effect of DC vaccination.

Although we observed grade 3 or 4 hematological toxicity, anorexia, fatigue, and alanine aminotransferase/aspartate aminotransferase elevation, the majority of adverse events were less than grade 2 (Table 2). There were no adverse events newly observed in this study compared with the previous studies employing GEM plus nab-PTX or FOLFIRINOX.[21,22] Therefore, we considered that a combined DC vaccination with GEM plus nab-PTX or FOLFIRINOX was safe and tolerable. A major concern using GEM plus nab-PTX or FOLFIRINOX regimen was toxicity to immune cells, because a hematological toxicity was more profound compared with GEM and/or S-1.[21,22] However, the results showed that the enhancement of WT1 or MUC1-specific immune responses was demonstrated to be independent of leukocytopenia. These results are consistent with our previous reports and eliminated a potential detrimental effect of chemotherapeutic agents on anti-tumor immunity.[29–31]

OK-432 has been used as an immunotherapeutic agent in various cancers.[36–38] Because OK-432 facilitates maturation of DCs and stimulates the secretion of type-1 helper T cell (Th1)-type cytokines such as IFNγ, tumor necrotizing factorα, interleukin-12 and interleukin-18 through toll-like receptor 4 signaling,[39] we employed OK-432 in the preparation of DCs in standard operating procedure (SOP) and used as an adjuvant for DC vaccine to improve clinical outcome in a similar manner to previous studies.[16,17,29–31,40] Considering AEs by OK-432, we administrated lower dose of OK-432 (0.5–2KE) compared with that used in the treatment for patients with various cancers (5–10KE).[37,38] The AEs directly related to OK-432 were transient mild fever and low grade erythema and/or induration at the injection sites, which is consistent with the frequently observed AEs in the previous DC vaccination trials employing OK-432[16,17,29–31,40] and the reported AEs in the treatment of cancer patients with OK-432.[36–38]

Many studies have demonstrated various prognostic factors related to the survival in patients with PDAC. These include PS, tumor localization, carbohydrate antigen 19–9, carcinoembryonic antigen, NLR, PLR, biochemical parameters such as albumin, lactate dehydrogenase, alkaline phosphatase, blood urea nitrogen, aspartate aminotransferase, bilirubin, C-reactive protein, and various molecular markers.[41] We found that NLR, PNI, and mGPS were the significant prognostic factors related to FPS and OS among clinico-laboratory factors examined, which is consistent with the previous studies[17,32,33] (Table 4). Inflammation associated with cancer is a well-known risk factor for the progression of various cancers and a critical factor for survival.[42] NLR, PLR, and mGPS have been demonstrated as indicators of systemic inflammatory response and have been identified as independent prognostic factors for both inoperable and surgically resected pancreatic cancer patients.[32,33,34]

Host immunity against tumors is prerequisite in immunotherapy. Therefore, host immune-related factors may offer a useful tool for predicting prognosis. There was a trend toward better prognosis in high absolute number of subset of lymphocytes such as $CD4^+$ T cells, $CD8^+$ T cells, type 1 helper T cells (Th1 cells), γδT cells, natural killer T cells (NKT cells), and low number of MDSCs. However, these factors did not reach statistical significance (Table 5). A univariate analysis revealed that only an absolute number of Treg prior to therapy was a prognostic factor for PFS and OS among various subsets of immune cells, indicating the importance of Treg in cancer immunotherapy.[43] Consistent with our findings, the prognostic significance of Treg number in PB or at tumor sites has been reported.[44,45] Liu et al. showed that a high initial Treg level or CD4/CD8 ratio before treatment was associated with a poor prognosis of the patients with unresectable pancreatic cancer who were treated with gemcitabine-based chemotherapy.[46]

We have demonstrated that a skin reaction after DC vaccination, which is considered to be a delayed type hypersensitivity reaction against WT1 and/or MUC1 peptide, is an independent treatment-related prognostic factor for patients with inoperable pancreatic cancer receiving WT1 and/or MUC1 peptide-pulsed DC vaccination together with a chemotherapy consisting of gemcitabine and/or S-1.[17] However, precise immunological baseline factors related to survival remain to be solved. In the present study, baseline skin reactions were not identified as an independent prognostic factor for both OS and PFS in a multivariate analysis, though it was extracted as a prognostic factor in a univariate analysis (Tables 5 and 6). Therefore, it is of particular interest that WT1 and MUC1 specific T cell numbers, detected by an IFNγ ELIspot assay prior to DC vaccination, emerged as independent prognostic factors for OS. The finding indicates that the tumor specific immunity prior to DC vaccination play an essential role and affects clinical outcome. Because a limited subgroup of patients will really benefit from this combined DC vaccination with chemotherapy, it would be critical to identify the patients who could have a better prognosis by the precise evaluation of tumor specific immunity prior to therapy.

The primary limitation of this study is the relatively small number of patients and the fact that it was a nonrandomized study. Although the existence of an add-on effect of DC vaccination to a concurrent chemotherapy was strongly suggested, it is too early to conclude the existence of an add-on effect. A phase III study is warranted to draw a definitive conclusion.

In conclusion, we showed the safety and the clinical benefits of vaccination with WT1 and/or MUC1 peptide-loaded DC and OK-432 with a concurrent chemotherapy with GEM plus nab-PTX or FOLFIRINOX in patients with advanced PDAC. WT1 or MUC1-specific IFNγ ELIspot number prior to DC vaccination was demonstrated to be an independent prognostic factor related to OS by the multivariate analyses, emphasizing the importance of a baseline anti-tumor immunity. These results suggest that WT1 and/or MUC1 peptide-loaded DC vaccination in combination with a chemotherapy might be a promising novel strategy for the treatment of patients with

relapsed or refractory PDAC who have better prognostic factors.

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ORCID

Masahiro Ogasawara http://orcid.org/0000-0002-7919-7146

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