Personalized peptide vaccination for advanced biliary tract cancer: IL-6, nutritional status and pre-existing antigen-specific immunity as possible biomarkers for patient prognosis

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Abstract. Considering that the prognosis of patients with advanced biliary tract cancer (BTC) remains very poor, with a median survival of less than 1 year, new therapeutic approaches need to be developed. In the present study, a phase II clinical trial of personalized peptide vaccination (PPV) was conducted in advanced BTC patients to evaluate the feasibility of this treatment and to identify potential biomarkers. A maximum of 4 human leukocyte antigen-matched peptides, which were selected based on the pre-existing host immunity prior to vaccination, were subcutaneously administered (weekly for 6 consecutive weeks and bi-weekly thereafter) to 25 advanced BTC patients without severe adverse events. Humoral and/or T cell responses specific to the vaccine antigens were substantially induced in a subset of the vaccinated patients. As shown by multivariate Cox regression analysis, lower interleukin-6 (IL-6) and higher albumin levels prior to vaccination and greater numbers of selected vaccine peptides were significantly favorable factors for overall survival [hazard ratio (HR)=1.123, 95% confidence interval (CI) 1.008-1.252, P=0.035; HR=0.158, 95% CI 0.029-0.860, P=0.033; HR=0.258, 95% CI 0.098-0.682, P=0.006; respectively]. Based on the safety profile and substantial immune responses to vaccine antigens, PPV could be a promising approach for refractory BTC, although its clinical efficacy remains to be investigated in larger-scale prospective studies. The identified biomarkers are potentially useful for selecting BTC patients who would benefit from PPV.

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Introduction

Biliary tract cancer (BTC) is one of the most aggressive types of cancer and has a very poor prognosis (1,2). Only 10% of newly diagnosed patients present with early-stage disease, which may be treated by a potentially radical excision of the tumor, and the remaining patients have unresectable disease with locally advanced and/or metastatic tumors. Recently, there have been substantial advances in treatment modalities, including systemic chemotherapies, for advanced BTC (1-4). For example, a randomized trial has suggested that cisplatin plus gemcitabine could be considered as a standard treatment option for patients with advanced BTC (3). In addition, a number of different targeted therapies for BTC have also been under investigation (1-4). Despite this progress, however, the prognosis of BTC patients remains very poor, with a median survival of less than 1 year. Therefore, further novel therapeutic approaches need to be developed.

We previously devised a new regime of peptide-based vaccination, known as 'personalized peptide vaccination (PPV)', in which vaccine antigens are selected and administered based on the pre-existing host immunity prior to vaccination (5-7). We reported favorable clinical and/or immune responses of this novel vaccination in various types of advanced cancer, including pancreatic, gastric, colorectal and prostate cancer, and glioblastoma (8-12). For example, a recently conducted randomized clinical trial of PPV for advanced prostate cancer patients showed a promising clinical outcome in the vaccinated group (11). In the present study, we addressed the feasibility of using PPV in advanced BTC patients in a small-scale phase II study. In addition, we identified potential biomarkers for predicting overall survival (OS) and selecting suitable patients for this treatment.

Patients and methods

Patients. Patients were eligible for inclusion in the present study if they had a histological diagnosis of BTC and showed positive humoral responses to at least two of the 31 different vaccine candidate peptides (Table I). Other inclusion criteria

Table I. Peptide candidates for cancer vaccination.

Symbol for peptide	Origin protein	Position of peptide	Amino acid sequence	HLA type
CypB-129	Cyclophilin B	129-138	KLKHYGPGWV	A2, A3sup[a]
Lck-246	p56 lck	246-254	KLVERLGAA	A2
Lck-422	p56 lck	422-430	DVWSFGILL	A2, A3sup
MAP-432	ppMAPkkk	432-440	DLLSHAFFA	A2, A26
WHSC2-103	WHSC2	103-111	ASLDSDPWV	A2, A3sup[a], A26
HNRPL-501	HNRPL	501-510	NVLHFFNAPL	A2, A26
UBE-43	UBE2V	43-51	RLQEWCSVI	A2
UBE-85	UBE2V	85-93	LIADFLSGL	A2
WHSC2-141	WHSC2	141-149	ILGELREKV	A2
HNRPL-140	HNRPL	140-148	ALVEFEDVL	A2
SART3-302	SART3	302-310	LLQAEAPRL	A2
SART3-309	SART3	309-317	RLAEYQAYI	A2
SART2-93	SART2	93-101	DYSARWNEI	A24
SART3-109	SART3	109-118	VYDYNCHVDL	A24, A3sup[a], A26
Lck-208	p56 lck	208-216	HYTNASDGL	A24
PAP-213	PAP	213-221	LYCESVHNF	A24
PSA-248	PSA	248-257	HYRKWIKDTI	A24
EGFR-800	EGF-R	800-809	DYVREHKDNI	A24
MRP3-503	MRP3	503-511	LYAWEPSFL	A24
MRP3-1293	MRP3	1293-1302	NYSVRYRPGL	A24
SART2-161	SART2	161-169	AYDFLYNYL	A24
Lck-486	p56 lck	486-494	TFDYLRSVL	A24
Lck-488	p56 lck	488-497	DYLRSVLEDF	A24
PSMA-624	PSMA	624-632	TYSVSFDSL	A24
EZH2-735	EZH2	735-743	KYVGIEREM	A24
PTHrP-102	PTHrP	102-111	RYLTQETNKV	A24
SART3-511	SART3	511-519	WLEYYNLER	A3sup[a]
SART3-734	SART3	734-742	QIRPIFSNR	A3sup[a]
Lck-90	p56 lck	90-99	ILEQSGEWWK	A3sup[a]
Lck-449	p56 lck	449-458	VIQNLERGYR	A3sup[a]
PAP-248	PAP	248-257	GIHKQKEKSR	A3sup[a]

[a]A3sup, HLA-A3 supertype (A3, A11, A31 and A33). HLA, human leukocyte antigen.

were as follows: age between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; positive status for human leukocyte antigen (HLA)-A2, -A24, -A3 supertype (A3, A11, A31 or A33), or -A26; life expectancy of at least 12 weeks; negative status for hepatitis B and C virus; and adequate hematological, hepatic and renal function. Exclusion criteria included pulmonary, cardiac or other systemic diseases; an acute infection; a history of severe allergic reactions; pregnancy or nursing; and other inappropriate conditions for enrollment as judged by clinicians. The protocol was approved by the Kurume University Ethics Committee, and was registered in the UMIN Clinical Trials Registry (UMIN 2907). Following a full explanation of the protocol, written informed consent was obtained from all patients prior to enrollment.

Clinical protocol. This was an open-label phase II study, in which the primary and secondary end-points were to identify biomarkers for OS and to evaluate the safety of PPV in BTC patients, respectively. In this study, 31 peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies (6-12), were employed for vaccination [12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for the HLA-A3 supertype (A3, A11, A31 or A33) and 4 peptides for HLA-A26] (Table I). The peptides were prepared under the conditions of Good Manufacturing Practice (GMP) by the PolyPeptide Laboratories (San Diego, CA, USA) and the American Peptide Company (Vista, CA, USA). The right peptides for vaccination to individual patients were selected, taking into consideration the pre-existing host immunity prior to vaccination, assessed by titers of IgG specific to each of the 31 different vaccine candidates, as reported previously (6-12). A maximum of 4 peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, were subcutaneously administered with incomplete Freund's adjuvant (Montanide ISA51; Seppic,

Paris, France) once a week for 6 consecutive weeks. After the first cycle of 6 vaccinations, up to 4 antigen peptides, which were re-selected according to the titers of peptide-specific IgG at every cycle of 6 vaccinations, were administered every 2 weeks. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (NCI-CTC Ver. 3.0). Complete blood counts and serum biochemistry tests were performed after every 6 vaccinations. The clinical responses were evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST) in the vaccinated patients, whose radiological findings by computed tomography (CT) scan or magnetic resonance imaging (MRI) were available prior to and following vaccinations.

Measurement of humoral and T cell responses specific to the vaccine peptides. The humoral responses specific to the vaccine peptides were determined by peptide-specific IgG titers using a bead-based multiplex assay with the Luminex 200 system (Luminex, Austin, TX, USA), as reported previously (13). If peptide-specific IgG titers to at least one of the vaccine peptides in the post-vaccination plasma were more than 2-fold higher than those in the pre-vaccination plasma, the changes were considered to be significant.

T cell responses specific to the vaccine peptides were evaluated by interferon (IFN)-γ ELISPOT assay (MBL, Nagoya, Japan) using peripheral blood mononuclear cells (PBMCs). Briefly, PBMCs ($2.5x10^4$ cells/well) were incubated in 384-well microculture plates (Iwaki, Tokyo, Japan) with 25 μl of medium (OpTmizer™ T Cell Expansion SFM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS (MP Biologicals, Solon, OH, USA), recombinant human interleukin (IL)-2 (20 IU/ml; Serotec, Oxford, UK) and 10 μM of each peptide. Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) after 3 days of culture. After incubation for the following 6 days, the cells were harvested and tested for their ability to produce IFN-γ in response to either the corresponding peptides or a negative control peptide from human immunodeficiency virus (HIV). Antigen-specific IFN-γ secretion after an 18-h incubation was determined by ELISPOT assay with the Zeiss ELISPOT reader (Carl Zeiss MicroImaging Japan, Tokyo, Japan). Antigen-specific T cell responses were evaluated by the difference between the spot numbers (mean of duplicate samples) in response to the corresponding peptides and those in response to the control peptide. The differences of at least 10 spot numbers per 10^5 PBMCs were considered significant. If the spot numbers in response to at least one of the vaccine peptides in the post-vaccination PBMCs were more than 2-fold higher than those in the pre-vaccination PBMCs, the changes were considered significant.

Measurement of C-reactive protein (CRP), serum amyloid A (SAA) and cytokines. The levels of CRP, SAA and IL-6 in the plasma were examined by ELISA using kits from R&D Systems (Minneapolis, MN, USA), Invitrogen and eBioscience (San Diego, CA, USA), respectively. Bead-based multiplex assays were used to measure Th1/Th2 cytokines, including IL-2, IL-4, IL-5 and IFN-γ (Invitrogen) with the Luminex 200 system. Frozen plasma samples were thawed, diluted and assayed in duplicate in accordance with the manufacturer's instructions. The mean of duplicate samples was used for statistical analysis.

Flow cytometric analysis of suppressive immune subsets in PBMCs. Suppressive immune subsets, myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Treg) in PBMCs were examined by flow cytometry. For analysis of MDSCs, PBMCs ($0.5x10^6$ cells) were stained with the following monoclonal antibodies for 30 min at 4˚C: anti-CD3-FITC, anti-CD56-FITC, anti-CD19-FITC, anti-CD33-APC, anti-HLA-DR-PE/Cy7 and anti-CD14-APC/Cy7 (all from Biolegend, San Diego, CA, USA). In the cell subpopulation negative for the lineage markers (CD3, CD19, CD56 and CD14) and HLA-DR, MDSCs were identified as positive for CD33. The frequency of MDSCs in the mononuclear cell gate defined by the forward scatter and side scatter was calculated. For analysis of Treg, PBMCs ($1x10^6$ cells) were stained with the cocktail of anti-CD4-FITC and anti-CD25-APC, and subsequently with anti-Foxp3-PE following fixation and permeabilization, according to the manufacturer's instructions (eBioscience). The frequency of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ cells was calculated. The samples were run on a FACSCanto II (BD Biosciences, San Diego, CA, USA), and data were analyzed using the Diva software (BD Biosciences).

Statistical methods. The two-sided Wilcoxon test was used to compare differences between pre- and post-vaccination measurements. OS time was calculated from the first day of peptide vaccination until the date of mortality or the last date when the patient was known to be alive. Predictive factors for OS were evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model. P-values <0.05 were considered to indicate a statistically significant difference. All the statistical analyses were conducted using the SAS version 9.1 software (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. Between November 2008 and December 2010, 25 BTC patients were enrolled in the present study. Table II shows the clinicopathological characteristics of the enrolled patients. There were 18 male and 7 female subjects, with a median age of 59 years, ranging from 37 to 79 years. Primary sites of BTC were 7 gallbladder carcinomas, 11 extrahepatic and 6 intrahepatic cholangiocarcinomas, and 1 periampullary carcinoma. All the patients had advanced-stage cancer (stage IVa, n=5; stage IVb, n=9; recurrent, n=11). Prior to enrollment, 22 patients failed to respond to 1 (n=13) or 2 (n=9) regimen(s) of chemotherapy, whereas the remaining 3 patients did not tolerate chemotherapy due to adverse events. The median duration of chemotherapy prior to the PPV was 4 months, ranging from 2 to 27 months. The performance status at the time of enrollment was grade 0 (n=20) or grade 1 (n=5). The numbers of peptides vaccinated to the patients at the first cycle of vaccination were 4 peptides in 19 patients, 3 in 5 patients and 2 in 1 patient. The median number of vaccinations was 10, with a range of 2 to 24. During

Table II. Characteristics of the enrolled patients.

Patient no.	Gender	Age (years)	PS	Disease type	Stage	Previous treatment (months)[a]	No. of vaccinations	Clinical response	OS (days)
1	M	59	0	ICC	R	GEM + S-1 (2)	18	SD	463
2	F	71	1	GBC	IVb	-	2	NA	57
3	F	59	1	GBC	IVb	GEM→GEM + CDDP (8)	4	NA	35
4	M	57	0	ECC	IVb	GEM + S-1 (3)	7	NA	116
5	M	75	0	GBC	IVb	GEM→GEM + S-1 (2)	5	NA	122
6	M	55	0	PAC	R	S-1→GEM (12)	14	SD	234
7	M	65	0	ECC	R	GEM→GEM + S-1 (4)	6	NA	102
8	M	73	1	ECC	R	GEM→S-1 (27)	3	NA	51
9	F	37	1	ECC	IVb	GEM + UFT→S-1 (7)	3	NA	48
10	F	69	0	ECC	R	GEM→S-1 (12)	24[b]	SD	455[c]
11	M	62	0	ECC	IVa	GEM→S-1 (6)	8	NA	177
12	M	49	0	GBC	R	GEM (6)	7	NA	111
13	F	56	0	ICC	R	-	16	SD	222
14	M	62	0	ECC	R	GEM + S-1(5)	12	PD	286
15	M	53	0	ICC	IVb	GEM (3)	6	SD	84
16	M	75	0	GBC	R	S-1 (2)	6	NA	292
17	M	79	0	ECC	IVb	S-1 (2)	12	NA	355[c]
18	M	59	0	ECC	IVb	GEM (2)	13	NA	207
19	F	56	0	GBC	IVb	GEM (2)	7	NA	92
20	M	71	0	ECC	R	GEM + S-1 (12)	11	NA	163[c]
21	M	51	0	ICC	R	GEM + S-1 (2)	12	SD	179[c]
22	M	66	0	ECC	IVa	GEM (3)	17[b]	SD	179[c]
23	M	52	1	ICC	IVa	5FU + CDDP→GEM + S-1 (14)	10	NA	101
24	M	41	0	ICC	IVa	GEM (4)	19[b]	PD	428[c]
25	F	48	0	GBC	IVa	-	14[b]	SD	125[c]

[a]Duration of previous chemotherapy; [b]under treatment; [c]patients alive. M, male; F, female; PS, performance status; ICC, intrahepatic cholangiocarcionma; ECC, extrahepatic cholangiocarcinoma; GBC, gallbladder carcinoma; PAC, periampullary carcinoma; R, recurrent; GEM, gemcitabine; CDDP, cisplatin; UFT, tegafur-uracil; SD, stable disease; PD, progressive disease; OS, overall survival; NA, not assessed.

the PPV, 20 of 25 patients were treated in combination with chemotherapy, but the remaining 5 patients did not tolerate combined chemotherapy (patients 2, 9, 12, 13 and 25).

Of the 10 vaccinated patients whose radiological findings were available prior to and following the first cycle of vaccination, none had a complete response (CR) or partial response (PR). The best response was stable disease (SD) in 8 (80%) patients. The remaining 2 patients (20%) had progressive disease (PD) (Table II).

Toxicities. The overall toxicities are shown in Table III. The most frequent adverse events were dermatological reactions at the injection sites (n=17), hematological toxicity (n=14) and cholangitis (n=11). Severe adverse events (grade 3) were as follows: injection site reaction (n=1), gastrointestinal hemorrhage (n=2), gastrointestinal stricture (n=1), cholangitis (n=11), anemia (n=1), hyperbilirubinemia (n=1) and elevation of ALT (n=1) and ALP (n=1). According to an assessment by the independent safety evaluation committee in this trial, all of these severe adverse events, except for 1 case with a grade 3 injection site reaction, were due to cancer progression or other causes, rather than to the vaccinations themselves.

Immune responses to the vaccine peptides. Both humoral and T cell responses specific to the vaccine peptides were analyzed in blood samples prior to and following vaccination (data not shown). Plasma samples were obtained from 25, 20 and 8 patients before and at the end of the first (6th vaccination) and second (12th vaccination) cycles of vaccination, respectively. The post-vaccination samples were not available in the patients who failed to complete the first or second cycle of 6 vaccinations due to disease progression. The IgG responses specific to at least one of the vaccine peptides were augmented in 7 of 20 patients (35%) and in 7 of 8 patients (88%) at the end of the first and second cycles of vaccination, respectively.

T cell responses to the vaccine peptides were measured by IFN-γ ELISPOT assay with PBMCs. PBMCs were available for this assay in 22, 17 and 7 patients prior to and at the end of the first and second cycle of vaccination, respectively. In the pre-vaccination samples, antigen-specific T cell responses were detectable in 5 patients (23%). Of the 17 patients who completed the first cycle of vaccination, 8 patients (47%) showed an induction of T cell responses to the vaccine peptides. At the end of the second cycle of vaccination, the antigen-specific T cell responses were induced in 4 of 7 patients (57%). It

Table III. Toxicities.

	Grade 1	Grade 2	Grade 3	Total
Injection site reaction	11	5	1	17
Gastrointestinal (GI)				
GI hemorrhage	0	0	2	2
GI stricture	0	0	1	1
Abdominal distension	0	1	0	1
Constipation	0	1	0	1
Ascites	1	0	0	1
Hepatobiliary				
Cholangitis	0	0	11	11
Pulmonary				
Pleural effusion	1	0	0	1
Cardiac general				
Hypertension	0	1	0	1
Blood/bone marrow				
Anemia	9	1	1	11
Leukocytopenia	1	0	0	1
Lymphopenia	2	0	0	2
Laboratory				
Hyperbilirubinemia	1	0	1	2
AST elevation	4	1	0	5
ALT elevation	1	1	1	3
ALP elevation	3	2	1	6
Hypoalbuminemia	4	3	0	7
Hyperglycemia	0	3	0	3
Hyponatremia	1	0	0	1
Hypokalemia	0	1	0	1
Hypercalcemia	1	1	0	2
Creatinine elevation	1	0	0	1

should be noted that 3 of the 4 patients with positive T cell responses at the end of the second cycle of vaccination showed reactivity to more than 2 peptides. Collectively, substantial increases in peptide-specific IgG titers and/or T cell responses following vaccination were observed in a subset of the vaccinated patients.

Cytokines and inflammation markers. We then measured several cytokines, including IL-2, IL-4, IL-5, IL-6, IFN-γ and the inflammation markers, CRP and SSA, in the plasma prior to and following the first cycle of vaccination. IL-6 was detectable in 17 of 25 patients (68%) prior to vaccination (median, 2 pg/ml; range, 0-21). Among the 20 plasma samples available at the end of the first cycle of vaccination, IL-6 levels were increased, decreased or unchanged in 12, 5 or 3 patients, respectively (median 3 pg/ml; range 0-43). There was no significant difference in the levels of IL-6 between pre- and post-vaccination samples (P=0.118, Wilcoxon test). Other cytokines, including IL-2, IL-4, IL-5 and IFN-γ, were rarely detectable in either pre- or post-vaccination plasma (data not shown).

The inflammation marker, CRP, was detectable in pre-vaccination plasma from all (100%) of the patients (median, 6.377 μg/ml; range, 0.043-8.891). Among the 20 plasma samples tested at the end of the first cycle of vaccination, plasma CRP levels were increased or decreased in 12 or 8 patients, respectively (median, 6.232 μg/ml; range, 1.331-17.332). Another inflammation marker, SAA, was also detected in pre-vaccination plasma from 21 (84%) of 25 patients (median, 113.486 μg/ml; range, 0-134.425). At the end of the first cycle of vaccination, plasma SAA levels were increased, decreased or unchanged in 12, 7 or 1 patients, respectively (median, 104.861 μg/ml; range, 0-138.917). There were no significant differences in the levels of CRP and SAA between pre- and post-vaccination samples (P=0.290 and P=0.252, respectively, Wilcoxon test).

Relationship between pre-vaccination clinical findings or laboratory data and OS. To identify potential biomarkers useful for selecting suitable patients for PPV, a Cox proportional hazards regression model was used with pre-vaccination clinical findings or laboratory data (Table IV). In the univariate analysis, IL-6, CRP, albumin, SAA and hemoglobin in pre-vaccination samples (P=0.002, P=0.004, P=0.008, P=0.031 and P=0.039, respectively), and the numbers of peptides selected for vaccination (P=0.039) were prognostic factors of OS. None of the other factors examined, such as age, gender, duration of previous chemotherapy, lymphocyte counts or frequencies of suppressive immune cell subsets (Treg and MDSCs) prior to vaccination, were statistically correlated with OS. Furthermore, multivariate Cox regression analysis was performed to define the clinical and laboratory features that were independently associated with OS by adjusting for possible confounding factors. Only the factors with a prognostic association in the univariate analysis, including IL-6, CRP, albumin, hemoglobin and the numbers of peptides selected for vaccination, were used for the multivariate analysis. SAA was not included for this analysis, since the levels of SAA were highly correlated with those of CRP (Pearson's correlation co-efficient 0.707; P=0.0002). As shown in Table IV, lower IL-6 and higher albumin levels in pre-vaccination samples and greater numbers of antigen peptides selected for vaccination were significantly favorable factors for OS [hazard ratio (HR) = 1.123, 95% confidence interval (CI) 1.008-1.252, P=0.035; HR=0.158, 95% CI 0.029-0.860, P=0.033; HR=0.258, 95% CI 0.098-0.682, P=0.006; respectively]. However, the other factors had no significant association.

Relationship between post-vaccination clinical findings or laboratory data and OS. To further identify potential post-vaccination markers for predicting patient prognosis, the univariate and multivariate Cox analyses were also carried out with post-vaccination clinical findings or laboratory data from the patients who completed the first cycle of 6 vaccinations (n=20). In the univariate analysis, levels of albumin, IL-6, CRP and hemoglobin (P=0.003, P=0.005, P=0.027 and P=0.031, respectively) and the number of vaccine peptides (P=0.033) were prognostic of OS. In addition, although not statistically significant, positive humoral responses to the vaccine peptides had a tendency to be associated with OS (P=0.089) and were also used for the multivariate Cox analysis. The multivariate analysis demonstrated that, among these factors with a potentially prognostic association in the univariate analysis, lower IL-6 levels and greater numbers of vaccine

Table IV. Univariate and multivariate analyses with pre-vaccination clinical findings and laboratory data.

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% CI)	P-value[a]	Hazard ratio (95% CI)	P-value[a]
Age	0.986 (0.944-1.030)	0.523		
Gender	1.673 (0.586-4.776)	0.336		
Duration of previous chemotherapy (months)	1.056 (0.965-1.154)	0.235		
Lymphocyte count (x10^3/mm^3)	0.639 (0.202-2.023)	0.446		
Hemoglobin (g/dl)	0.618 (0.392-0.976)	0.039		
Albumin (g/dl)	0.158 (0.041-0.616)	0.008	0.158 (0.029-0.860)	0.033
IL-6 (pg/ml)	1.159 (1.055-1.274)	0.002	1.123 (1.008-1.252)	0.035
CRP (μg/ml)	1.533 (1.143-2.056)	0.004		
SAA (μg/ml)	1.014 (1.001-1.027)	0.031		
MDSC (%)	1.140 (0.823-1.580)	0.432		
Treg (%)	0.823 (0.561-1.206)	0.317		
No. of selected peptides	0.395 (0.163-0.953)	0.039	0.258 (0.098-0.682)	0.006

[a]P-values determined by the Cox proportional hazard regression model. CI, confidence interval; IL-6, interleukin-6; CRP, C-reactive protein; SAA, serum amyloid A; MDSC, myeloid-derived suppressor cells; Treg, $CD4^+CD25^+Foxp3^+$ regulatory T cells.

Table V. Univariate and multivariate analyses with post-vaccination clinical findings and laboratory data.

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% CI)	P-value[a]	Hazard ratio (95% CI)	P-value[a]
Elevation of CTL responses	0.530 (0.166-1.691)	0.284		
Elevation of humoral responses	0.364 (0.114-1.165)	0.089		
Hemoglobin (g/dl)	0.668 (0.463-0.965)	0.031		
Albumin (g/dl)	0.173 (0.055-0.544)	0.003		
IL-6 (pg/ml)	1.112 (1.033-1.198)	0.005	1.152 (1.052-1.261)	0.002
CRP (μg/ml)	1.217 (1.023-1.448)	0.027		
SAA (μg/ml)	1.008 (0.995-1.021)	0.234		
No. of vaccinated peptides	0.271 (0.082-0.899)	0.033	0.120 (0.027-0.540)	0.006

[a]P-values determined by the Cox proportional hazard regression model. CI, confidence interval; IL-6, interleukin-6; CRP, C-reactive protein; SAA, serum amyloid A.

peptides were significantly favorable factors for OS (HR=1.152, 95% CI 1.052-1.261, P=0.002; HR=0.120, 95% CI 0.027-0.540, P=0.006; respectively) (Table V). However, the other post-vaccination factors were not significantly associated with OS.

Discussion

For patients with advanced or recurrent BTC that are ineligible for surgery, various regimens of chemotherapeutic agents have been investigated (1-4). For example, a combination of chemotherapeutic agents, such as gemcitabine and cisplatin, has recently demonstrated a promising result (3). However, further treatment modalities for refractory patients who are unresponsive to or relapse following such regimens remain to be established. This is the first clinical report of refractory BTC patients who received PPV. Immune responses to the vaccine antigens, which have been reported to be significantly associated with clinical responses in previously conducted clinical trials of PPV (6,14), were substantially induced in a subset of the vaccinated patients. Toxicity of PPV mainly involved skin reactions at the injection sites, and no severe adverse events were observed. Based on the positive immune responses to vaccine antigens and the safety profile, PPV could be further investigated as one of the promising approaches for refractory BTC.

The most unique aspect of PPV is the 'personalized' selection of antigen peptides ideal for individual patients in consideration of the pre-existing host immunity prior to vaccination (5-7). In view of the heterogeneity and complexity of host immune responses against tumors, this approach appears to be more rational than vaccination with non-personalized 'universal' tumor antigens. Notably, in the present study, the number of selected and vaccinated peptides was significantly associated with OS in the multivariate analysis, suggesting that greater numbers of peptides would be required for better clinical responses, possibly due to the heterogeneity and complexity of host immune responses against tumors.

Cancer vaccines do not always elicit beneficial immune or clinical responses in treated patients. Therefore, identification of biomarkers for predicting clinical responses in vaccinated patients would be a significant issue in the clinical application of cancer vaccines (5,15-17). At present, however, there is little information available regarding predictive biomarkers in patients undergoing cancer vaccines. In this study, the multivariate analysis demonstrated that lower IL-6 and higher albumin values, which may reflect less inflammation and better nutritional status, prior to vaccination were significantly favorable factors for OS. IL-6 is a multifunctional cytokine that regulates various aspects of immune responses, acute phase reactions and hematopoiesis. In particular, IL-6 has been reported to be deeply involved in cancer development, such as tumor cell growth and cancer-associated inflammation (18).

There have been a number of studies describing the correlation between IL-6 levels and prognosis in various types of cancer (19-22). IL-6 has also been reported to be one of the critical cytokines for inducing suppressive immune cell subsets. For example, MDSCs and Th17, which are known to modulate antitumor immunity, were shown to be generated from their precursors in the presence of IL-6 and other cytokines (23-25). Although the role of IL-6 in the immune response to cancer vaccines remains to be clarified, it is possible that the blockage of IL-6 signaling would be beneficial for enhancing the therapeutic efficacy of cancer vaccines.

In conclusion, the present study demonstrated that PPV induced substantial immune responses to vaccine antigens without severe adverse events in advanced BTC patients. In addition, the multivariate analysis suggested that lower plasma IL-6 and better nutritional status prior to vaccination and pre-existing immune responses to greater numbers of antigens may contribute to better responses to PPV. Therefore, the evaluation of these factors prior to vaccination may be useful for selecting patients who would benefit from PPV and defining eligibility and/or exclusion criteria for molecular-based personalized immunotherapy in BTC patients. Nevertheless, since this was a small study with a limited number of patients, all of whom received PPV, the clinical efficacy of PPV, as well as the clinical utility of the identified factors in refractory BTC patients remain to be confirmed in future larger-scale prospective trials conducted in defined patient populations with or without receiving PPV.

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