Investigation of factors associated with reduced clinical benefits of personalized peptide vaccination for pancreatic cancer

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Abstract. The aim of the present study was to determine the factors associated with reduced clinical benefits of personalized peptide vaccination (PPV) for pancreatic cancer. Phase II PPV clinical trials comprising 309 (8 non-advanced and 301 advanced-stage) patients with pancreatic cancer were conducted. Two to four peptides were selected among a set of 31 different peptides as vaccine candidates for personalized peptide vaccination based on human leukocyte antigen types and preexisting peptide-specific IgG levels, and subcutaneously injected. The selected peptides were subcutaneously injected. Of the 309 patients, 81 failed to complete the 1st PPV cycle due to rapid disease progression, and their median overall survival [2.1 months; 95% confidence interval (CI), 1.8-2.7] was significantly shorter than that of the remaining 228 patients (8.4 months; 95% CI, 8.4-9.9; P<0.01). 'Immune boosting' was defined when IgG levels before vaccination increased more than 2-fold after vaccination. Immune boosting was observed in the majority of patients with PPV irrespective of whether or not they received concomitant chemotherapy. Additionally, patients demonstrating immune boosting exhibited longer survival rates. Although the positive-response rates and peptide-specific IgG levels in pre- and post-vaccination samples differed among the 31 peptides, patients exhibiting immune boosting in response to each of the vaccinated peptides demonstrated longer survival times. Pre-vaccination factors associated with reduced clinical benefits were high c-reactive protein (CRP) levels, high neutrophil counts, lower lymphocyte and red blood cell counts, advanced disease stage and the greater number of chemotherapy courses prior to the PPV treatment. The post-vaccination factors associated with lower clinical benefits were PPV monotherapy and lower levels of immune boosting. In conclusion, pre-vaccination inflammatory signatures, rather than pre- or post-vaccination immunological signatures, were associated with reduced clinical benefits of personalized peptide vaccination (PPV) for pancreatic cancer.

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Introduction

The incidence of pancreatic cancer, which is one of the most aggressive cancers and has a short overall survival (OS), is increasing year by year (1-3). There have been substantial advances in the therapeutic modalities for advanced pancreatic cancer, including carbon beam ion radiotherapy, systemic chemotherapies using gemcitabine (GEM), tegaful-gimeracil-oteracil potassium (S-1) and oxaliplatin, irinotecan, fluorouracil, leucovorin (Folfirinox), as well as an EGFR-inhibitor erlotinib. Immune checkpoint inhibitors have been approved only for pancreatic cancer patients with a high burden of microsatellite instability (MSI-high), who make up a very small subset (~1%) of the total cases (4). Therefore, the development of new approaches is needed. The field of cancer immunotherapy has drastically moved forward during these three decades since Boon and his colleagues reported for the first time a tumor-associated antigen, MAGE-A1, recognized by cytotoxic T lymphocyte (CTL) in 1991. Cancer vaccines, including peptide-based cancer vaccines, may be a promising approach. However, early trials of these vaccines did not realize sufficient clinical benefits to warrant approval for advanced cancer patients, including PC patients (5-7). We therefore developed a novel immunotherapeutic approach, the personalized peptide vaccination (PPV), in which human leukocyte antigen (HLA)-matched peptides are selected and administered based on the pre-existing immunity before vaccination (8,9). Randomized clinical trials of

PPV for patients with chemotherapy-naïve prostatic cancer or chemotherapy-resistant bladder cancer showed clinical benefits (10,11). The PPV also showed clinical benefits for some recurrent glioblastoma patients (12). However, PPV trials in patients with advanced pancreatic cancers failed to provide a clear clinical benefit (13-16). In the present study, we attempted to identify factors such as biomarkers, disease stage, the number of previously conducted chemotherapy regimens, associated with the lower clinical benefits in these previous phase II clinical trials of PPV, which collectively enrolled 309 pancreatic cancer patients.

Materials and methods

Peptides and protocols of clinical study. Thirty-one candidate peptides were available for PPV. All 31 peptides were cytotoxic T lymphocyte (CTL) epitopes restricted to the HLA-A2, -A24, or -A3 supertypes (A3, A11, A31, or A33), or HLA-A26 of HLA-class Imolecules (12.13). Twelve peptides were for the HLA-A2, 14 for the HLA-A24, and 9 for the HLA-A3 supertypes, and 4 were matched for HLA-A26 of cancer patients as reported previously (Table SI). The peptides were prepared under conditions of Good Manufacturing Practice using a Multiple Peptide System (San Diego, CA). Patients were vaccinated with 2 to 4 peptides based on human leukocyte antigen (HLA) type and pre-existing immunity by measuring peptide-specific immunoglobulin G (IgG) levels. Each of the selected peptides was mixed with incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France) and injected subcutaneously into the inguinal, abdominal, or lateral thigh areas as 1.5 ml emulsion (3 mg/each peptide).

All protocols were approved by the ethical committee of Kurume University at first followed by the regional ethical committee (Fukuoka clinical research board <Number 718004>, and then registered in the UMIN Clinical Trials Registry of Japanese government. Detailed protocols are presented online only (https://upload.umin.ac.jp/cgi-open-bin/ctr/index.cgi?function=02) where the protocols were written by Japanese with brief translation to English. In brief, there were 3 different protocols with regard to the vaccination intervals. The patients who became resistant to the standard systemic therapies (n=180) received 6 injections of PPV at 1-week intervals (the 1st cycle) followed by 6 injections at 2-week intervals (the 2nd cycle) (protocol PRT1; UMIN registration numbers: 000001482, 000001881, 000006295, 000019390). Cancer patients in the early or any other stages except those who became resistant to the standard systemic therapies (n=109) received 4 injections of PPV at 1-week intervals and then 4 injections at 2-week intervals (the 1st cycle), followed by 4 injections at 2-week intervals and then 4 injections at 4-week intervals (the 2nd cycle) (protocol PRT2; UMIN registration numbers: 000006297, 000011593, 000029789). In these two protocols, patients received 1.5 ml emulsion (3 mg/each peptide) of 2 to 4 peptides at each visit for the vaccination. The third protocol was used for cancer patients who lived in the northern parts of Japan or in other countries (n=20) and thus both the patients and family needed to stay in Kurume at least two days; they received 4 injections of PPV at 4-week intervals (the 1st cycle) followed by the same schedule for the 2nd cycle (protocol PRT3; UMIN registration numbers: 000006927, 000011230). In this third protocol, patients received 3.0 ml emulsion (6 mg/each peptide) of 2 to 4 peptides at each visit for the vaccination, with half the dose injected into either side of the body. In all three protocols, patients who wished to continue the PPV after the 2nd cycle received the vaccination at 2- to 12-week intervals until withdrawal of consent or unacceptable toxicity. All these studies were in accordance with the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice guidelines, and the studies were conducted in an outpatient setting. Written-informed consent to participate in the clinical trial and to use their data for research and publication purposes was obtained from all individual participants before their inclusion in the study.

Patients. The phase 2 clinical trials of PPV were conducted from November 2008 to March 2018 at the Cancer Vaccine Center of Kurume University and Kurume University Hospital. A total of 309 patients with pancreatic cancer were enrolled, 301 patients with advanced-stage disease (258 with stage IV and 43 with recurrent disease) and 8 with non-advanced stage disease (stages I-III). The clinical outcomes for some of these patients have been partly reported previously (15,16). Eligibility criteria at the time of entry were a pathologically confirmed diagnosis of pancreatic cancer for those patients for whom a tumor sample was available (n=145) or clinically diagnosed pancreatic cancer for those without a tumor sample (n=160), positive IgG responses specific to at least 2 of the HLA-class-IA matched peptides in pre-vaccination plasma, positive status for the HLA-A2, -A24, or -A3 supertypes (A3, A11, A31, or A33) or positive status for HLA-A26, age of ≧20 years, Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1, life expectancy of at least 12 weeks, and adequate bone marrow function, hepatic function, and renal function. Exclusion criteria were acute infection, a history of severe allergic reactions, or other systemic diseases. The histological diagnoses for the 145 patients with available tumor samples consisted of adenocarcinoma (n=126), invasive ductal carcinoma (10), intra-ductal papillary mucinous carcinoma (IDMC) (5), mucinous cystadenocarcinoma (3), or neuroendocrine tumor (1). In all the protocols, concomitant chemotherapy regimens were permitted, including but not limited to gemcitabine (GEM), TS-1 (an oral fluoropyrimidine derivative), GEM+ nab-paclitaxel (nabPTX), GEM+TS-1, and leucovorin+5-fluorouracil+irinotecan+oxaliplatin (FOLFIRINOX). All patients provided written informed consent for study participation and data collection.

Peptide-specific immune responses. Peripheral blood (30 ml) was taken from patients before and after each cycle of vaccinations. Plasma was separated after centrifugation and frozen until use. The IgG responses specific to the vaccine peptides were measured in plasma by a Luminex system as reported previously (8-11).

Statistical analysis. The Kaplan-Meier method, log-rank test, Cox proportional hazards analysis, Student's t-test, chi-square test, and Fisher's exact test were used for the statistical analyses. OS was calculated as the time in months from the date of study enrollment to death or to the date of last

contact. The data-cutoff for OS was June 2018. Time-to-event endpoints were analyzed using the Kaplan-Meier method, and between-group comparisons for OS were conducted using the log-rank test. All reported P-values were two-sided, and P-values of <0.05 were considered significant. JMP version 12 or SAS version 9.4 software (SAS Institute Inc.) was used to perform all analyses.

Results

Baseline characteristics. The baseline characteristics of the 309 pancreatic cancer patients are shown in Table I. There were 171 males and 138 females, and the median age was 64 years. HLA-status was HLA-A24 in 180, HLA-A2 in 130, and HLA-A3 supertypes (A3, A11, A31, A33) in 161 patients, and HLA-A26 in 58 patients. Performance status (PS) was 0 in 241 and 1 in 68 patients. Clinical stages were stage I in 3, II in 2, III in 3, and IV in 258 patients, and there were 43 recurrent cases. The number of chemotherapeutic courses prior to the vaccination was 0, 1, 2, 3, or 4 in 28, 136, 108, 29, or 8 patients, respectively. Chemotherapy regimens administered during the 1st to 2nd courses of PPV were none (n=59 patients), gemcitabine (GEM) (59), S-1 (65), GEM+S-1 (40), GEM+ nab-paclitaxel (14), and others (71), respectively. The median number of vaccinations was 9, with a range of 1 to 60. Informed consent was obtained from all patients prior to entering the study.

Eighty-one of 309 patients (26%) failed to complete the 1st cycle of PPV due to rapid disease progression. All 81 of these patients were in the advanced disease stages. Significant differences were seen between the 81 patients who did not complete the 1st cycle and the remaining 228 patients who did complete the 1st cycle in regard to PS (worse in the former group), number of chemotherapy courses prior to PPV (larger in the former), and the percentage of patients receiving chemotherapy combined with PPV (lower in the former) (Table I). In addition, the white blood cell and neutrophil numbers along with c-reactive protein (CRP), a typical inflammatory protein, prior to study entry were significantly higher in the 81 patients who did not complete the 1st cycle, whereas the red cell and lymphocyte numbers were significantly higher in the 228 patients who did complete the 1st cycle (Table II).

Adverse events (AEs). The numbers of grade 1, 2, 3, 4 or 5 AEs were 1,018, 555, 190, 14 and 86, respectively (Table SII). The most frequent adverse event was injection site reaction (grade 1: n=403; grade 2: n=55; grade 3: n=8). Frequently observed grade 3 AEs were elevation of GGT (n=24), anemia (21), lymphopenia (20), injection site reaction (8), ascites (7), glucose intolerance (7), and anorexia (7). Grade 4 AEs were anemia (n=4), hepatobiliary disorders (n=3), lymphopenia (2), leukocytopenia (1), neutropenia (1), colonic obstruction (1), duodenal stenosis (1), and hypercalcemia (1). Frequently observed grade 5 AEs were neoplasms (44), multi-organ failure (17), hepatic failure (4), and respiratory failures (3). According to the assessment by an independent safety evaluation committee in this trial, all of these severe AEs, except injection site reaction (8), were related to the cancer progression or the combination chemotherapies.

Immune responses. Pre-vaccination peptide-specific IgG titers to each of the 31 peptides were measured using a Luminex system with a cut-off level of 10 fluorescence intensity units (FIU) taken as a detectable level of IgG as reported previously (8-12). The positive rate of antibody in the patients' plasma (n=309) was largely different among the 31 peptides, ranging from 12 to 90% with a median positive rate of 56%; Table III lists the peptides in order from highest IgG positivity to lowest. The magnitudes of IgG titers for the 31 peptides among the patients showing detectable levels were also different from each other, with the median FIU being 35 (range: 23-132) among the 31 peptides (Table III).

Post-vaccination peptide-specific lgG levels were measured at the end of both the 1st cycle and 2nd cycle in plasma from the 228 patients who completed at least the 1st cycle of vaccination. It was considered to be a positive immune response when the post-vaccination IgG titer at the end of either the 1st or 2nd cycle was two times higher than the pre-vaccination titer (8-10). Six peptides (PSMA-624, Lck-208, EZH2-735, MRP3-503, UBE2V-85 and Lck-422) with <10 cases of evaluable patients were excluded in the following analysis to avoid a possible bias. Under these circumstances, the percentage of patients showing positive IgG responses among the 228 patients who completed at least the 1st cycle differed greatly for the 25 peptides, ranging from 25 to 79%, with a median rate of 55% (Table III). The magnitudes of IgG titers of the 25 peptides among the patients showing the positive responses were also very different, with a median FIU of 3,278 among the 25 peptides, which was 89-fold greater than the pre-vaccination level (37 FIU, as shown above).

Effect of chemotherapy on the PPV-induced immune boosting. We also investigated whether chemotherapy suppressed the vaccination-induced immune boosting by comparing the rate and magnitude of IgG boosting between the PPV patients with and those without chemotherapy (Fig. 1). IgG boosting was observed in post-vaccination plasma in 23 of 27 (85%) PPV patients without chemotherapy (10 of 10 patients (100%) who declined chemotherapy of their own will and 13 of 17 patients (76%) who could not tolerate chemotherapy), 42 of 45 (93%) with GEM, 5 of 7 (71%) with GEM + nabPTX, 27 of 28 (96%) with GEM+TS-1, 43 of 48 (90%) with TS-1, and 63 of 65 (97%) with the other chemotherapies (Fig. 1). We further investigated the n-fold increase of IgG boosting from the pre-vaccination to the post-vaccination IgG titers using the IgG levels shown in Fig. 1. The increase was 7.0-fold in 27 PPV patients without chemotherapy (13.5-fold in the 10 patients who declined chemotherapy of their own will, and 2.9-fold in the 17 patients who could not tolerate chemotherapy), 3.3-fold in the 45 patients with GEM, 27.9-fold in the 7 patients with GEM+nabPTX, 2.9-fold in the 29 patients with GEM+TS-1, 2.8-fold in the 48 patients with TS-1, and 11.6-fold in the 65 patients who received other chemotherapies. These results suggested that the combined chemotherapy did not suppress PPV-induced boosting of peptide-specific IgG from the viewpoint of either the positive rate or the magnitude of IgG boosting.

Overall survival (OS). The median OS of 309 patients was 5.8 months (M) with a 95% confidence interval (CI) of 5.2-6.7 M from the 1st vaccination, while it was 17.6 M with a

Table I. Characteristics of the enrolled patients (n=309).

Variable	Characteristics of patients (n=309)	Patients who completed the first cycle of PPV (n=228)	Patients who did not complete the first cycle of PPV (n=81)	P-value
Median age (range), years	64 (33-83)	64 (33-83)	64 (39-79)	0.40[a]
Sex				0.24[b]
Male	171	97	40	
Female	138	131	41	
HLA status				
HLA-A24	180	132	48	0.90[b]
HLA-A2	130	96	34	1.00[b]
HLA-A3 family	161	120	41	0.80[b]
HLA-A26	58	40	18	0.41[b]
Clinical stage (surgery)				0.67[b]
I	3	3	0	
II	2	2	0	
III	3	4	0	
IV	258	190	68	
Recurrence	43	29	13	
Performance status				0.01[b]
0	241	185	56	
1	66	43	23	
2	2	0	2	
Numbers of chemotherapy prior to PPV				<0.01[b]
0	28	23	5	
1	136	108	28	
2	108	78	30	
3	29	18	11	
4	8	1	7	
Combination treatment (up to the 2nd cycle)				<0.01[b]
None	59	34	25	
GEM	59	45	14	
GEM+TS-1	40	28	12	
TS-1	65	48	17	
GEM+nabPTX	14	7	7	
Other chemotherapies	72	66	6	
Number of vaccinations				<0.01[a]
Median (range)	9 (1-60)	12 (4-60)	3 (1-7)	
Median OS (months)				
From diagnosis	17.6	19.5	13.6	<0.01[c]
From PPV	5.8	8.4	2.1	<0.01[c]

P-values were determined using [a]Studen's t test, [b]Fisher's exact test and [c]Kaplan-Meier method. HLA, human leukocyte antigen; PPV, personalized peptide vaccination; GEM, gemcitabine hydrochloride; TS-1, tegafur/gimeracil/oteracil potassium; nabPTX, paclitaxel; OS, overall survival.

95% CI of 16.0-19.5 M from the initial diagnosis (Table I). The median time from the 1st vaccination of the 81 patients who failed to complete the 1st cycle of PPV due to rapid disease progression was much shorter than that of the remaining 228 patients who completed the 1st cycle (2.1 M, 95% CI: 1.8-2.7 vs. 8.4 M, 95% CI: 8.4-9.9; P<0.01), although that from the 1st diagnosis was not different between the 81 patients who failed to complete the 1st cycle and the remaining 228 patients (11 vs. 11 M) (Table I).

The 309 patients were also divided into those with non-advanced (stages I-III) and those with advanced (stage IV or recurrence) stage disease to better understand the role of prophy-

Table II. Laboratory markers of the enrolled patients (n=309).

Variable	Characteristics of all patients (n=309)	Patients who completed the first cycle of PPV (n=228)	Patients who did not complete the first cycle of PPV (n=81)	P-value
Pre-vaccination cell counts				
White blood cells	5,570	5,115	6,520	<0.01
Red blood cells	354	358	332	<0.01
Lymphocytes	1,346	1,390	1,190	<0.01
Platelets	22	21.5	22.2	0.16
Neutrophils	3,369	3,138	4,443	<0.01
% neutrophils	64	62	69	<0.01
Pre-vaccination CRP (mg/dl)	0.33	0.21	1.20	<0.01
Pre-vaccination IgG (FIU)				
To 31 peptides	603	616	504	0.18
To vaccinated peptides	2,339	2,235	2,545	0.56
Post-vaccination IgG (FIU)				
To 31 peptides	-	1,584	-	-
To vaccinated peptides	-	4,612	-	-
Post-vaccination immune boosting (%)	-	92.1	-	-
Median OS (months)				
Immune boosting +	-	9.2 (n=208)	-	<0.01
Immune boosting -	-	4.9 (n=20)	-	-

P-values were calculated using a Students' t-test. CRP, c-reactive protein; FIU, fluorescence intensity unit; OS, overall survival.

lactic or therapeutic PPV for pancreatic cancer, respectively. The median OS of the 8 patients with stages I to III disease from the 1st vaccination or from the initial diagnosis was 62.6 M (95% CI: 11.2-not reached) (Fig. 2A) or 72.3 M, (95% CI: 16.1-not reached) (data not shown), respectively. All 8 of these patients were over 60 years old (range: 61 to 83 years). Six and two patients were histologically diagnosed as having adenocarcinoma and IDMC, respectively. Seven patients and one patient received RO or R1 surgery, respectively. Recurrence was observed in 4 patients, and progression free survival and OS from the 1st vaccination were 42 and 63 M in the stage I adenocarcinoma case, 38 and >39 M in the stage III IDMC case, 6 and 53 M in the stage III adenocarcinoma case, and 6 and 11 M in the stage III adenocarcinoma case, respectively. The remaining 4 patients with PPV alone were free from recurrence.

The median OS of the 301 patients with advanced-stage disease (stage IV or recurrence) was 5.6 M (95% CI: 5.6-6.4) (Fig. 2A) from the 1st vaccination. Among them, no significant difference in the median OS was seen between the stage IV (n=258) and recurrent cases (n=43) (5.6 M, 95% CI: 5.0-6.3 vs. 7.2 M, 95% CI: 4.0-9.8; P=0.33) (data not shown). No significant difference in the median OS was found among the different types of HLA-class I (180A24+ patients: 5.7 M, 95% CI: 5.0-6.7 M; 130A2+ patients: 5.8 M, 95% CI: 5.1-7.7; 161A3 supertypes+ patients: 5.8 M, 95% CI: 4.8-6.7; 43 A26+ patients: 4.8 M, 95% CI: 3.9-10.0) (data not shown). In contrast, the median OS in the patients under PTR1 (n=180, 5.3 M, 95% CI: 4.6-6.1 M) was shorter than that of the patients under PRT2 (n=109, 7.7 M, 95% CI: 5.6-10.0) or PRT3 (n=20, 6.5 M, 95% CI: 3.7-12.1) (P<0.001), primarily since the vast majority of the patients under PRT1 failed to respond to all the available standard chemotherapies prior to entry into the PPV study (data not shown).

Effect of chemotherapy on OS. We investigated the effect of pre-vaccination chemotherapy status on OS. The median OS values of the 301 patients with advanced cancer were inversely correlated with the number of chemotherapy regimens conducted prior to the vaccination (Fig. 2B). The median OS values of the patients with 0 (n=20), 1 (136), 2 (108), 3 (29), or 4 (8) courses of chemotherapy prior to the vaccination were 9.4 M (95% CI: 4.9-33.1), 5.9 M (95% CI: 4.9-8.5), 5.6 M (95% CI: 4.6-6.5), 4.7 M (95% CI: 3.3-8.5), and 1.9 M (0.6-2.8), respectively (P<0.001).

We then investigated the effect of combination chemotherapies on the OS of the 301 patients with advanced cancer. The median OS values of patients who refused (n=19) or could not tolerate (n=33) combination chemotherapy were 5.4 M (95% CI: 1.9-9.6) and 2.9 M (95% CI: 1.8-3.4) (P=0.03), respectively. Those for the patients receiving the various chemotherapies were as follows: GEM (n=59) (6.1 M, 95% CI: 4.6-7.3), S-1 (n=65) (4.9 M, 95% CI: 4.0-7.8), GEM and TS-1 (n=40) (5.4 M, 95% CI: 4.6-9.4), GEM and nab-paclitaxel (n=14) (17.3 M, 95% CI: 3.4-24.1), and other chemotherapy regimens, including 6 cases of FORIFIRI (n=71) (7.9 M, 95% CI: 5.8-11.5) (data not shown). The median OS of the patients who received PPV alone for at least the 1st to 2nd cycles of PPV either because they refused the treatment of their own will or because they could not tolerate chemotherapy (n=52) (3.1 M, 95% CI: 2.0-4.1 M) was significantly shorter than that of the patients who received PPV in combination with chemotherapy (n=249) (6.2 M, 95% CI: 5.5-7.4 M) (P=0.01) (Fig. 2C).

Table III. Pre- and post-vaccination IgG responses to each of the 31 peptides and their correlation to patient OS.

	Pre-vaccination IgG					Post-vaccination IgG responses (at the end of 1 or 2 cycles)		Correlation between vaccination and OS (at the end of 1 or 2 cycles)		
Peptides	Positive cases, n (%)	Negative cases, n (%)	Median FIU of positive cases	Vaccinated cases, n (%)	Dropped cases, n (%)	Positive/ Negative, n (%)	Median FIU of Positive cases	Positive cases (median OS)	Negaive cases (median OS)	P-value
SART2-93	279 (90)	30 (10)	54	125 (72)	35 (28)	30/60 (33)	1,500	30 (13.1 M)	60 (6.4 M)	<0.01
Lck-486	258 (83)	51 (17)	44	86 (79)	18 (21)	46/22 (68)	11,301	46 (12.0 M)	22 (4.4 M)	<0.01
Lck-488	267 (86)	42 (14)	58	121 (72)	34 (28)	51/36 (59)	13,342	51 (12.3 M)	36 (4.9 M)	<0.01
Lck-90	254 (82)	55 (18)	44	77 (78)	17 (22)	27/33 (45)	1,499	27 (14.3 M)	33 (5.0 M)	<0.01
SART3-734	257 (83)	52 (17)	132	97 (77)	22 (23)	31/44 (41)	7,928	31 (10.1 M)	44 (6.2 M)	0.04
PSA-248	242 (78)	67 (22)	42	18 (78)	4 (22)	11/3 (79)	13,221	11 (13.6 M)	3 (4.2 M)	<0.01
SART3-511	228 (74)	81 (26)	39	50 (82)	9 (18)	14/27 (34)	537	14 (14.3 M)	27 (7.2 M)	0.02
SART3-309	235 (76)	74 (24)	31	40 (77)	9 (23)	18/13 (58)	4,306	18 (12.4 M)	13 (5.2 M)	<0.01
WHSC2-141	224 (72)	85 (28)	37	42 (79)	9 (21)	18/15 (55)	14,760	18 (12.5M)	15 (5.8 M)	<0.01
CypB-129	223 (72)	86 (28)	29	59 (80)	12 (20)	22/25 (47)	1,523	22 (9.3 M)	25 (5.5 M)	<0.01
Lck-246	217 (70)	92 (30)	45	42 (79)	9 (21)	18/15 (55)	3,467	18 (12.0 M)	15 (4.9 M)	<0.01
WHSC2-103	212 (69)	97 (31)	35	94 (77)	22 (23)	26/46 (36)	647	26 (11.9 M)	46 (6.7 M)	<0.01
SART3-302	210 (68)	99 (32)	80	53 (75)	13 (25)	31/9 (78)	20,126	31 (10.9 M)	9 (7.2 M)	0.26
MRP3-1293	187 (61)	122 (39)	30	38 (79)	8 (21)	18/12 (60)	8,963	18 (12.5 M)	12 (5.0 M)	<0.01
EGF-R-800	179 (58)	130 (42)	28	21 (71)	6 (29)	9/6 (60)	391	9 (13.1 M)	6 (7.0 M)	0.12
PAP-213	169 (55)	140 (45)	31	25 (76)	6 (24)	11/8 (58)	2,772	11 (12.3 M)	8 (4.6 M)	0.10
Lck-449	166 (54)	143 (46)	23	16 (94)	1 (6)	5/10 (33)	18,286	5 (11.9 M)	10 (4.9 M)	0.03
ppMAPkkk-432	150 (49)	159 (51)	42	39 (62)	15 (38)	6/18 (25)	497	6 (12.0 M)	18 (7.9 M)	0.10
HNRPL-140	152 (49)	157 (51)	38	29 (83)	5 (17)	14/10 (58)	1,965	14 (16.6 M)	10 (9.8 M)	<0.01
PAP-248	174 (56)	135 (44)	43	33 (85)	5 (15)	14/14 (50)	2,485	14 (10.1 M)	14 (7.4 M)	0.56
UBE2V-43	152 (49)	157 (51)	36	30 (77)	7 (23)	17/6 (74)	28,486	17 (15.9 M)	6 (5.3 M)	<0.01
SART3-109	142 (46)	167 (54)	30	51 (78)	11 (22)	19/21 (48)	1,896	19 (10.1 M)	21 (5.4 M)	0.08
HNRPL-501	140 (45)	169 (55)	37	47 (72)	13 (28)	21/13 (62)	5,696	21 (10.3 M)	13 (3.6 M)	<0.01
SART2-161	118 (38)	191 (62)	31	18 (72)	5 (28)	5/8 (38)	3,278	5 (17.2 M)	8 (6.0 M)	0.26
PSMA-624	111 (36)	198 (64)	23	8 (62)	3 (38)	2/3 (40)	12,213	2 (22.5 M)	3 (2.3 M)	0.28
PTHrP-102	102 (33)	207 (67)	24	22 (82)	4 (18)	8/10 (44)	397	8 (5.1 M)	10 (9.2 M)	0.62
Lck-208	82 (27)	227 (73)	23	13 (69)	4 (31)	1/8 (11)	2,016	1 (5.1 M)	8 (7.9 M)	0.28
EZH2-735	67 (22)	242 (78)	22	5 (80)	1 (20)	2/2 (50)	20,059	2 (-M)	2 (6.2 M)	0.43
MRP3-503	58 (19)	251 (81)	35	10 (80)	2 (20)	3/5 (38)	8,487	3 (25.4 M)	5 (8.1 M)	0.50
UBE2V-85	46 (15)	263 (85)	27	6 (100)	0 (0)	4/2 (67)	3,026	4 (48.7 M)	2 (14.2 M)	0.11
Lck-422	36 (12)	273 (88)	28	9 (89)	1 (11)	1/7 (13)	78	1 (12.0 M)	7 (9.6 M)	0.89

Student's t test was used to clarify whether there was a significant difference in OS. OS, overall survival; M, months.

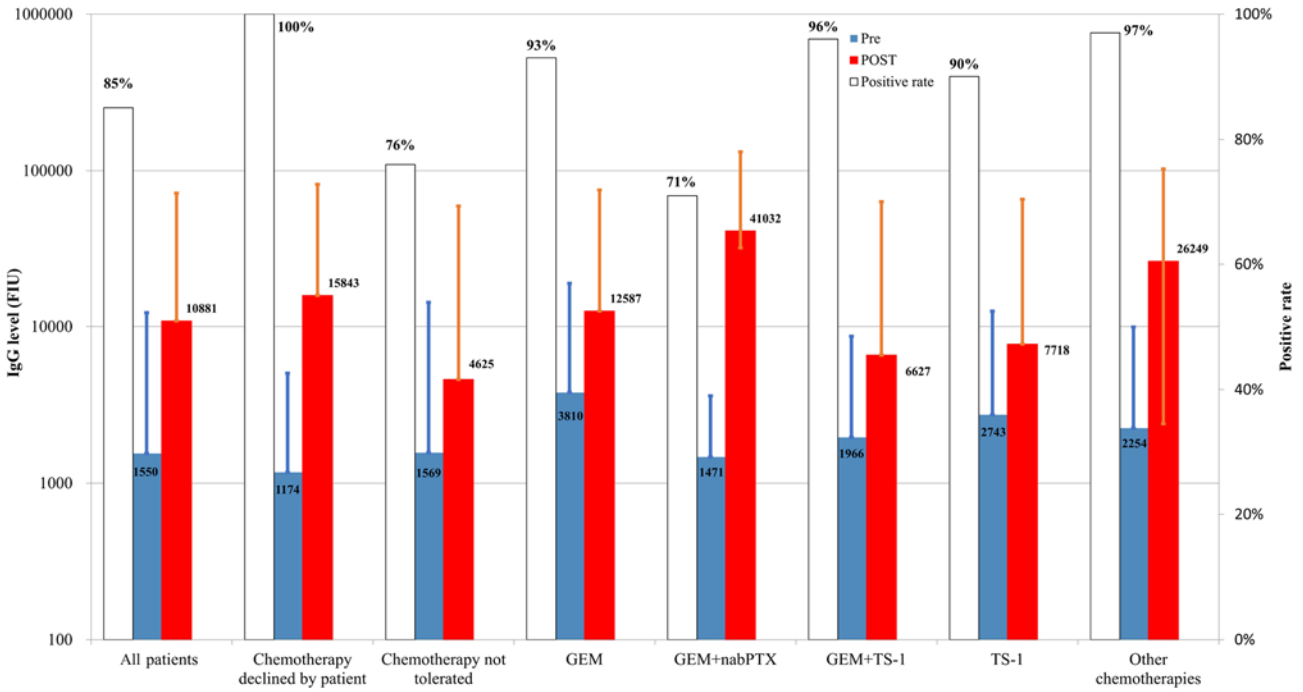

Figure 1. Effect of chemotherapy on PPV-induced immune boosting. Positive rates of IgG boosting in patients receiving PPV without chemotherapy, and those with chemotherapy are presented. The pre- and post-vaccination IgG levels are also presented.

PPV combined with chemotherapy might be more appropriate for patients who had previously undergone a smaller number of chemotherapy regimens.

Correlation between the immune boosting and OS. The median OS of the 208 patients who exhibited IgG boosting (9.2 M, 95% CI: 7.3-10.1) was significantly (P<0.01) longer than that of the 20 patients who did not show IgG boosting (4.9 M, 95% CI: 2.8-5.6) (Table II), confirming the results reported previously (8-14). We also examined whether this was also the case for each of the 31 peptides in the 309 patients with pancreatic cancer. Six (PSMA-624, Lck288, EZH2-735, MRP3-503, UBE2v-43, and Lck422) of the 31 peptides that were used in only a few cases (<10 tested cases) were excluded from this analysis to avoid a possible bias (Table III). The median OS of the patients that exhibited IgG boosting against each of the 17 of 25 peptides (65.4%) that were used for >10 cases was significantly longer (P<0.05) than that of the patients with no IgG boosting (Table III). No significant difference was found in the remaining 7 peptides.

Biomarkers. Finally, we investigated the correlation between the OS and pre-vaccination CRP level or neutrophil numbers in each of the 309 patients (Fig. S1). The higher CRP levels or neutrophil numbers seemed to be associated with shorter OS. Indeed, the median OS of the patients having higher CRP levels or higher neutrophil numbers (median or higher than median value) was significantly shorter than that of the patients with lower values for these parameters (Fig. 3A and B), respectively. The opposite was true in the case of lymphocyte numbers (Fig. 3C). Similar results were obtained in the 228 patients who completed at least the 1st cycle of the vaccination (data not shown). In contrast, only a higher CRP level, but not either higher neutrophil numbers or lower lymphocyte numbers, was an unfavorable biomarker at the statistically significant level for the 81 patients who failed to complete the 1st cycle of the vaccination (data no shown).

We also investigated the correlation between the OS and either pre- or post-vaccination IgG levels against the vaccinated peptides in all 309 patients, but significant levels of correlation were not observed (data not shown). Then, we examined the correlation between the OS and the IgG levels for each of the vaccinated peptides in each of the 228 patients who completed at least the 1st cycle of the vaccination (Fig. S2). The higher levels of the increased IgG seemed to be associated with longer OS. Indeed, the median OS of the patients with higher increased IgG levels (median or higher than the median value) was significantly longer than that of their counterparts with lower IgG levels (10.3 M, 95% CI: 7.7-12.0 vs. 7.0 M, 95% CI: 5.6-8.6) (Fig. 4).

Discussion

One of the unexpected results of this study was that as many as 81 of the 301 patients with advanced-stage disease failed to complete the 1st cycle of PPV due to a rapid disease progression, regardless of the fact that the median OS of these 81 patients from the 1st diagnosis was not different from that of the remaining 228 patients (11 vs. 11 M, respectively). Pre-vaccination biomarkers to discriminate the former group from the latter group were PS (worse in the former group), chemotherapy regimens prior to PPV (larger in the former), neutrophil or lymphocyte numbers (higher or lower in the former group), and CRP (higher in the former group). We previously reported that these factors were unfavorable for the OS of cancer patients receiving PPV (11-16). In addition, we reported that only the CRP level, but not either

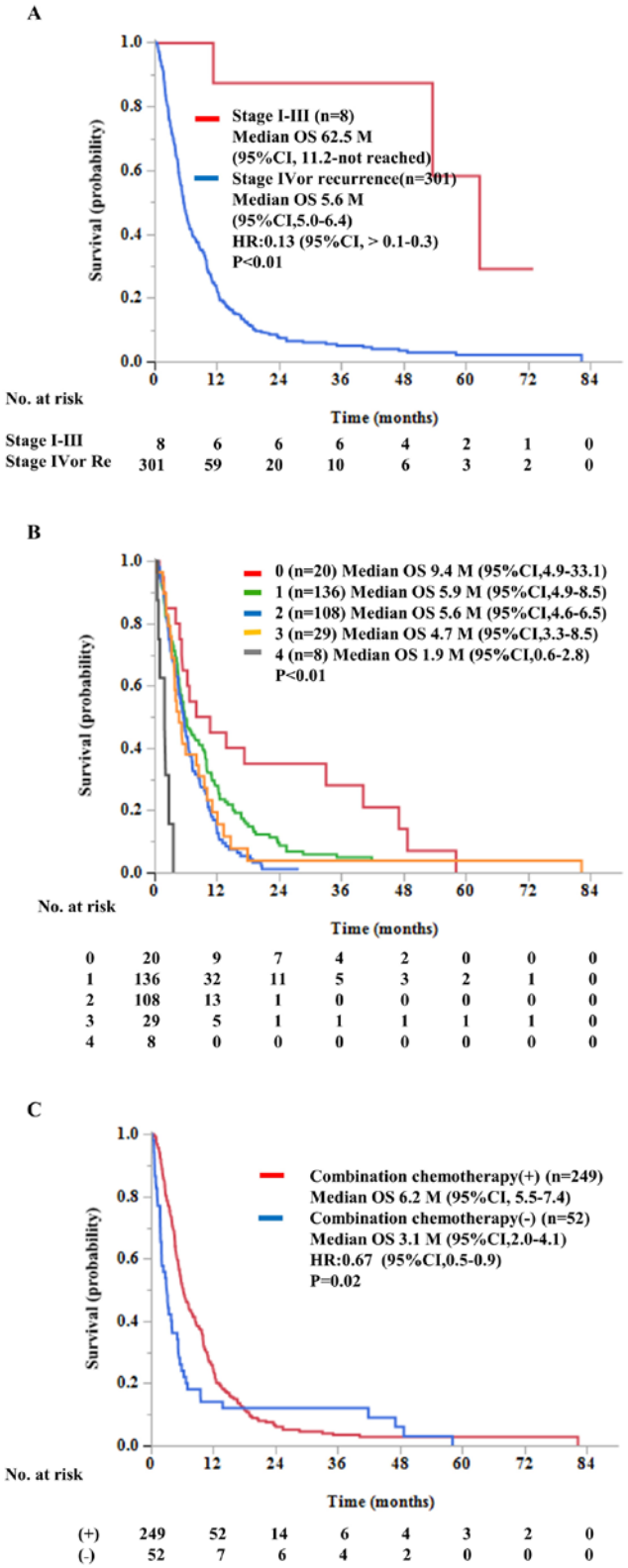

Figure 2. OS of the patients with PPV. (A) The median OS of 8 (stages I to III) or 301 patients (stage IV or recurrence) from the 1st vaccination. (B) The median OS of the 301 patients with advanced cancer with 0, 1, 2, 3, or 4 different types of chemotherapy regimens prior to the vaccination. (C) The median OS of patients with PPV with (n=249) or without (52) combined chemotherapy. OS, overall survival; PPV, personalized peptide vaccination; CI, confidence interval; HR, hazard ratio; M, month.

neutrophil or lymphocyte numbers, was a unfavorable biomarker in both the 228 patients who completed at least the 1st cycle of the vaccination and the 81 patients who failed to complete the 1st cycle. Therefore, a higher pre-vaccination CRP level might be a more useful factor associated with lower clinical benefits of PPV for pancreatic cancer. A suitable cut-off level to discriminate non-responders from responders might be <0.2 mg/dl, the baseline range at our institution, since the median OS of patients with higher CRP (≥0.2 mg/dl) (n=194) levels was significantly shorter than that of the 115 patients within the normal range (4.3 M, 95% CI: 3.7-4.9 vs. 11.1 M, 95% CI: 9.6-12.5; P<0.01, HR: 2.89) (data not shown).

Both the positive rate of antibody and the magnitude of IgG titers in pre-vaccination samples were largely different among the 31 peptides. These diversities among the peptides were also observed in the post-vaccination samples (the positive IgG responses and magnitude of IgG titers). However, it was commonly observed that the patients showing immune boosting to each of the vaccinated peptides showed longer survival than those without such boosting, suggesting that the vast majority of these 31 peptides maintained their ability to prolong clinical benefits through immune boosting. Therefore, the diversities among the peptides might not be a risk factor associated with lower clinical benefits of PPV.

IgG boosting was observed in the post-vaccination plasma from the majority of the patients who completed the 1st cycle of the vaccination irrespective of whether their PPV regimen was combined with a chemotherapy regimen. We previously reported that neither GEM nor TS-1 suppressed the PPV-induced immune boosting in patients with advanced pancreatic cancer (13,14) or other advanced cancers (17-20), respectively. The previous results showed that the rate of increase of IgG levels were higher in patients not receiving chemotherapy than in patients receiving chemotherapy. This result seems to be contradictory, but we think that these seemingly contradictory data could be at least due to that the immunity of 10 patients who declined chemotherapy was relatively kept enough to be activated by PPV, but that of 17 patients who could not tolerate chemotherapy was too suppressed or exhausted to be activated by PPV. All these results suggested that the addition of chemotherapy did not suppress PPV-induced boosting of peptide-specific IgG from the viewpoint of the positive IgG response rate or the magnitude of IgG boosting as compared to those in patients with PPV alone. This issue, however, needs to be confirmed by means of a clinical trial with a large number of patients.

Taken together, our results revealed that the following six pre-vaccination factors were associated with lower clinical benefits of PPV for pancreatic cancer patients: Higher levels of CRP, higher numbers of neutrophils, lower numbers of lymphocytes and red blood cells, advanced disease stages, and larger numbers of pre-vaccination chemotherapy regimens. The sole post-vaccination unfavorable factor was PPV monotherapy. The concomitant administration of various regimens of chemotherapy did not suppress either the PPV-induced immune boosting or clinical benefits.

The vast majority of the 309 pancreatic cancer patients enrolled in this trial were in the advanced stages, and only 8 of them (2.7%) were in the early stages and entered the PPV trial in order to prevent recurrence. Among these 8 patients, the 4 patients who underwent PPV alone were free from recurrence. The magnitude of PPV-induced IgG boosting for these 8 patients was significantly higher than that of the 301 patients

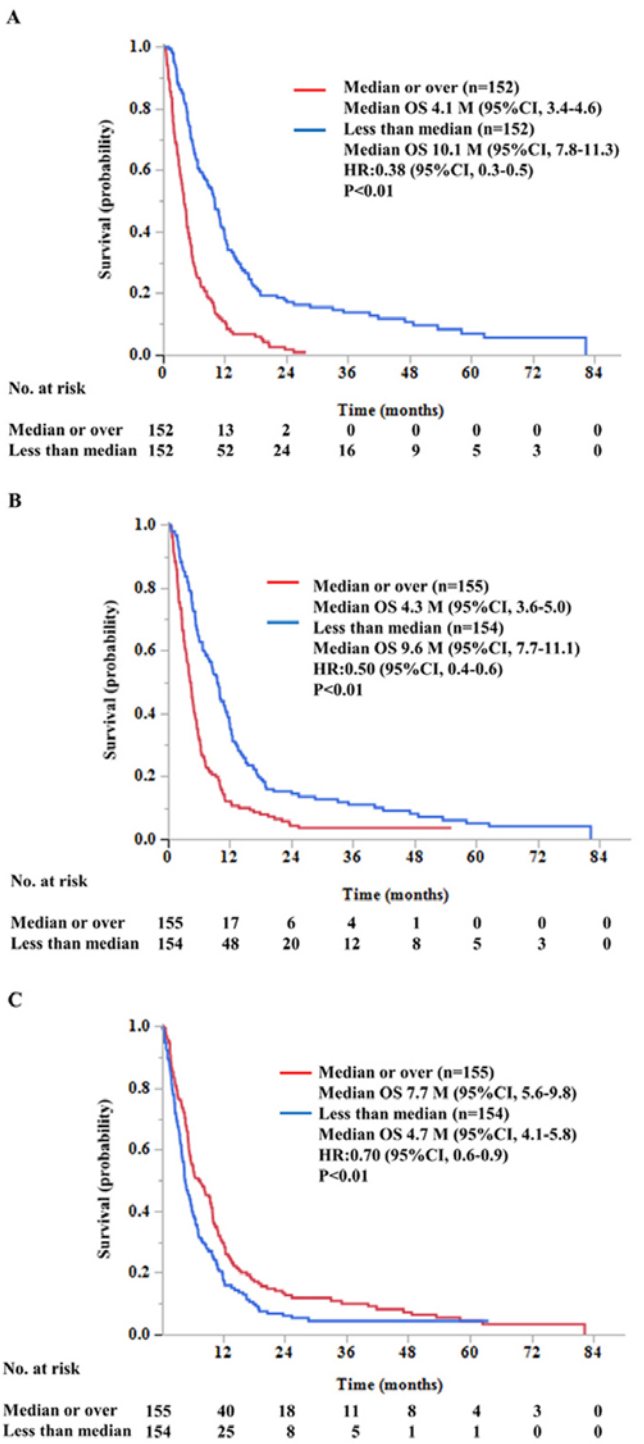

Figure 3. Correlation between the OS and pre-vaccination CRP levels, neutrophil number or lymphocyte number. The first three panels present the median OS of the patients having (A) higher (median or higher value) CRP levels, (B) higher neutrophil numbers or (C) higher lymphocyte numbers when compared with their counterparts with values below the median among the total group of 309 patients. OS, overall survival; CRP, c-reactive protein; CI, confidence interval; HR, hazard ratio; M, month.

with advanced-stage cancer (P<0.01) by Fisher's exact test (data not shown). It is generally recognized from an immunological point of view that cancer vaccines are more appropriate for prevention of recurrence than for treatment of advanced cancers. However, none of the previously conducted prophylactic cancer vaccine trials, including the MAGRIT study using

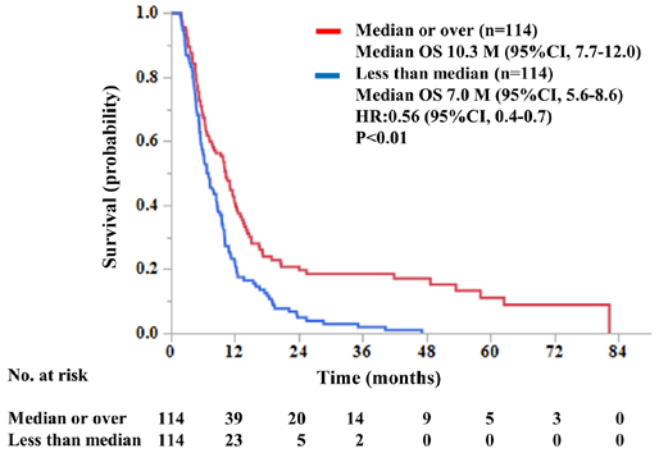

Figure 4. Correlation between the OS and the increased levels of IgG against the vaccinated peptides. The 228 patients who completed at least the first cycle of the vaccination were subdivided into 114 patients exhibiting a greater increase (median or >median level) in IgG against the vaccinated peptides. The remaining 114 patients demonstrated no increase or a lower increase (<median level). OS, overall survival; CI, confidence interval; HR, hazard ratio; M, month.

MAGE3 antigen, demonstrated clear clinical benefits (6,21,22). In addition, recent advances in surgical and chemotherapeutic approaches have led to considerable increases in survival in patients with pancreatic cancer, particularly in those with non-advanced stages (23-25). Our results also showed that PPV might be more affordable for patients with early stages of pancreatic cancer than those with advanced stages, although this issue shall be addressed in the next stage of clinical study of PPV with a large number of patients with stage I-III disease.

Our results showed that pre-vaccination inflammatory signatures, rather than post-vaccination immunological signatures, were associated with lower clinical benefits of personalized peptide vaccination (PPV) for pancreatic cancer. The major limitation of the present study, however, is the retrospective nature of the analysis of the 309 pancreatic cancer patients under PPV treatment. Thus, the present results, while informative, are far from definitive.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YU, TY, KI and SY treated the patients at the Cancer Vaccine Center, Kurume University. YU designed six studies (UMIN registration nos. 000001881, 000006297, 000006295, 000019390, 000029789 and 000011593). MY designed three

studies (UMIN registration nos. 000001482, 000006927 and 000011230). YU, DM, MU, SS, AY TS and KO acquired patient samples. YU, SS, AY, TS and SY analyzed patient data. YU, KO and KI drafted the manuscript and confirmed the authenticity of all the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All studies were conducted in an outpatient setting in accordance with the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice guidelines. Written-informed consent to participate in the clinical trial and for application of the data to research and publication purposes was obtained from all individual participants before their inclusion in the study.

Patient consent for publication

All patients consented to the publication of their research results.

Competing interests

Tetsuro Sasada received a grant from BrightPath Biotherapeutics Co. Akira Yamada is a part-time executive of BrightPath Biotherapeutics Co. Kyogo Itoh received research funding from Taiho Pharmaceutical Company. The remaining authors declare that they have no competing interests.

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