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Randomized phase II/III clinical trial of elpamotide for patients with advanced pancreatic cancer: PEGASUS-PC Study

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Key words

Advanced pancreatic cancer, elpamotide, immunotherapy, peptide vaccine, phase II/III

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Gemcitabine is a key drug for the treatment of pancreatic cancer; however, with its limitation in clinical benefits, the development of another potent therapeutic is necessary. Vascular endothelial growth factor receptor 2 is an essential target for tumor angiogenesis, and we have conducted a phase I clinical trial using gemcitabine and vascular endothelial growth factor receptor 2 peptide (elpamotide). Based on the promising results of this phase I trial, a multicenter, randomized, placebo-controlled, double-blind phase II/III clinical trial has been carried out for pancreatic cancer. The eligibility criteria included locally advanced or metastatic pancreatic cancer. Patients were assigned to either the Active group (elpamotide + gemcitabine) or Placebo group (placebo + gemcitabine) in a 2:1 ratio by the dynamic allocation method. The primary endpoint was overall survival. The Harrington–Fleming test was applied to the statistical analysis in this study to evaluate the time-lagged effect of immunotherapy appropriately. A total of 153 patients (Active group, *n* = 100; Placebo group, *n* = 53) were included in the analysis. No statistically significant differences were found between the two groups in the prolongation of overall survival (Harrington–Fleming *P*-value, 0.918; log–rank *P*-value, 0.897; hazard ratio, 0.87, 95% confidence interval [CI], 0.486–1.557). Median survival time was 8.36 months (95% CI, 7.46–10.18) for the Active group and 8.54 months (95% CI, 7.33–10.84) for the Placebo group. The toxicity observed in both groups was manageable. Combination therapy of elpamotide with gemcitabine was well tolerated. Despite the lack of benefit in overall survival, subgroup analysis suggested that the patients who experienced severe injection site reaction, such as ulceration and erosion, might have better survival.

Pancreatic cancer is the fifth leading cause of cancer mortality in Japan, with an estimated 29 916 deaths in 2012;[1] there were an estimated 35 628 deaths attributable to pancreatic cancer in the USA in 2009.[2] Prognosis remains dismal, with a 5-year survival of 7% in Japan for all five disease stages.[3]

Gemcitabine has been the standard therapy for experimental regimens in advanced pancreatic cancer for over a decade, and there has been minimal progress to improve survival rates for patients treated with a gemcitabine-based combination regimen since the late 1990s.[4–10] Overall survival has been significantly prolonged with combination therapies, such as gemcitabine plus erlotinib, a combination of oxaliplatin, irinotecan, fluorouracil, and leucovorin, and gemcitabine plus nab-paclitaxel. In these therapies, patients experienced skin rash, febrile neutropenia, and peripheral neuropathy/myelosuppression, respectively.[11–13] Because of the significant toxicities associated with these therapies, these treatments must be limited to patients with good performance status, and the regimens require close monitoring. A therapeutic program for advanced pancreatic cancer that improves survival rates without severe adverse effects is acutely necessary.

Vascular endothelial growth factor is a pro-angiogenic molecule that plays a key role in the pathogenesis of many cancers.[14] In particular, the growth of pancreatic cancer depends prominently on angiogenesis.[15] It is known that VEGF promotes tumor growth, invasion, and metastases through activation of the MAPK pathway.[16,17] In addition, VEGF and its receptors, VEGFR1 and VEGFR2, are coexpressed in pancreatic cancer, and their expression correlates with a poor prognosis,[18–22] suggesting that VEGF could have autocrine effects on microvascular endothelial

cells.[23,24] Preclinical data suggest that inhibition of VEGF attenuates pancreatic cancer growth and metastasis.[25,26] Thus, VEGF represents an attractive therapeutic target in human pancreatic cancer.

Bevacizumab is a recombinant humanized mAb that binds to VEGF-A, and blocks interaction with its receptors. Bevacizumab has been shown to improve outcomes in combination with other chemotherapies in a number of advanced malignancies,[27–32] however, its role in advanced pancreatic cancer remains controversial, and is limited to investigational use.

Elpamotide, an epitope peptide derived from the amino acid sequence of VEGFR2, has been previously characterized by induction of peptide-specific CTLs that are capable of killing VEGFR2-expressing human endothelial cells.[33] A crucial molecule associated with neovascularization, VEGFR2 is highly expressed in newly induced tumor blood vessels but not in normal blood vessels. Elpamotide is expected to exert anticancer activity through a novel mechanism of action that differs from that of bevacizumab, although both of them target tumor vascularization.

Immunotherapy is a growing field of treatment for cancer, and over 100 clinical trials have been carried out around the world for cancer vaccines. In 2009, a vaccine against prostate cancer, sipuleucel-T, was approved by the FDA following a study that showed prolonged survival results.[34] In 2010, ipilimumab, a mAb that activates the immune system by targeting CTL-associated protein 4, was also approved by the FDA.[35] These studies provided evidence that a stimulated immune response of cancer patients can have a clinically positive effect in cancer treatments, and established immunotherapy as the fourth cancer treatment method following surgery, radiotherapy, and chemotherapy. Despite these advances in immunotherapies, a peptide-based cancer vaccine has not been successfully developed to date.

A phase I study combining elpamotide with gemcitabine was carried out between 2006 and 2008 for patients in Japan with advanced pancreatic cancer.[36] The combined administration of elpamotide with gemcitabine was associated with prolonged survival time (8.7 months) compared with data from the gemcitabine monotherapy group (5.65 months) in a randomized study comparing gemcitabine with 5-fluorouracil.[37] Based on the promising results of the phase I trial, we have carried out a randomized phase II/III clinical trial using elpamotide for patients with advanced pancreatic cancer.

Materials and Methods

Study design. Patients were randomly allocated to either the Active group (elpamotide + gemcitabine) or the Placebo group (placebo + gemcitabine) at 2:1 ratio. Patients were randomly assigned by the dynamic allocation method considering disease extent (locally advanced versus metastatic disease) and institution as allocation adjustment factors. All patients received i.v. gemcitabine (1000 mg/m^2) on days 1, 8, and 15 as one cycle, which was repeated every 4 weeks. Patients in the Active group (n = 100) received a s.c. injection of emulsified elpamotide (2.0 mg/mL/body) every week; patients in the Placebo group (n = 53) received a placebo (1.0 mL/body) emulsion without elpamotide. Treatment was double-blinded, and continued until disease progression was determined by investigators.

Study drug. Elpamotide (RFVPDGNRI) is an epitope peptide derived from the amino acid sequence of VEGFR2 restricted to HLA-A*24:02. Elpamotide (1.0 mg) dissolved in 1.0 mL saline was mixed with Montanide ISA51 VG (Seppic, Paris, France) (1.0 mL) to form a water-in-oil emulsion immediately before injection. Saline was mixed with Montanide ISA51 VG to form an emulsion as a placebo. Both therapies were given s.c.

Eligibility criteria. Eligible patients were 20–80 years old, with locally advanced or metastatic pancreatic cancer that was histologically or cytologically diagnosed as adenocarcinoma or adenosquamous carcinoma, with no prior chemotherapy or radiotherapy for pancreatic cancer, and had the HLA-A*24:02 genotype. Entry criteria also included an Eastern Cooperative Oncology Group performance status of 0 or 1, a life expectancy longer than 3 months, and adequate or acceptable function of bone marrow (white blood cell count of ≥3500/mm^3, neutrophil count of ≥2000/mm^3, platelet count of ≥100 000 /mm^3, hemoglobin ≥9.0 g/dL), liver (serum bilirubin concentration of ≤2.0 mg/dL, and both aspartate aminotransferase and alanine aminotransferase levels in serum of ≤150 IU/L), and kidney (serum creatinine concentration of ≤1.5 mg/dL). Patients were excluded if they had symptomatic brain metastases, active bleeding, malignant ascites requiring drainage, or serious medical conditions such as uncontrolled hypertension, arrhythmia, or heart failure. Individuals were excluded if they had serious illness or concomitant non-malignant disease that was more than grade 3 according to the Common Terminology Criteria for Adverse Events, version 3.0, and difficult to control by medication. Patients were followed for 24 months after their enrolment.

Assessments. Physical examinations, complete blood counts, and biochemistry tests were checked weekly before treatment administration. All adverse events were assessed according to the Common Terminology Criteria for Adverse Events, version 3.0. Computed tomography and/or MRI was carried out at 4 and 8 weeks after the first dosage and every 8 weeks thereafter until disease progression. Tumor response was assessed by the Diagnostic Radiology Committee according to the Response Evaluation Criteria in Solid Tumor, version 1.1.

Cytotoxic T-lymphocyte-related gene expression. The mRNA expressions of CTL-related genes (*PRF1*, *ITGAL*, *B2M*, *ICAM1*, *LTBR*, *FAS*, *CD3E*, *CD3G*, *CD247*, *GZMB*, *FASLG*, *CD3D*, and *TRA@*) were measured before and after vaccination (days 8 and 29). The mRNA samples of PBMCs were analyzed using the MassARRAY System (Sequenom, San Diego, CA, USA) as previously described.[38] *ACTB*, *GAPDH*, *RPL13A*, *TBP*, and *YWHAZ* were evaluated as internal standard genes. The sequences of the primers are summarized in Tables S1–S3. Quantitative gene analysis was carried out using MassARRAY Quantitative Gene Expression 3.4 (Sequenom, San Diego, CA, USA).

Statistical analysis. The primary endpoint was OS, defined as the time from date of random assignment to the date of death from any cause. Secondary end-points were PFS, disease control rate, and safety. Progression-free survival was counted from the date of random assignment to the date of death without progression, or of progression as confirmed by the Diagnostic Radiology Committee. Survival estimations were carried out using the Kaplan–Meier method and the H-F test, with the weight proportional to cumulative death probability, and was used for statistical analysis of the time-lagged effect of immunotherapy. Log–rank analysis was also carried out. To evaluate the immune functions during vaccine treatment, the changes of CTL-related gene expression (days 1–8, days 8–29, and days 1–29) were compared. Relations of treatment groups and each change were evaluated using the Wilcoxon rank sum test when

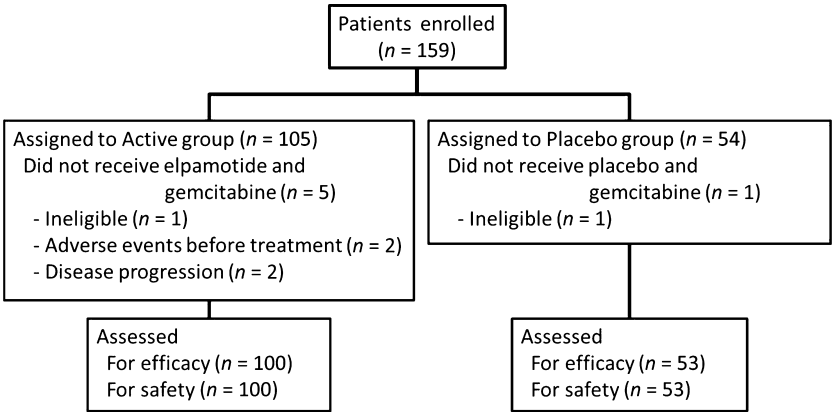

Fig. 1. Flow diagram of a phase I clinical trial of gemcitabine and elpamotide (Active group) *versus* gemcitabine and placebo (Placebo group) for treatment of pancreatic cancer.

changes were treated as continuous values. Relations between treatment groups and each change were evaluated using Fisher's exact test when changes were dichotomized into two groups at median. In addition, to find genes with different distributions in accordance with strong ISR, each distribution was compared by Wilcoxon rank sum test treating strong ISR as a group. All comparisons were undertaken by each treatment group. All statistical analyses were carried out with SAS software, version 9.1.3 (SAS Institute, Cary, NC, USA).

Sample size was estimated with the assumption that effects would be observed from the time point of 50% cumulative survival rate. Assuming a type I error α (two-sided) level of 5% and a power of 80% or more for 50–60% reduction of hazard, sample size was estimated at 100 patients for the Active group and 50 patients for the Placebo group, using the Cox proportional hazards model.

The protocol was approved by institutional review boards of all participating institutions, and the study results were validated by the Independent Data Monitoring Committee. All patients signed written informed consent before inclusion.

This study was registered with the UMIN Clinical Trials Registry before the enrolment of the first subject (registration no. UMIN000001664; URL, https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&type=summary&recptno=R000002006&language=J).

Results

Patients. One hundred and fifty-nine patients from 25 sites in Japan were randomly assigned to the Active group or the Placebo group at the ratio of 2:1 between January 2009 and January 2010. Five patients in the Active group and one patient in the Placebo group did not receive treatment due to an ineligible status determined after enrolment, such as adverse events or disease progression observed before the commencement of the study drug administration. Two patients were excluded before treatment initiation due to adverse events. One patient experienced cerebral infarction and the other experienced neutropenia. The remaining 153 patients were included in the full analysis set and used to assess treatment efficacy and safety (Fig. 1).

Table 1 shows the characteristics of the 153 enrolled and treated patients. There were no statistically significant differences between the two groups in baseline characteristics.

Study treatment. The median treatment period was 113.5 days (range, 1–715 days) for elpamotide and 113.0 days (range, 1–715 days) for gemcitabine in the Active group, and 112.0 days (range, 1–749 days) for placebo and 106.0 days (range, 1–742 days) for gemcitabine in the Placebo group. The main reason for treatment discontinuation was disease progression (79.0% in the Active group and 75.47% in the Placebo group) and the second reason was adverse effects (12.0% in the Active group and 9.43% in the Placebo group). The dose intensities were 87.31% for elpamotide and 78.07% for gemcitabine in the Active group, and 85.93% for placebo and 70.67% for gemcitabine in the Placebo group.

Efficacy. The analysis of OS was based on 135 deaths (88.2%) among the 153 patients. Figure 2(a) shows the OS of both groups. No statistically significant differences were found between the groups in the prolongation of OS (H-F *P*-value, 0.918; log–rank *P*-value, 0.897; HR, 0.87 [95% CI, 0.486–1.557]). Median survival time is 8.36 months (95%

Table 1. Clinical characteristics of pancreatic cancer patients treated with elpamotide + gemcitabine (Active group) or placebo + gemcitabine (Placebo group)

Variable	Active (n = 100)	Placebo (n = 53)	*P*-value
Age, years			
Median	63.5	65.0	0.371†
Range	38–80	36–80	
Gender, *n* (%)			
Male	62 (62.0)	31 (58.5)	0.729‡
Female	38 (38.0)	22 (41.5)	
PS (ECOG), *n* (%)			
0	76 (76.0)	36 (67.9)	0.284§
1	24 (24.0)	17 (32.1)	
Extent of disease, *n* (%)			
Locally advanced	27 (27.0)	14 (26.4)	1.000‡
Metastatic	73 (73.0)	39 (73.6)	
Tumor type, *n* (%)			
Adenocarcinoma	98 (98.0)	52 (98.1)	1.000‡
Adenosquamous carcinoma	2 (2.0)	1 (1.9)	
Pancreas excision, *n* (%)			
No	93 (93.0)	51 (96.2)	0.719‡
Yes	7 (7.0)	2 (3.8)	
Lymphocyte, *n* (%)			
<18%	32 (32.0)	17 (32.1)	1.000‡
≥18%	68 (68.0)	36 (67.9)	

†*t*-test; ‡Fisher's exact test; §Mantel test.

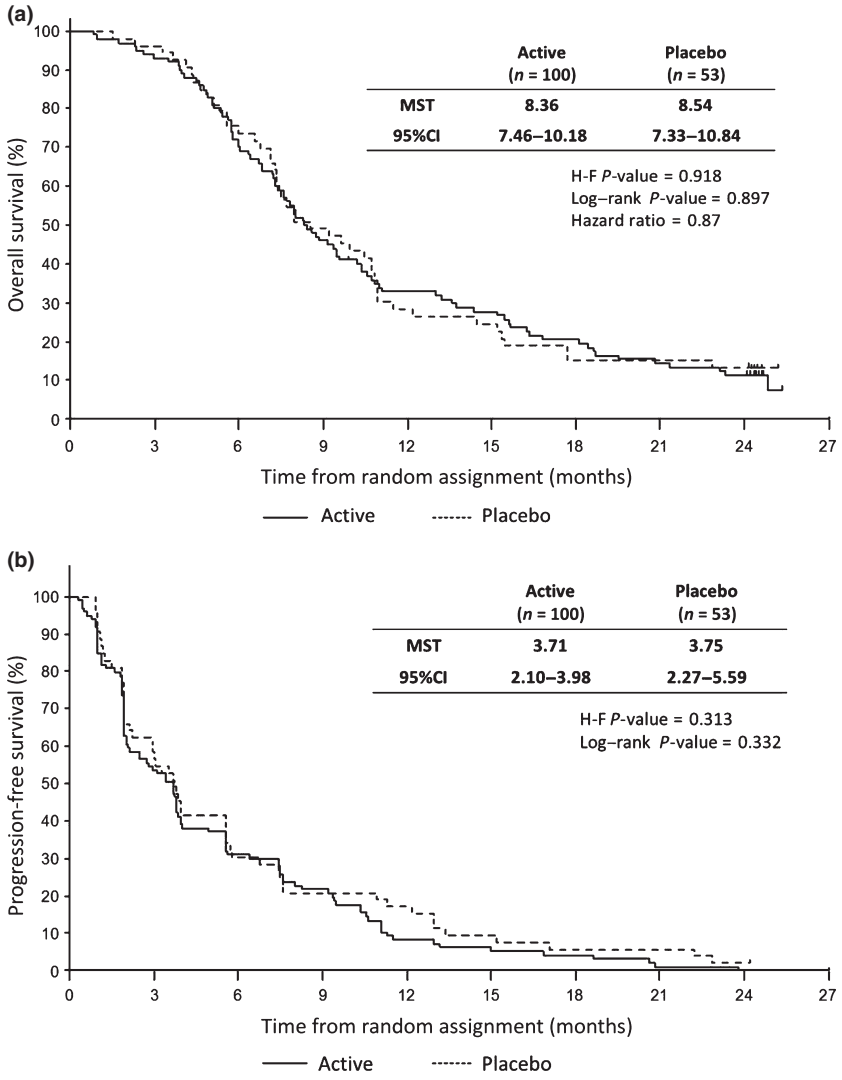

Fig. 2. Kaplan–Meier estimates of overall survival (a) and progression-free survival (b) in pancreatic cancer patients treated with gemcitabine and elpamotide (Active group) or gemcitabine and placebo (Placebo group), according to treatment group. CI, confidence interval; H-F, Harrington–Fleming test; MST, median survival time.

CI, 7.46–10.18) in the Active group, and 8.54 months (95% CI, 7.33–10.84) in the Placebo group.

The analysis of PFS was based on 150 events (98.0%) among the 153 patients. The median PFS length was 3.71 months (95% CI, 2.10–3.98) in the Active group and 3.75 months (95% CI, 2.27–5.59) in the Placebo group (Fig. 2b). There were no significant differences found between the two groups (H-F *P*- value, 0.313; log–rank *P*-value, 0.332).

The disease control rate was 59.6% (95% CI, 49.3–69.3) in the Active group and 60.4% (95% CI, 46.0–73.5) in the Placebo group.

Safety. Table 2 shows the most common adverse events related to the study drug with an incidence >30%, and the major grade 3 or worse adverse events related to the study drug. The incidence of hematologic toxicity was high, but was not significantly different between the two groups. The incidence of non-hematologic toxicity was generally mild in both groups. Patients in the Active group had slightly higher incidence of lower grade fever and increased aspartate aminotransferase (AST) compared to patients in the Placebo group. There were no deaths related to the protocol treatment.

Although an ISR was observed in both groups the higher frequency and severity were observed in the Active group compared with the Placebo group in almost symptoms (Table 3).

Subgroup analysis. Subgroup analysis according to the degree of ISR was carried out. The patient group with severe ISR (ulceration and erosion), which were observed in the Active group only, showed significantly prolonged survival compared with other groups (Fig. 3). Median survival time of patients with and without severe ISR was 15.67 months (95% CI, 7.59–24.84) and 8.28 months (95% CI, 7.26–9.59) in the Active group, and 8.54 months (95% CI, 7.33–10.84) in the Placebo group, respectively. The HR in OS of patients with severe ISR compared with patients without severe ISR in the Active group was 0.80 (95% CI, 0.39–1.64), and in the Placebo group was 0.90 (95% CI, 0.62–1.31).

Although non-severe ISR were observed in both groups, the tendency of correlation between the incidence of induration and OS was different in each group. One-year survival rate of patients with and without induration was 48.2% and 7.9% in the Active group, 25.0% and 32.0% in the Placebo group, respectively.

As ulceration, erosion, and some induration at the injection site could be considered as a kind of immune response that indicates the induction of CTL by elpamotide, we analyzed the OS in each group using strong ISR (ulceration, erosion, and induration) as a stratification factor. The one-year survival rate of patients with and without strong ISR was 46.8% and 5.9%

Table 2. Summary of adverse events (AE) related to the study drug (incidence of all AE ≥30% and major grade 3–4 AEs) in pancreatic cancer patients treated with elpamotide + gemcitabine (Active group) or placebo + gemcitabine (Placebo group)

Drug-related AE	Active (n = 100)		Placebo (n = 53)		P-value (Mantel test)
	All No. (%)	Grade 3–4 No. (%)	All No. (%)	Grade 3–4 No. (%)	
Hematologic					
Leukopenia	84 (84.0)	31 (31.0)	41 (77.4)	23 (43.4)	0.118
Thrombocytopenia	76 (76.0)	15 (15.0)	46 (86.8)	8 (15.1)	0.338
Neutropenia	74 (74.0)	48 (48.0)	42 (79.2)	30 (56.6)	0.430
Hemoglobin decreased	63 (63.0)	17 (17.0)	37 (69.8)	8 (15.1)	0.654
Hematocrit decreased	43 (43.0)	7 (7.0)	24 (45.3)	3 (5.7)	0.135
Erythropenia	43 (43.0)	7 (7.0)	24 (45.3)	3 (5.7)	0.277
Lymphopenia	41 (41.0)	25 (25.0)	24 (45.3)	13 (24.5)	0.321
Non-hematologic					
Injection site induration	62 (62.0)	2 (2.0)	28 (52.8)	0 (0.0)	0.596
Nausea	49 (49.0)	1 (1.0)	28 (52.8)	0 (0.0)	0.706
Anorexia	47 (47.0)	8 (8.0)	29 (54.7)	5 (9.4)	0.360
Injection site erythema	43 (43.0)	0 (0.0)	16 (30.2)	0 (0.0)	0.854
AST increased	33 (33.0)	3 (3.0)	11 (20.8)	0 (0.0)	0.015
Fever	31 (31.0)	0 (0.0)	11 (20.8)	0 (0.0)	0.010
ALT increased	30 (30.0)	0 (0.0)	17 (32.1)	1 (1.9)	0.413
Vomiting	28 (28.0)	1 (1.0)	16 (30.2)	2 (3.8)	0.173
Malaise	26 (26.0)	1 (1.0)	16 (30.2)	2 (3.8)	0.531
γ-GTP increased	20 (20.0)	5 (5.0)	10 (18.9)	5 (9.4)	0.380
Hypoalbuminemia	18 (18.0)	3 (3.0)	12 (22.6)	0 (0.0)	0.267
Hyponatremia	11 (11.0)	5 (5.0)	4 (7.5)	1 (1.9)	0.489
Injection site ulceration	10 (10.0)	4 (4.0)	0 (0.0)	0 (0.0)	–
Interstitial pneumonia	3 (3.0)	2 (2.0)	3 (5.7)	0 (0.0)	0.249

Grades of adverse events were defined according to the Common Terminology Criteria for Adverse Events (version 3.0). AST, aspartate aminotransferase.

in the Active group, and 25.0% and 32.0% in the Placebo group. The result of interaction analysis between the incidence of strong ISR and OS showed a significant difference between the two groups (H-F, homogeneity of X^2 across strata = 10.05, P = 0.006; log–rank, homogeneity of X^2 across strata = 9.45, P = 0.008).

Expression of CTL-related genes. To evaluate the immune functions during vaccine treatment, the expressions of CTL related genes in PBMCs were measured. Any significant increase of gene expression after vaccine treatments was not observed in elpamotide group compared to placebo group (Data not shown). However, when the relations with ISR were analyzed, *CD247* levels before treatment showed significant association with severe ISR in elpamotide group (P = 0.0213), while no related genes was associated in placebo group.

Discussion

The toxicity observed in this study was manageable. Although interstitial pneumonia was detected in three patients from each group, all patients recovered with appropriate treatment. Overall, this study showed that the combination of elpamotide and gemcitabine is safe and well tolerated in this cohort.

In this study, no statistical difference was found in the OS rate or PFS rate between the Active group and the Placebo group. However, a subgroup of patients who could benefit from the

Table 3. Injection site reaction (ISR) in pancreatic cancer patients treated with elpamotide + gemcitabine (Active group) or placebo + gemcitabine (Placebo group)

ISR	Active (n = 100)			Placebo (n = 53)		
	Grade 1 No. (%)	Grade 2 No. (%)	Grade 3 No. (%)	Grade 1 No. (%)	Grade 2 No. (%)	Grade 3 No. (%)
Erosion	0 (0.0)	2 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Erythema	34 (34.0)	9 (9.0)	0 (0.0)	13 (24.5)	3 (5.7)	0 (0.0)
Induration	53 (53.0)	7 (7.0)	2 (2.0)	24 (45.3)	4 (7.5)	0 (0.0)
Pain	17 (17.0)	5 (5.0)	0 (0.0)	4 (7.5)	0 (0.0)	0 (0.0)
Pruritus	12 (12.0)	1 (1.0)	0 (0.0)	7 (13.2)	1 (1.9)	0 (0.0)
Rash	1 (1.0)	1 (1.0)	0 (0.0)	0 (0.0)	1 (1.9)	0 (0.0)
Swelling	4 (4.0)	7 (7.0)	0 (0.0)	1 (1.9)	1 (1.9)	0 (0.0)
Ulceration	0 (0.0)	6 (6.0)	4 (4.0)	0 (0.0)	0 (0.0)	0 (0.0)

There were no ISRs graded higher than Grade 3.

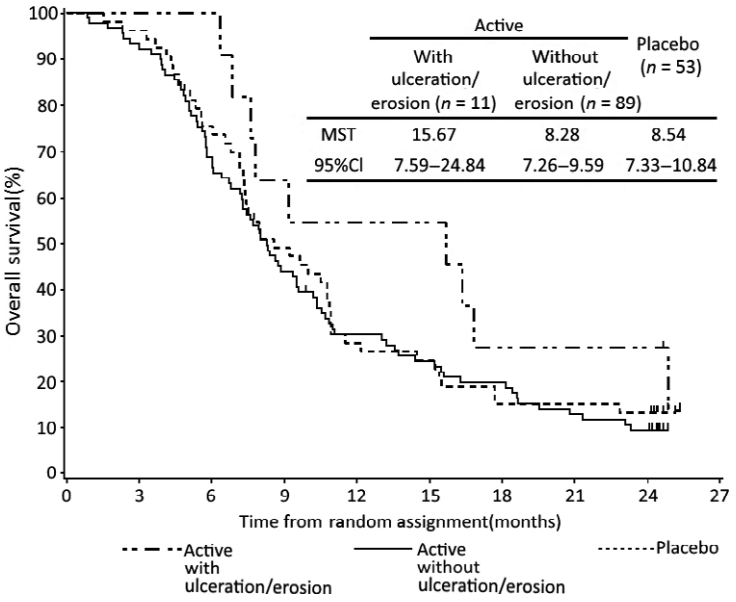

Fig. 3. Kaplan–Meier estimates of overall survival in patients with pancreatic cancer treated with gemcitabine and elpamotide (Active group), according to ulceration at the injection site. CI, confidence interval; H-F, Harrington–Fleming test; MST, median survival time.

peptide vaccine would be identified according to the biomarkers, as the clinical efficacy of peptide vaccines depends on the immunological response in the individual patients.

Past clinical trials of peptide vaccines with single-arm treatment indicated better clinical outcomes in patients who showed strong ISR, which suggested that ISR would be the indicator of immune response induced by peptide vaccine.[39] Montanide is known to cause ISR by itself. Our non-clinical study in rabbits showed ISR in both the Active group and Placebo group (OncoTherapy Science Inc., unpublished data). In this study, although ISR was observed in both groups, it was more frequent and severe in the Active group compared with the Placebo group, suggesting that the strong immune response induced by elpamotide caused strong ISR in addition to that developed by Montanide. Strong ISR, such as ulceration and erosion, were observed in the Active group only. These facts suggested that strong ISR would be a good indicator for immunological response and a stratification factor for clinical outcome.

Vascular endothelial growth factor receptor 2 is highly expressed in endothelial cells of new vessels and plays an important role in wound healing.[40,41] Impaired wound healing is often observed in patients treated by anti-angiogenic therapies targeting VEGFR2. It can be hypothesized that severe ISR observed in the Active group of patients could have been caused by the delayed recovery from ISR induced by Montanide as a result of targeting VEGFR2.[42–44]

We therefore focused on severe ISR as an indicator of immunological response and undertook a *post hoc* analysis using severe ISR as a stratification factor.

As shown in Figure 3, prolonged survival was observed in the subgroup with severe ISR compared with those who did not show severe ISR among the Active group. Median survival of the patients without severe ISR in the Active group was comparable with that of the Placebo group. This suggests that the patients with severe ISR benefited from treatment with elpamotide whereas the patients without severe ISR did not. Severe ISR may be considered just a result of frequent vaccinations, which indicates that the patients survived long enough to receive vaccinations many times. However, the duration from beginning of vaccination to the occurrence of ISR is approximately the median of the treatment period, regardless of the treatment period, indicating that the length of treatment period is not a main cause of severe ISR.

Therefore, we hypothesized that severe ISR, such as ulceration and erosion, and strong ISR, such as induration at the injection site, could be the indicators for immune response induced by elpamotide, and analyzed survival in each group using these factors for stratification. As no severe ISR developed in the Placebo group, only induration was applied for the analysis of this group.

Comparison of 1-year survival rate between the subgroup with severe/strong ISR and without severe/strong ISR within the same treatment group produced totally different results between the Active group and the Placebo group. Patients with strong or more severe ISR showed prolonged survival compared with those without strong ISR in the Active group, however, there was no difference in the Placebo group.

In this study, we did not evaluate the CTL induction by enzyme-linked immunospot assay or ELISA for γ-interferon detection. In addition, significant increase of CTL-related gene expression in PBMCs after vaccine treatment was not observed. Therefore the verification of the interaction between ISR and CTL induction is difficult at this time. However, our analysis showed high expression level of *CD247,* which encodes the CD3zeta chain, before vaccination was significantly associated with severe ISR in the elpamotide group. Many past reports indicated that the CD3zeta chain is an important molecule for antitumor immunity and downregulation of its expression level was observed in cancer patients.[45,46] These results may support our hypothesis that strong ISR is an indicator for immune response induced by elpamotide because these results probably indicate that patients with severe ISR had a better background for induction of CTLs.

Taken together, these subgroup analyses suggested that elpamotide could be effective for patients who show a strong immunological response, that is, severe or strong ISR after treatment with peptide vaccine. However, the number of patients who showed strong or severe ISR was not large. This could be counted as a cause of statistical insignificance in the OS between the Active group and the Placebo group in this study.

One possibility to overcome this limit would be a cocktail of peptides that induces the immunological response against multiple molecules.

A previous clinical study using a cocktail of peptides, in which the peptides were derived from the amino acid sequences of VEGFR1 and VEGFR2 and three kinds of peptides derived from different tumor-specific antigens, were administered to patients with metastatic colon cancer. It was reported that induction of CTLs against at least one peptide was observed in all patients. In addition, the survival time was significantly longer ($P = 0.027$) in patients with CTLs induced against three or more peptides, compared with the patients with fewer kinds of CTLs.[47] These results indicated that the combination of multiple peptides in a therapy could adapt with the tumor heterogeneity, thus be more effective than a monopeptide cancer vaccine.

Predictive biomarkers to define the patients who could benefit from peptide vaccine prior to treatment and the application of combination therapy with immune checkpoint inhibitors should also be considered to improve the efficacy of peptide vaccine.

Moreover, one should pay attention to the negative result of bevacizumab and gemcitabine (the CALGB80303 trial), a randomized, phase III study in advanced pancreatic cancer that showed no difference in OS between the two treatment arms.[48] Thus, the future perspective could be suggested as follows. First, a combination of targets in angiogenesis including VEGFR1, VEGFR2, and/or other angiogenic factors, including fibroblast growth factor 1, is essential for improving the clinical benefits as this process has multiple stakeholders. Second, one should consider anti-angiogenesis vaccination and antitumor vaccination using tumor-specific antigens with standard chemotherapy. Finally, a change in the target population should be considered to those who may have the potential for a better response, such as pretreatment elevation of CD247, or intervention to increase CD247 before vaccination.

The subgroup analysis according to severe/strong ISR suggests important concepts for future trials. Delayed type hypersensitivity testing using small amounts of active peptide vaccine should be added for future trials. There is a possibility that patients without severe ISR also induced VEGFR2-specific CTLs without any survival benefit. The concept of antitumor strategies other than the VEGFR2 pathway might also be important to suppress tumor growth.

Although the results of this study did not meet the primary endpoint of OS, this study has confirmed the potential of peptide-based cancer vaccines. Subgroup analyses strongly suggested that patients with a strong immunological response might benefit from peptide vaccine treatment. A phase III clinical trial using a cocktail of peptides against tumor-specific molecules and neo-vessel markers in patients with pancreatic cancer after gemcitabine failure has already been finished and its results are being analyzed.

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Disclosure Statement

Y. Ohashi has served as a consultant for OncoTherapy Science, Inc. T. Tsunoda, who is a part-time lecturer at the School of Medicine, Wakayama Medical University, holds stock in OncoTherapy Science, Inc. The other authors have no conflicts of interest.

Abbreviations

CI	confidence interval
H-F	Harrington–Fleming test
HLA	human leukocyte antigen
HR	hazard ratio
ISR	injection site reaction
OS	overall survival
PFS	progression-free survival
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Polymerase chain reaction primers used in this study.

Table S2. Extended primers used in this study.

Table S3. Competitive primers used in this study.