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標準治療抵抗性膠芽腫に対するペプチドワクチンの第Ⅲ相臨床研究に関する研究

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研究要旨
本研究では、進行、再発、難治がんに対する新規の治療法確立を最終目的として、まず HLA-A24 陽性のテモゾロミド治療抵抗性膠芽腫患者を対象としたテーラーメイドペプチドワクチン投与の有効性と安全性を検証する臨床試験を第 III 相プラセボ対照二重盲検比較試験として実施して医薬品承認を目指す。その後適応拡大を目指す。我々が開発したテーラーメイドがんペプチドワクチン研究は、各種がん、とりわけ膠芽腫への探索的臨床研究と企業による治験において良好な成績が得られ、その特色・独創性は世界から注目されている。当該研究によりがんペプチドワクチンが実用化された暁には多くのがん患者の福音となると思われる。

A.　研究目的

研究目的は膠芽腫に対して新規抗がん剤としてのがんペプチドワクチン療法の確立である。具体的には HLA-A24 陽性のテモゾロミド治療抵抗性膠芽腫患者を対象としたテーラーメイドペプチドワクチン投与の有効性と安全性を検証する臨床試験を第 III 相プラセボ対照二重盲検比較試験として実施して医薬品承認をめざす。

B.　研究方法

本研究では、テモゾロミド治療抵抗性の HLA-A24 陽性の膠芽腫癌患者を対象とし、BSC 下にプラセボ群を対照として、ペプチドワクチン投与（以下 ITK-1 と記載）群の臨床効果を検証する多施設共同無作為割付第Ⅲ相二重盲検比較臨床試験を実施する。本試験の主要評価項目は全生存期間である。

本被験薬は被験者の末梢血を用いた ITK-1 抗ペプチド抗体価検査に従い、12 剤から 2～4 剤を選択して投与する治療法であるため、薬剤部に対する盲検性の確保は困難であるが、治験薬調製後の乳化製剤は外観上からは判別不能であるため、治験責任（分担）医師への盲検性の確保は可能となる。また、被験者にも割付を開示しないため、プラセボ効果などのバイアスを排除できること、また、プラセボ投与割り当て患者がプラセボ投与を知る事で、落胆し、治験より早期脱落する事を回避することができる。更に本試験の主要評価項目は全生存期間であり、治験責任（分担）医師によるバイアスが入り込む余地は考えにくいことから、より信頼性の高い成績が得られると考えられる。従って、ITK-1 群とプラセボ群の 2 群からなる多施設共同無作為割付二重盲検比較試験を設定した。これらのことより、上記の設定にて当該治験の目的が達成され得る。ペプチド製剤の選択方法として、まず被験者の末梢血検体を用いて、ペプチド製剤 12 種類への「抗体検査」を実施し、抗ペプチド抗体価が高かった順位に従って、2～4 種類以上のペプチド製剤またはプラセボの投与数を決定する。ただし、GP-108、GP-109、GP-110 のペプチド（第 2 選択ペプチド）については膠芽腫患者では発現頻度が低いので、他の 9 種のペプチド製剤（第 1 選択ペプチド）を優先して選択する。

第 1 選択ペプチド	GP-101, GP-104, GP-105, GP-106, GP-107, GP-111, GP-112, GP-113, GP-114
第 2 選択ペプチド	GP-108, GP-109, GP-110

倫理面への配慮

臨床試験（治験）に先立ち、安全性を担保する為の安全性薬理試験並びに必要不可欠なラットを用いた各種毒性試験を GLP 基準に基づき実施し、ヒトにおける臨床試験の実施に問題は無いと判断された。

本研究は患者を対象とした介入試験であり、薬事法下の医師主導治験である。「ヘルシンキ宣言」ならびに「医薬品の臨床試験の実施の基準に関する省令（GCP）」を遵守して実施される。治験実施計画書及び患者同意説明文書は医薬品医療機器総合機構による治験相談は実施済みであり、各実施医療機関の IRB においても科学的及び倫理的な面からの審査・承認を経て、治験届出後に治験が開始された。さらに公的登録サイト（UMIN、JAPIC）に登録して行う。被験者からの同意取得に当たっては同意説明文書を用いて試験の内容、予想される不利益・危険性、同意撤回の自由等を説明する。被験者が説明内容を十分に理解したことを確認し

た上で、本試験への参加について本人の自由意志による同意を文書にて取得する（インフォームドコンセント)。また、試験開始後も、GCP に基づくモニタリングおよび監査が実施される。

C.　研究成果

①　臨床試験全体の研究成果については総合研究報告書に記載してあるためのそちらを参照してください：

②　統計学的研究成果：(1) 遅延効果のある薬剤の評価など比例ハザードを仮定できない生存時間の群間比較で用いられる Harrington-Fleming 検定下での症例数設計を行った。(2) 臨床的・倫理的観点から試験中止を検討するための中間解析の統計的検討を行った。(3) 動的割付のプログラミングを作成した。これらの研究成果の詳細はプロトコル及び統計解析計画書に記述した。

D.　考察

申請時研究計画では初年度前半に医師主導治験を開始して後半には登録予定症例（110例）の5%を目標とし、2年及び3年目には各々40%と90%を目指す計画である。しかし、臨床試験計画書の固定が予定より数カ月、遅延した。遅延の主な原因は、①10治験実施施設間での臨床試験デザインを巡り統一見解をえるために長期間を要したこと及び②医薬品医療機器総合機構(PMDA)相談において研究グループとPMDAの意見調整に多くの時間を要したことがあげられる。この数カ月の遅延については、平成24年1月からの症例登録を加速させて研究期間終了時までには解消すべく努力する。

E.　自己評価

申請時研究計画に沿って概ね順調に経過していると自己評価している。治験開始数カ月の遅延は、今後症例登録を加速させて研究期間終了時までには解消すべく努力する。

F.　研究発表

①. 論文発表

無し

②　学会発表

無し

G.　知的財産権の出願・登録状況(予定を含む)

無し

Ⅲ．研究成果の刊行に関する一覧表

雑誌

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Matsumoto K, Noguchi M, Satoh T, Tabata K, Fujita T, Iwamura M, Yamada A, Komatsu N, Baba S, Itoh K.	A phase I study of personalized peptide vaccination for advanced urothelial carcinoma patients who failed treatment with methotrexate, vinblastine, adriamycin and cisplatin.	BJU International	108	831-838	2011
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Ⅳ．研究成果の刊行物・別冊

Personalized peptide vaccination in patients with refractory non-small cell lung cancer

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Abstract. Since the prognosis of non-small cell lung cancer (NSCLC) remains poor, the development of novel therapeutic approaches, including cancer vaccines, is highly desirable. In the current study, we conducted a phase II study of personalized peptide vaccination (PPV), in which a maximum of 4 peptides were selected based on pre-existing humoral immune responses and administered subcutaneously (weekly for 6 consecutive weeks and bi-weekly thereafter) in refractory NSCLC patients. Forty-one refractory NSCLC patients (4 stage IIIb, 22 stage IV and 15 recurrent), who had failed to respond to chemotherapy and/or targeted therapy (median number of regimens, 3; median duration, 10 months), were enrolled. Median overall survival (OS) was 304 days with a one-year survival rate of 42% in the enrolled patients. The main toxicity of PPV was skin reactions at the injection sites, but no serious adverse events were observed. In order to identify potential biomarkers for predicting OS, pre-vaccination and post-vaccination clinical findings and laboratory data were retrospectively assessed and evaluated by multivariate Cox regression analysis. Among the pre-vaccination factors examined, high C-reactive protein (CRP) level was a significant predictor of unfavorable OS [hazard ratio (HR)=10.115, 95% confidence interval (CI)=2.447-41.806, P=0.001]. Among the post-vaccination factors, high CRP level and low frequency of $CD3^+CD26^+$ cells were significant predictors of unfavorable OS (HR=23.127, 95% CI=2.919-183.233, P=0.003; HR=0.952, 95% CI=0.917-0.989, P=0.012). Taken together, our results suggest the feasibility of PPV for the treatment of refractory NSCLC. Evaluation of the identified factors before or at an early stage of vaccination could be potentially useful for selecting NSCLC patients who would likely have better prognosis following PPV.

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Key words: non-small cell lung cancer, peptide vaccine, biomarker

Introduction

Non-small cell lung cancer (NSCLC) is one of the most common causes of cancer death worldwide. Although recent advances in chemotherapy and/or targeted therapy have helped to improve the clinical outcomes of patients with refractory NSCLC (1-5), their prognosis still remains very poor with a median survival time of 6-8 months. Therefore, development of novel therapeutic approaches, including cancer vaccines, would be highly desirable.

We developed a new approach of peptide-based vaccination, named personalized peptide vaccination (PPV), in which vaccine antigens are selected and administered based on pre-existing host immunity before vaccination (6-14). We have shown promising results of PPV in various types of advanced cancers (6-9). For example, a recently conducted randomized clinical trial of PPV for patients with advanced prostate cancer suggested a potentially favorable clinical outcome in the vaccinated group (9). However, to improve clinical efficacy further, prognostic biomarkers that would make it possible to select patients for whom cancer vaccines would be appropriate remain to be identified. In the present investigation, we conducted a small-scale phase II study to identify potential biomarkers that would be useful for prediction of overall survival (OS) before or at an early stage of vaccination in refractory NSCLC patients. Our results suggested the feasibility of PPV for refractory NSCLC. The identified factors would be informative for predicting the subpopulation of NSCLC patients, who would likely have better prognosis following PPV.

Patients and methods

Patients. Patients with a histological diagnosis of NSCLC were eligible for inclusion in the present study, if they had failed to respond to previous chemotherapy and/or targeted therapy. They also had to show positive humoral responses to at least two of the 31 different candidate vaccine peptides (Table I), determined by both HLA class I type and the titer of IgG against each peptide. The other inclusion criteria, as well as the exclusion criteria, were not largely different from those of other previously reported clinical studies (6-9): patient age

between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 1 or 2; positive status for HLA-A2, -A3, -A11, -A24, -A26, -A31, or -A33; life expectancy of at least 12 weeks; negative status for hepatitis virus B and C; adequate hematologic, renal, and hepatic function. Patients with lymphocyte counts of <1000 cells/μl were excluded from the study, since we had previously reported that pre-vaccination lymphopenia is a predictor of unfavorable OS in cancer patients receiving PPV (12). Other exclusion criteria included pulmonary, cardiac, or other systemic diseases; acute infection; a history of severe allergic reactions; pregnancy or nursing; or 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 no. 1839). After a full explanation of the protocol, written informed consent was obtained from all patients before enrollment.

Clinical protocol. This was an open-label phase II study in which the primary and secondary endpoints were to identify potential biomarkers for OS and to evaluate the safety of PPV in NSCLC patients, respectively. Thirty-one peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies (6-9, 13), were employed for vaccination [12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (-A3, -A11, -A31, and -A33), 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) and American Peptide Company (Vista, CA). Appropriate peptides for vaccination in individual patients were selected in consideration of pre-existing host immunity before vaccination, assessed from the titers of IgG specific to each of the 31 different vaccine candidates, as described previously (14). Combined chemotherapy and/or targeted therapy were allowed during the vaccination period, unless patients were unable to tolerate combined chemotherapies or declined them (Table II). A maximum of 4 peptides (3 mg/each peptide), which were selected on the basis of HLA typing and peptide-specific IgG titers, were administrated subcutaneously 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 in 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 Ver3). Complete blood counts and serum biochemistry tests were performed after every 6 vaccinations. The clinical responses were evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) in the vaccinated patients, for whom computed tomography (CT) scan or magnetic resonance imaging (MRI) data were available before and after the first cycle of vaccinations.

Measurement of humoral and T cell responses. The humoral responses specific to each of the 31 candidate peptides (Table I), including those employed and not employed for vaccination, were determined by the peptide-specific IgG levels using the Luminex system (Luminex, Austin, TX), as reported previously (14). If the plasma titers of peptide-specific IgG in response to at least one of the vaccinated peptides after vaccination were >2-fold higher than those before vaccination, the changes were considered to be significant.

T cell responses specific to the vaccine peptides were evaluated by interferon (IFN)-γ Elispot using peripheral blood mononuclear cells (PBMCs), which were separated by density gradient centrifugation from peripheral blood (30 ml) with Ficoll-Paque Plus (GE Healthcare; Uppsala, Sweden) and stored frozen until analysis. After thawing, 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) containing 10% FBS (MP Biologicals, Solon, OH), IL-2 (20 IU/ml; AbD Serotec, Kidlington, UK), and each peptide (10 μM). Half of the medium was removed and replaced with new medium containing a corresponding peptide (20 μM) after culture for 3 days. After incubation for a further 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) sequence (SLYNTYATL for HLA-A2; RYLRQQLLGI for HLA-A24; RLRDLLLIVTR for HLA-A3 supertype; EVIPMFSAL for HLA-A26). Antigen-specific IFN-γ secretion after 18 h of incubation was determined by Elispot, in accordance with the manufacturer's instructions (MBL, Nagoya, Japan). All assays were carried out in triplicate, and analyzed with the Zeiss Elispot reader (Carl Zeiss MicroImaging Japan, Tokyo, Japan). Antigen-specific T cell responses were evaluated by the difference between the numbers of spots produced in response to each corresponding peptide and that produced in response to the control peptide; a difference of at least 30 spots per 10^5 PBMCs was considered positive.

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

Flow cytometric analysis of immune subsets among PBMCs. A suppressive immune subset, myeloid-derived suppressor cells (MDSCs), among PBMCs were examined by flow cytometry. For analysis of MDSCs, PBMCs ($0.5x10^6$) suspended in PBS containing 2% FBS were incubated with the following monoclonal antibodies (Abs) 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. In the cell subset negative for lineage markers (CD3, CD19, CD56, CD14) and HLA-DR, MDSCs were identified as positive for CD33. The frequency of MDSCs in the lymphocyte gate defined by forward scatter and side scatter was calculated. In addition, the expression of CD26 in PBMCs was also analyzed, since the expression level of this gene assessed by cDNA microarray analysis has been shown to be predictive of OS in patients with prostate cancer receiving PPV (Sasada *et al*, unpublished data).

Table I. Peptide candidates for cancer vaccination.

Symbol for peptide	Protein	Position of peptide	Amino acid sequence	HLA type
CypB-129	Cyclophilin B	129-138	KLKHYGPGWV	A2, A3sup[a]
Lck-246	p56Lck	246-254	KLVERLGAA	A2
Lck-422	p56Lck	422-430	DVWSFGILL	A2, A3sup
MAP-432	ppMAPkkk	432-440	DLLSHAFFA	A2, A26
WHSC2-103	WHSC2	103-111	ASLDSDPWV	A2, A3sup, 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, A26
Lck-208	p56Lck	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	p56Lck	486-494	TFDYLRSVL	A24
Lck-488	p56Lck	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
SART3-734	SART3	734-742	QIRPIFSNR	A3sup
Lck-90	p56Lck	90-99	ILEQSGEWWK	A3sup
Lck-449	p56Lck	449-458	VIQNLERGYR	A3sup
PAP-248	PAP	248-257	GIHKQKEKSR	A3sup

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

PBMCs were stained with anti-CD26-PE and anti-CD3-FITC Abs. The frequency of the $CD26^+$ subset among $CD3^+$ cells was calculated. The samples were run on a FACSCanto II (BD Biosciences, San Diego, CA), and data were analyzed using the Diva software package (BD Biosciences). All Abs were purchased from Biolegend (San Diego, CA).

Statistical analysis. The two-sided Wilcoxon test was used to compare differences between pre- and post-vaccination measurements at a significance level of $P<0.05$. OS time was calculated from the first day of peptide vaccination until the date of death or the last date when the patient was known to be alive. The survival curve was estimated by the Kaplan-Meier method. Predictive factors for OS were evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model. Statistically significant ($P<0.05$) variables in the univariate analysis were included in the multivariate analysis. Spearman rank correlation index was also utilized to choose the variables for multivariate analysis. All statistical analyses were conducted using the JMP version 8 or SAS version 9.1 software package (SAS Institute Inc., Cary, NC).

Results

Patient characteristics. Between December 2008 and October 2010, 41 patients with refractory NSCLC were enrolled in this study. Table II shows the clinicopathological characteristics of the enrolled patients. There were 19 male and 22 female subjects with a median age of 63 years, ranging from 37 to 76 years. Histologically, the tumors comprised 32 adenocarcinomas, 5 squamous cell carcinomas, 2 adenosquamous cell carcinomas, 1 large cell carcinoma, and 1 pleomorphic carcinoma. The

Table II. Characteristics of the enrolled patients with refractory NSCLC (n=41).

Patient no.	Histology	HLA type	Gender	Age	Stage	PS	Previous treatment		Combined therapy	No. of vaccination	Treatment response	OS (days)
							No. of regimens	Period (months)				
1	Ad	A24	F	67	IV	0	1	2	CBDCA + PTX	24	SD	683
2	Ad	A26	F	56	R	0	5	16	S-1	24	SD	691
3	Ad	A11/A31	M	70	IV	0	1	5	-	6	PD	58
4	Ad	A24	F	69	IV	1	4	4	-	15	PD	225
5	Adsq	A2/A24	M	68	IIIb	0	3	5	Erlotinib	7	PD	95
6	Adsq	A24/A33	F	52	R	0	2	18	Erlotinib	6	NA	467
7	Ad	A2/A33	M	63	IV	0	1	1	-	4	NA	41
8	Ad	A2/A24	F	53	R	1	5	24	GEM	9	PD	159
9	Pleo	A24	M	55	R	0	2	6	DOC	3	NA	41
10	Ad	A2/A26	M	50	R	0	1	11	CBDCA + PTX	6	NA	422
11	Ad	A2/A24	M	57	IIIb	0	1	6	-	18	SD	354
12	Ad	A24	M	72	IV	0	1	4	-	22	SD	596[b]
13	Sq	A11/A33	F	53	IV	0	2	8	Gefitinib	6	SD	573[b]
14	Ad	A26	M	75	R	0	2	10	-	17	SD	366
15	Ad	A2	F	59	IV	0	3	10	Gefitinib	8	PD	291
16	Ad	A2	F	54	IV	1	4	24	CDDP + PEM	2	NA	304
17	Ad	A24	F	72	IV	0	1	25	-	11	SD	266
18	Ad	A2/A33	F	69	R	0	6	23	-	5	NA	51
19	Ad	A2/A31	F	76	R	0	3	4	-	6	NA	503[b]
20	Ad	A2/A11	M	61	IV	0	1	4	DOC	6	NA	431
21	Ad	A2/A11	F	65	R	0	1	3	Gefitinib	20[a]	SD	412[b]
22	Ad	A2/A11	M	50	IV	0	1	2	-	14	NA	356
23	Ad	A24/A33	M	67	R	0	3	9	-	17	SD	398[b]
24	Ad	A2/A3	M	70	IV	0	2	12	-	6	NA	230
25	Ad	A24/A33	F	68	IV	1	4	9	-	7	PD	81
26	Ad	A26/A33	F	65	IV	0	6	30	-	5	NA	208
27	Ad	A2/A26	F	70	IV	0	3	21	Erlotinib	11	SD	258
28	Ad	A24/A26	M	53	R	0	4	13	-	11	NA	189
29	Ad	A24	M	54	IV	0	5	13	-	8	PD	77
30	Ad	A24	M	37	R	0	2	10	PEM	14	PD	239[b]
31	Sq	A2/A24	M	64	IIIb	0	3	6	VNR	14[a]	NA	232[b]
32	Ad	A2/A24	F	59	R	0	3	43	Gefitinib	16[a]	SD	251[b]
33	Ad	A24	F	73	IIIb	0	10	72	-	11	PD	246[b]

Table II. Continued.

Patient no.	Histology	HLA type	Gender	Age	Stage	PS	Previous treatment		Combined therapy	No. of vaccination	Treatment response	OS (days)
							No. of regimens	Period (months)				
34	Sq	A2/A24	F	62	IV	0	1	2	-	4	NA	50
35	Ad	A26/A33	F	54	IV	0	3	17	Gefitinib	14[a]	NA	239[b]
36	Sq	A24/A11	M	60	IV	0	3	12	-	15[a]	NA	237[b]
37	LCC	A24/A26	M	70	IV	0	6	19	-	14[a]	SD	190[b]
38	Sq	A2	M	66	R	1	3	6	-	10	PD	127
39	Ad	A2/A30	F	57	IV	0	4	32	PEM + Gefitinib	13[a]	PD	181[b]
40	Ad	A24/A26	F	44	R	0	3	23	Erlotinib	12	SD	176[b]
41	Ad	A2/A26	F	57	IV	0	2	11	-	12[a]	SD	176[b]

[a]Under treatment, [b]patients alive. NSCLC, non-small cell lung cancer; Ad, adenocarcinoma; Adsq, adenosquamous carcinoma; LCC, large cell carcinoma; Ple, Pleomorphic carcinoma; Sq, squamous cell carcinoma: M, male; F, female; R. recurrent; PS, performance status: CBDCA, carboplatin; PTX, paclitaxel; GEM, gemcitabine; DOC, docetaxel; CDDP, cisplatin; PEM, pemetrexed; VNR, vinorelbine; SD, stable disease; PD, progressive disease; NA, not assessed; OS, overall survival.

patients' cancers were at the refractory stage (stage IIIb, n=4; stage IV, n=22; recurrent, n=15) when they had failed to respond to one (n=11), two (n=7), three (n=11), or >4 (n=12) regimen(s) of chemotherapy, targeted therapy, and/or a combination of them. The median duration of these preceding regimens prior to PPV was 10 months, ranging from 1 to 72 months. Performance status at the time of enrollment was grade 0 (n=36) or grade 1 (n=5). The numbers of peptides used for vaccination of the patients during the first cycle were 4 peptides in 31 patients, 3 in 5 patients, and 2 in 5 patients. Among the 41 patients, 35 completed the first cycle of 6 vaccinations, whereas the remaining 6 patients failed to do so due to rapid disease progression. The median number of vaccinations was 11, with a range of 2 to 24. Among the 25 vaccinated patients for whom both pre- and post-vaccination radiological findings were available, none had a complete response (CR) or partial response (PR). The best response, seen in 14 patients, was stable disease (SD); the remaining 11 patients had progressive disease (PD).

Toxicities. Toxicities are shown in Table III. The most frequent adverse events were skin reactions at the injection sites (n=28) and hypoalbuminemia (n=21). One grade 4 serious adverse event (SAE), anemia, was noted. Grade 3 SAEs comprised injection site reaction (n=2), fever (n=1), hemoptysis (n=1), anemia (n=1), lymphopenia (n=1), and thrombocytopenia (n=1). According to evaluation by the independent safety evaluation committee for this trial, all of these SAEs, except for two cases of grade 3 injection site reaction, were concluded to be not directly associated with the vaccinations, but with cancer progression or other causes.

Immune responses to the vaccine peptides. Both humoral and T cell responses specific to the vaccine peptides were analyzed using blood samples obtained before and after the PPV. Plasma samples were obtained from 41, 35 and 18 patients before vaccination and at the end of the first (6 vaccinations) and second (12 vaccinations) cycles, respectively. Due to disease progression, 6 patients failed to complete the first cycle of 6 vaccinations. For monitoring of humoral immune responses, peptide-specific IgG reactive with each of the 31 different peptides, including those employed and not employed for vaccination, were measured by bead-based multiplex assay. The IgG responses specific to at least one of the vaccine peptides were augmented in 17 of 35 patients (49%) and in all of the 18 patients (100%) examined at the end of the first and second cycles of vaccination, respectively (data not shown).

T cell responses to the vaccine peptides were measured by IFN-γ Elispot assay. PBMCs from 36, 32 and 9 patients were available for this assay before and at the end of the first (6 vaccinations) and second (12 vaccinations) cycles, respectively. In the pre-vaccination samples, antigen-specific T cell responses were detectable in only 8 patients (22%). Among the 32 patients at the end of the first cycle of vaccinations, 11 (34%) showed T cell responses to the vaccine peptides. Among the 9 samples at the end of the second cycle of vaccinations, T cell responses were observed in 5 patients (56%) (data not shown).

Collectively, an increase of peptide-specific IgG titers was observed in about half and in all of the vaccinated patients at the end of the first and second cycles, respectively. In contrast,

Table III. Toxicities.

Toxicity type	Grade 1	Grade 2	Grade 3	Grade 4
Skin reactions at injection sites (n=28)	10	16	2	0
Constitutional symptom				
Fever (n=3)	1	1	1	0
Pulmonary/upper respiratory				
Dyspnea (n=3)	1	2	0	0
Hemoptysis (n=1)	0	0	1	0
Blood/bone marrow				
Anemia (n=11)	9	0	1	1
Leukocytopenia (n=7)	5	2	0	0
Neutropenia (n=4)	3	1	0	0
Lymphopenia (n=12)	10	1	1	0
Thrombocytopenia (n=2)	1	0	1	0
Laboratory				
Hyperbilirubinemia (n=3)	1	2	0	0
AST elevation (n=3)	2	1	0	0
ALT elevation (n=4)	3	1	0	0
Hypoalbuminemia (n=21)	17	4	0	0
Creatinine elevation (n=1)	1	0	0	0

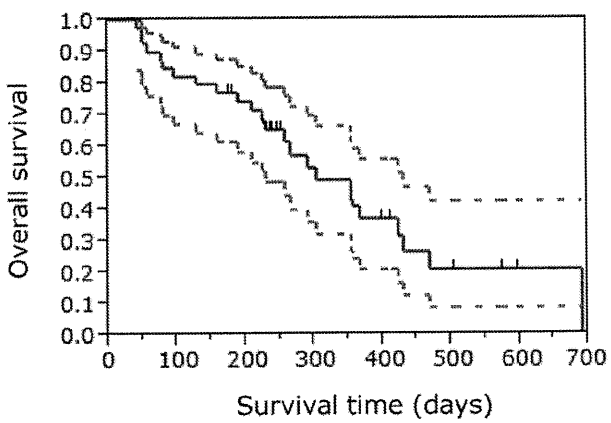

Figure 1. Kaplan-Meier survival analysis in the NSCLC patients receiving PPV. The median overall survival of patients who received PPV (n=41, solid line) was 304 days and the one-year survival rate was 42%. Dotted lines show 95% confidence intervals.

antigen-specific T cell responses were induced in only limited patients even after vaccination.

Cytokines and inflammation markers. We then measured cytokines (IL-2, IL-4, IL-5, IL-6, IFN-γ) and inflammation markers (CRP and SSA) in plasma before and at the end of the first cycle of vaccinations. IL-6 was detectable in 23 of 41 patients before vaccination, with a median level of 1 pg/ml, ranging from 0 to 103 pg/ml. Among the 35 plasma samples available at the end of the first cycle of vaccination, IL-6 levels were increased, decreased, and unchanged in 13, 7, and 15 patients, respectively. There was no significant difference in the level of IL-6 before and after vaccination (P=0.614, Wilcoxon test). However, the 22 patients who showed a decrease or no change in IL-6 levels after vaccination had a tendency to have a better prognosis than the remaining 13 patients who showed an increase in IL-6 (P=0.068, log-rank 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 the majority of patients (40 of the 41 patients), with a median level of 0.39 mg/dl (ranging from 0 to 1.11 mg/dl). Among the 35 plasma samples tested at the end of the first cycle of vaccination, plasma CRP levels were increased and decreased in 30 and 5 patients, respectively. Another inflammation marker, SAA, was also detected in pre-vaccination plasma from the majority of patients (40 of the 41 patients), with a median level of 6.21 mg/dl (ranging from 0 to 14.12 mg/dl). Among the 35 plasma samples available at the end of the first cycle of vaccination, plasma SAA levels were increased and decreased in 25 and 10 patients, respectively. There were significant increases in the levels of CRP (P<0.001, Wilcoxon test) as well as SAA (P=0.005, Wilcoxon test) after vaccination, compared with those before vaccination. However, there were no significant associations between changes in CRP or SAA levels and clinical outcomes in the vaccinated patients (data not shown).

Flow cytometric analysis of immune subsets among PBMCs. Immune cell subsets among both pre-vaccination and post-vaccination PBMCs were examined by flow cytometry. The median frequency of MDSCs among pre- and post-vaccination PBMCs was 0.4% (range, 0.1-3.4%, n=33) and 0.3% (range, 0.1-2.0%, n=33), respectively. There was a significant decrease in the frequencies of MDSCs after vaccination (P=0.002, Wilcoxon

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

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Age	1.006 (0.963-1.051)	0.786		
Gender	0.633 (0.281-1.428)	0.271		
Duration of previous treatment (months)	0.985 (0.934-1.039)	0.589		
Number of previous regimens	1.017 (0.807-1.282)	0.889		
Frequency of lymphocytes (%)	0.945 (0.898-0.993)	0.026		
Hemoglobin (g/dl)	0.826 (0.629-1.083)	0.167		
Albumin (g/dl)	0.220 (0.086-0.563)	0.002		
IL-6 (pg/ml)	1.021 (1.003-1.039)	0.020		
CRP (mg/dl)	9.375 (2.350-37.403)	0.002	10.115 (2.447-41.806)	0.001
Frequency of MDSCs (%)	1.089 (0.512-2.318)	0.825		
Frequency of $CD3^+CD26^+$ (%)	0.966 (0.914-1.021)	0.219		

CI, confidence interval; CRP, C-reactive protein; MDSCs, myeloid-derived suppressor cells.

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

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
Skin reactions at injection sites	0.861 (0.287-2.585)	0.789		
Increase in T cell responses	0.708 (0.227-2.203)	0.551		
Increase in humoral responses	1.042 (0.407-2.669)	0.932		
Frequency of lymphocytes (%)	0.953 (0.909-0.999)	0.048		
Hemoglobin (g/dl)	0.745 (0.546-1.017)	0.064		
Albumin (g/dl)	0.169 (0.064-0.445)	<0.001		
IL-6 (pg/ml)	1.055 (1.023-1.087)	<0.001		
CRP (mg/dl)	13.250 (2.095-83.794)	0.006	23.127 (2.919-183.233)	0.003
Frequency of MDSCs (%)	0.833 (0.183-3.785)	0.813		
Frequency of $CD3^+CD26^+$ (%)	0.956 (0.916-0.998)	0.042	0.952 (0.917-0.989)	0.012

CI, confidence interval; CRP, C-reactive protein; MDSCs, myeloid-derived suppressor cells.

test). The median frequency of $CD3^+CD26^+$ cells among pre- and post-vaccination PBMCs was 18.8% (range, 7.4-47.0%, n=35) and 18.3% (range, 3.3-61.8%, n=35), respectively. There was no significant difference in the frequencies of $CD3^+CD26^+$ cells (P=0.965, Wilcoxon test) before and after vaccination. There were no significant associations between changes in the frequencies of MDSCs or $CD3^+CD26^+$ cells and clinical outcomes in the vaccinated patients (data not shown).

Relationship between clinical findings or laboratory data and OS. The median OS for the 41 patients was 304 days, with a one-year survival rate of 42% (Fig. 1). The Cox proportional hazards model was used to identify factors that were significantly associated with OS from clinical findings or laboratory data before vaccination. Univariate analysis using pre-vaccination data showed that albumin, CRP, SAA, IL-6, and the frequency of lymphocytes in whole blood (P=0.002, P=0.002, P=0.004, P=0.020, and P=0.026, respectively) were significantly predictive of OS (Table IV). However, none of other factors examined, including age, gender, performance status, duration of chemotherapy or target therapy before vaccination, number of previous regimens, or other laboratory data (hemoglobin, creatinine, frequencies of regulatory T cells, MDSCs, or $CD3^+CD26^+$ cells), were significantly correlated with OS (data not shown). In addition, multivariate Cox regression analysis was performed to evaluate the influence of each of the factors that had been shown to be significantly associated with OS in the univariate analysis (P<0.05), after adjusting for possible confounding factors. Albumin, CRP, IL-6, and the frequency of lymphocytes in whole blood were included in the multivariate Cox regression analysis. SAA was excluded in this analysis, since the level of SAA was highly correlated with that of CRP (Spearman rank

correlation coefficient, 0.819; P<0.001). As shown in Table IV, higher CRP level in pre-vaccination plasma was significantly predictive of unfavorable OS [hazard ratio (HR)=10.115, 95% confidence interval (CI)=2.447-41.806, P=0.001]. However, the other factors showed no significant association.

Similarly, the Cox proportional hazards model was used to identify factors associated with OS from clinical findings or laboratory data at the end of the first cycle of vaccination. Univariate analysis showed that albumin, IL-6, SAA, CRP, frequency of $CD3^+CD26^+$ cells, and frequency of lymphocytes in whole blood were predictive of OS at the end of the first cycle of vaccination (P<0.001, P<0.001, P=0.004, P=0.006, P=0.042, and P=0.048, respectively) (Table V). None of the other factors, including other laboratory data, increase in IgG or T cell responses to the vaccine peptides, and skin reactions at the injection sites, were significantly correlated with OS. Albumin, IL-6, CRP, frequency of $CD3^+CD26^+$ cells, and frequency of lymphocytes were included in the multivariate Cox regression analysis. SAA was excluded in this analysis, since the level of SAA was highly correlated with that of CRP (Spearman rank correlation coefficient, 0.698; P<0.001). Multivariate Cox regression analysis demonstrated that higher CRP level and lower frequency of $CD3^+CD26^+$ cells in post-vaccination samples were predictive of unfavorable OS (HR=23.127, 95% CI=2.919-183.233, P=0.003; HR=0.952, 95% CI=0.917-0.989, P=0.012) (Table V).

Discussion

Since only a subset of patients obtain clinical benefits from peptide-based cancer vaccines, it would be critical to identify biomarkers for selection of suitable patients (15-17). With regard to post-vaccination biomarkers, we have shown that an increase in peptide-specific IgG responses after PPV is well associated with improved OS in patients with certain types of cancers (12,18). In addition, several factors, including cytotoxic T lymphocytes (CTL) responses, Th1 responses, delayed type hypersensitivity (DTH), and autoimmunity, have also been reported to be associated with clinical responses in some clinical trials (16,17,19,20), although these results have not always been reproducible. Notably, there are currently no validated pre-vaccination biomarkers, predictive of clinical responses, in widespread use. Therefore, in the present study, we searched for clinically useful predictive markers for PPV in patients with NSCLC. Multivariate analysis of pre-vaccination factors showed that higher level of plasma CRP was predictive of unfavorable OS. Among post-vaccination factors, higher level of plasma CRP and lower frequency of $CD3^+CD26^+$ cells were predictive of unfavorable OS. Although more data are still needed to validate our findings, evaluation of the factors identified here could be useful for selecting patients with NSCLC who would potentially benefit from cancer vaccines.

Elevated CRP level was shown to be also a predictor of unfavorable OS in NSCLC patients receiving chemotherapy or targeted therapy (21,22), suggesting that it might not necessarily be unique to vaccinated patients. In contrast, the frequency of $CD3^+CD26^+$ cells among PBMCs has not been reported previously as a biomarker in NSCLC patients. CD26 is a cell surface glycoprotein that functions as a proteolytic enzyme, dipeptidyl peptidase IV, and plays a critical role in signal transduction (23). Since it is highly expressed on activated T cells (23), increased frequency of $CD3^+CD26^+$ might reflect the immune activation induced by vaccination. The role of $CD26^+$ activated T cells induced by PPV in NSCLC thus remains to be determined.

MDSCs are a heterogeneous population of immature myeloid cells that inhibit the functions of other immune cells and promote tumor progression (24,25). MDSCs can facilitate tumor growth by inducing angiogenesis at tumor sites or by suppressing anti-tumor immune cells, such as antigen-specific T cells (24,25). Notably, the frequencies of MDSCs were significantly decreased after PPV. In addition, the patients who showed a decrease or no change in IL-6 after vaccination had a tendency to have better outcome. IL-6 is a multifunctional cytokine that regulates various aspects of cancer development, such as tumor cell growth and suppression of anti-tumor immune cells, including CTL and NK cells (26). The roles of these immune suppressive cells and/or cytokine, MDSCs and IL-6, in immune responses to cancer vaccines remain to be examined.

The prognosis of refractory NSCLC patients remains very poor, with a median survival time of 6-8 months (1-5). In contrast, the median OS of the 41 NSCLC patients who received PPV was 304 days (>10 months), with a one-year survival rate of 42%, in the current study. The main toxicity of PPV was skin reactions at the injection sites, but no SAEs were observed. Our previous trials of PPV for various types of cancers have also confirmed its safety (13). Considering the disease conditions of the patients enrolled in the current study, all of whom had already been resistant to or ineligible for conventional chemotherapeutic and targeted agents before enrollment, our findings suggest the feasibility of PPV for refractory NSCLC, even though OS was not the main objective of the current study. Nevertheless, since this is a retrospective study with a limited number of patients, clinical utility of PPV should be further verified in larger-scale, prospective trials conducted in defined patient populations with or without receiving PPV.

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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 cholangiocarcinoma; 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