Table 4A. Comparison of immune responses between short- and long-term survivors

Short-term survivors (n = 23)							
		Anti-peptide cellular response			Anti-peptide IgG response		
Pts no.	Peptide	Pre	Post (sixth)	Increased response	Pre	Post (sixth)	Increased response
1	SART3-109	0	NT	NA	492	1221	≥2
	Lck-208	0	NT	NA	11	18	negative
	Lck-488	0	NT	NA	15	20	negative
	SART3-315	0	NT	NA	30	27	negative
2	SART3-109	53	183	≥2	456	3123	≥2
	Lck-488	159	0	negative	320	310	negative
	ART1-170	1312	0	negative	<10	<10	negative
	SART3-315	77	189	≥2	<10	<10	negative
3	SART2-161	899	0	negative	36	38	negative
	Lck-208	323	108	negative	<10	<10	negative
	Lck-486	101	0	negative	118	144	negative
	SART3-315	53	69	negative	35	30	negative
4	SART3-109	41	NT	NA	22	14	negative
	Lck-208	67	NT	NA	<10	<10	negative
	Lck-486	78	NT	NA	107	92	negative
	ART4-75	79	NT	NA	NT	NT	NA
5	CypB-172	212	NT	NA	<10	1211	≥10
	HNRL-501	477	NT	NA	<10	18	≥10
	ppMAPkkk-294	0	NT	NA	12	13	negative
6	PAP-213	159	0	negative	34	39	negative
	PSA-248	55	0	negative	273	2138	≥2
	SART3-315	449	0	negative	<10	<10	negative
	PSA-152	516	61	negative	<10	<10	negative
7	UBE-43	0	NT	NA	308	NT	NA
	UBE-208	223	NT	NA	73	NT	NA
	PSCA-21	0	NT	NA	143	NT	NA
	EGFR-479	74	NT	NA	68	NT	NA
8	UBE-43	0	NT	NA	544	NT	NA
	PSCA-21	56	NT	NA	358	NT	NA
	PTHrP-42	0	NT	NA	176	NT	NA
	Her2/neu-484	0	NT	NA	227	NT	NA
9	SART3-302	608	NT	NA	229	19363	≥10
	Lck-422	0	NT	NA	14	215	≥2
	WHSC2-103	0	NT	NA	48	70	negative
	UBE2V-43	0	NT	NA	35	59	negative
10	SART3-109	5561	0	negative	274	283	negative
	Lck-488	0	0	negative	98	96	negative
	MRP3-1293	0	0	negative	78	76	negative
	PAP-213	0	0	negative	68	69	negative

NA, not available; NT, not tested. [a]Values indicate IFNγ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFNγ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFNγ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 10.

Table 4A. Comparison of immune responses between short- and long-term survivors (continued)

11	SART3-309	NT	NT	NA	199	537	≥2
	CypB-129	NT	NT	NA	804	530	negative
	UBE-43	NT	NT	NA	41	28	negative
	HNRL-501	NT	NT	NA	35	26	negative
12	SART2-93	0	0	negative	12	12	negative
	Lck-208	68	0	negative	15	11	negative
	Lck-486	123	348	≥2	21	<10	negative
	CypB-91	0	0	negative	15	11	negative
13	CypB-172	488	1000	≥2	151	<10	negative
	Lck-422	0	0	negative	12	<10	negative
	MAP-294	0	0	negative	41	21	negative
	HNRL-501	0	0	negative	15	16	negative
14	SART3-109	0	2045	≥10	9524	7283	negative
	Lck-208	0	2246	≥10	0	0	negative
	Lck-488	118	184	negative	70	86	negative
	PSA-248	0	0	negative	8	11	negative
15	SART3-109	0	0	negative	561	780	negative
	PAP-213	0	0	negative	112	125	negative
	PSA-248	0	0	negative	251	271	negative
	PSA-152	109	0	negative	29	<10	negative
16	SART3-302	0	931	≥10	251	223	negative
	CypB-172	0	0	negative	312	350	negative
	Lck-246	0	4326	≥10	186	199	negative
	ppMAPkkk-294	0	0	negative	132	126	negative
17	Her2/neu-553	0	NT	NA	31	NT	NA
	EZH2-291	0	NT	NA	26	NT	NA
	PTHrP-102	0	NT	NA	10	15	negative
	PSA-248	0	NT	NA	45	822	≥10
18	PAP-213	0	1289	≥10	534	18980	≥10
	PSA-248	0	0	negative	103	9855	≥10
	Her2/neu553	0	302	≥10	59	89	negative
19	SART3-109	0	0	negative	879	930	negative
	Lck-488	0	0	negative	641	663	negative
	PAP-213	0	191	≥10	143	138	negative
20	SART3-302	0	0	negative	11	10	negative
	UBE2V-43	0	0	negative	40	43	negative
	HNRL-501	0	0	negative	10	12	negative
	EZH2-569	0	130	≥10	38	39	negative
21	SART3-109	0	0	negative	287	7040	≥10
	Lck-486	0	0	negative	232	334	negative
	PAP-213	0	131	≥10	91	21230	≥10
	EZH2-291	753	0	negative	341	14258	≥10

NA, not available; NT, not tested. [a]Values indicate IFNγ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFNγ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFNγ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 10.

Table 4A. Comparison of immune responses between short- and long-term survivors (continued)

22	SART2-161	0	4923	≥10	24	90	≥2
	SART3-109	318	0	negative	141	151	negative
	Lck-486	0	0	negative	39	41	negative
	MRP3-1293	262	0	negative	30	30	negative
23	SART3-109	0	NT	NA	945	7675	≥2
	Lck-486	0	NT	NA	18	18	negative
	MRP3-1293	0	NT	NA	16	16	negative

NA, not available; NT, not tested. [a]Values indicate IFNγ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFNγ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFNγ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFNγ levels/post (sixth vaccination) IFNγ levels ≥ 10.

molecules, as has been reported on T cell receptor-engineered $CD4^+$ T cells.[34] Although we have no data on the association between HLA class II types in the vaccinated patients and anti-peptide IgG responses in the current study, this important issue will be addressed in further studies. Biological roles of peptide-specific IgG also need to be elucidated in the near future.

Increases IgG responses to the vaccinated peptides in patients showing longer survival could be, at least in part, in reflection of their better immune-competence with regard to helper T cell functions and subsequent B cell responses, although biomarkers predictable of better immune-competence with regard to favorite clinical benefits in response to peptide vaccinations are presently unclear. This issue is now under investigation and our preliminary results suggest that serum levels of C-reactive protein could be one of them (Noguchi M, et al. unpublished results). At the literature level, a number of prognostic factors have been evaluated with respect to their roles in determining the treatment strategy and ability to predict the response to therapy. Recent reports have shown some significant prognostic factors for CRPC patients. Smaletz et al. reported that performance status, lactate dehydrogenase (LDH), PSA and alkaline phosphate were significant prognostic factors of overall survival in HRPC patients.[35] Halabi et al. reported that performance status, Gleason sum, LDH, alkaline phosphatase, PSA, hemoglobin and visceral metastases were associated with survival in CRPC patients.[36] Unlike these reports, we identified the number of lymphocytes before vaccination and IgG responses after vaccination. These factors were not included in the other reports because most patients in the above studies were treated without specific active immunotherapy.

To address whether or not the long-term survived HRPC patients shown in **Table 4A and B** were different from "better performing, more likely to survive" patients who are not treated with cancer vaccines, we compared the results shown in this study with those of the TAX327 study of docetaxel-based regimens without the vaccine treatment, as a well known historical control, primarily because the disease conditions of HRPC patients in the TAX327 study were similar to those of this study subjects.[13,37] Namely, in the TAX327 study, a randomized, nonblinded, multinational phase III study involving 1,006 men with HRPC, they had a median survival of 16 to 20 months.[13,37] In that study, there were 800 deaths (80%) of 1,006 patients within 18 months of follow-up.[38] Therefore, long-term survivors for more than 30 months (900 days) shown in **Table 4A and B** could be considered to benefit from the peptide vaccination, and thus could be different from better performing HRPC patients who received the standard therapy without cancer vaccines. Of note, the beneficial roles of our personalized peptide vaccination have been also clearly demonstrated in the recently conducted randomized trial in consideration of the pre-existing host immunity.[39] Although several papers[2,4,39] have been reported on the relationships between lymphocyte counts and survival in advanced cancers, there have been no publications regarding antibody responses after peptide vaccinations and survival in cancer patients. Because all of our data were derived from the cancer patients that might have received a survival benefit from vaccinations, we cannot know whether the patients who were able to mount an antibody response and who were not lymphopenic were in fact more likely to control the cancer (and survive longer) even if they did not receive the vaccine. To address this issue, we will need to examine anti-peptide IgG responses after vaccinations with antigen peptides that do not affect patient survival. However, it would be very difficult for us to obtain such data.

One might have a question whether the IgG responses to the vaccinated peptides are unique to the peptides used in this study or widely observed in peptide vaccines conducted in other groups. Unfortunately, to our knowledge, no other groups have examined anti-peptide IgG responses after peptide vaccinations in the literature. Therefore, it would be impossible for us to decide whether the IgG responses that we detected in this study are unique to our peptide vaccines or not. Also, we do not know at the present time whether anti-peptide IgG responses are useful in general as an indicator of survival in cancer patients without vaccinations, because all of our data were derived from the cancer patients that received peptide vaccinations. Of note, however, the methods to identify the peptides used in this study are largely different from those by other groups. We at first established tumor-specific CTL clones and lines in culture of patients' PBMCs and autologous tumor cell lines, followed by identification of genes

Table 4B. Comparison of immune responses between short- and long-term survivors

Long-term survivors (n = 20)							
		Anti-peptide cellular response			Anti-peptide IgG response		
Pts no.	Peptide	Pre	Post (sixth)	Increased response	Pre	Post (sixth)	Increased response
24	SART2-93	83	0	negative	23	106	≥2
	SART3-109	0	922	≥10	252	11618	≥10
	Lck-488	116	85	negative	120	337	≥2
	PSMA-624	154	0	negative	58	276	≥2
25	Lck-208	71	0	negative	<10	<10	negative
	ART1-170	101	74	negative	<10	35	≥10
	ART4-75	101	0	negative	24	510	≥10
	CypB-84	141	0	negative	<10	<10	negative
26	CypB-129	0	0	negative	43	149	≥2
	Lck-246	0	0	negative	1155	22853	≥10
	HNRL-501	120	729	≥2	<10	51	≥10
	EIF-51	169	0	negative	NT	NT	NA
27	SART3-109	0	0	negative	1107	26809	≥10
	SART3-315	0	0	negative	<10	151	≥10
	Lck-208	60	0	negative	169	142	negative
	PSA-152	106	0	negative	81	114	negative
28	SART3-109	0	700	≥10	57	67	negative
	Lck-488	194	0	negative	90	94	negative
	MRP3-1293	108	0	negative	20	37	negative
	PSMA-624	133	0	negative	55	66	negative
29	SART2-161	143	0	negative	27	15	negative
	SART3-109	0	1032	≥10	263	418	negative
	Lck-488	208	0	negative	81	56	negative
	PSA-248	0	0	negative	70	78	negative
30	UBE2V-43	0	720	≥10	48	5083	≥10
	EIF4EBP-51	261	294	negative	23	83	≥2
	PSA-170	565	0	negative	62	32	negative
	EGF-R-479	0	0	negative	71	67	negative
31	SART3-109	80	3502	≥10	121	115	negative
	Lck-486	0	0	negative	45	35	negative
	SART1-690	0	0	negative	301	426	negative
	SART2-899	677	147	negative	98	260	≥2
32	MAP-432	64	480	≥2	178	1259	≥2
	Lck-246	0	720	≥10	11	451	≥10
	Lck-422	0	130	≥10	13	15	negative
	UBE-43	0	0	negative	15	1534	≥10
33	SART3-309	59	0	negative	142	179	negative
	CypB-172	324	0	negative	129	121	negative
	WHSC-103	70	0	negative	<10	<10	negative

NA, not available; NT, not tested. [a]Values indicate IFN-γ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFN-γ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 10.

Table 4B. Comparison of immune responses between short- and long-term survivors (continued)

	WHSC-141	0	0	negative	<10	<10	negative
34	SART3-315	0	0	negative	NT	NT	NA
	PSA-248	0	0	negative	29	4413	≥10
	PSM-624	0	0	negative	<10	<10	negative
	PAP-213	0	0	negative	41	25783	≥10
35	SART2-93	51	912	≥2	99	934	≥2
	Lck-488	108	61	negative	74	721	≥2
	PSA-152	0	0	negative	10	900	≥10
	PSA-248	0	0	negative	717	1058	negative
36	SART3-109	0	0	negative	184	228	negative
	Lck-208	56	0	negative	23	28	negative
	PAP-213	57	1802	≥10	13	379	≥10
	SART3-315	158	1121	≥2	NT	NT	NA
37	SART3-302	0	1417	≥10	40	11118	≥10
	SART3-309	0	0	negative	108	424	≥2
	PSA-170	0	0	negative	21	1221	≥10
	PSA-178	0	0	negative	32	1889	≥10
38	SART3-302	0	0	negative	309	15523	≥10
	CypB-129	0	0	negative	91	858	≥2
	PSMA-441	0	1163	≥10	NT	NT	NA
	PSMA-711	0	0	negative	NT	NT	NA
39	SART3-109	0	282	≥10	134	9562	≥10
	Lck-486	449	126	negative	14	12	negative
	PSA-248	157	172	negative	12	14507	≥10
	PTHrP-102	209	119	negative	16	11256	≥10
40	SART2-161	81	0	negative	1433	1451	negative
	SART3-109	0	0	negative	5368	24796	≥2
	PSA-248	0	0	negative	47	3854	≥10
	EZH2-291	0	784	≥10	2027	6674	≥2
41	SRAT3-109	0	0	negative	170	992	≥2
	Lck-488	0	0	negative	54	30278	≥10
	MRP3-1293	312	0	negative	21	3996	≥10
	PSA-248	78	0	negative	25	29669	≥10
42	CypB-129	464	0	negative	348	468	negative
	HNRL-501	0	436	≥10	859	1298	negative
	EIF-51	0	102	≥10	714	6797	≥2
	EZH2-569	0	899	≥10	2501	305	negative
43	CypB-129	140	0	negative	26	38	negative
	UBE-43	141	3424	≥10	27	1910	≥10
	EZH2-569	313	417	negative	18	446	≥10
	Her2-484	69	0	negative	<10	15	≥10

NA, not available; NT, not tested. [a]Values indicate IFN-γ production of peripheral blood mononuclear cells (PBMCs) reactive to the corresponding peptide (pg/mL). A two-tailed Student's t-test was employed for the statistical analyses. A well was considered positive when the level of IFN-γ production in response to a corresponding peptide was significantly higher ($p < 0.05$) than that in response to an HIV peptide, and also when the mean amount of IFN-γ production in response to a corresponding peptide was > 50 ng/ml compared with that to an HIV peptide. [b]Plasma levels of peptide-specific IgG were measured using the Luminex™ system as previously reported.[12] Values indicate fluorescence intensity units (FIU) of IgG antibodies reactive to the corresponding peptide. Positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 2 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 2. In addition, positive immune responses were defined as either pre-IgG levels/post (sixth vaccination) IgG levels ≥ 10 or pre-IFN-γ levels/post (sixth vaccination) IFN-γ levels ≥ 10.

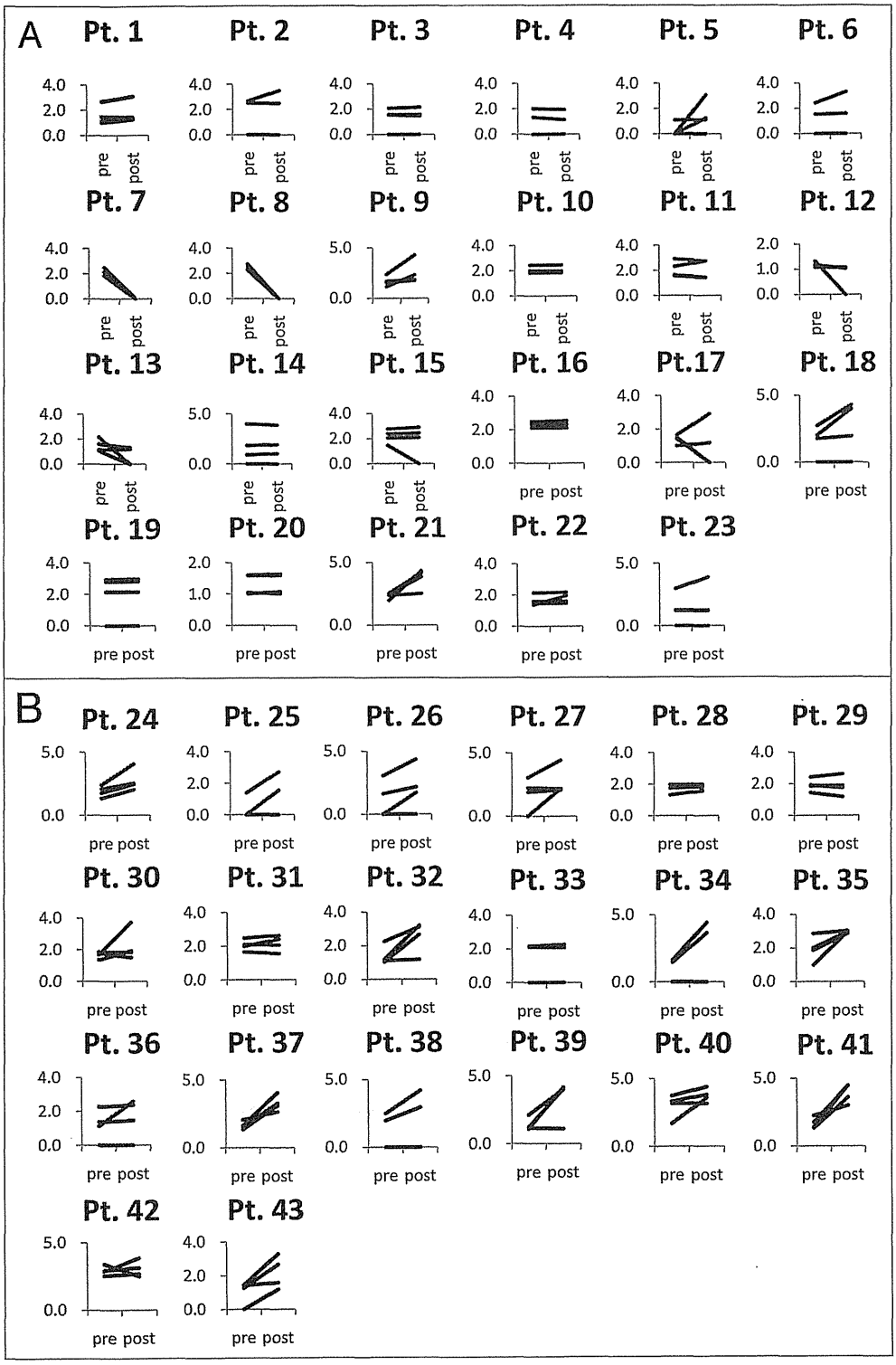

Figure 2. Changes of IgG levels reactive to each of the vaccinated peptides during pre- and post-vaccination periods (sixth) for short-term survivors (A) and long-term survivors (B). The vertical bars denote log 10 scores in order to better represent n-fold increases in IgG levels. NA, not available.

encoding tumor associated antigens by means of cDNA expression cloning technique reported by Boon et al.[40] Among many peptide candidates coded by these antigens, the peptides capable of inducing CTL reactive to tumor cells in HLA-class IA-restricted and peptide-specific manners were screened by incubation of PBMCs from cancer patients. Interestingly, many of these identified peptides were also recognized by pre-vaccination plasma IgG of cancer patients as reported previously.[41] Subsequently, to save limited source of patients' PBMCs, a large numbers of peptide candidates holding the motifs for binding to HLA-class IA molecules were at first tested for their ability to react to pre-vaccination patients' IgG, followed by testing their ability to induce HLA-class IA-restricted and peptide-specific CTL reactive to tumor cells in patients' PBMCs. Therefore, the peptides employed in this study mainly selected by their ability to be recognized by both cellular and humoral immunity. As far as we know, no other clinical trials of peptide-based cancer vaccine provided such peptides; other groups used the peptides capable of inducing only CTL without paying attention to their reactivity to IgG.

In conclusion, we have shown that IgG response is superior to CTL response as an immunological biomarker that is predictive of the overall survival of advanced cancer patients under treatment with personalized peptide vaccination. These results might provide new insights to better understand biomarkers of cancer vaccine for advanced cancer patients. Application of these results for the other types of cancer vaccine using common proteins or common peptides in a non-personalized manner could be worthy to consider.

Patients and Methods

Study population. This study was conducted through the serial collection of blood samples from 500

consecutive patients positive for HLA-A24, -A2 or -A3 supertypes with advanced cancer, who entered into phase I, I/II and II clinical trials for personalized peptide vaccination at 8 institutions (Kurume University Hospital, Kinki University Hospital, Okayama University Hospital, Hokkaido University Hospital, Niigata University Hospital, Kitasato University Hospital, Kansai Medical University Hospital and Yamaguchi University Hospital, Japan) between October 2000 and October 2008. The ethics review committee of each institution accepted the present project and blood samples were collected at baseline (before vaccination), at sixth vaccination, and during the follow-up period after written informed consent was obtained. All 500 patients suffered from advanced cancer originating in the prostate (n = 174), colon and rectum (n = 74), pancreas (n = 50), stomach (n = 42), brain (n = 33), uterus (n = 28), lung (n = 22), kidney (n = 13), skin (n = 12), breast (n = 11), bladder and urinary tracts (n = 10) and elsewhere (n = 31) (**Table 1A and B**). The safety, immune responses and clinical responses in most of those studied had been reported previously.[6,13-29] The exceptions were the results of vaccinations against bladder cancer, breast cancer, some pancreatic cancer cases, and those from HLA-A3 supertype-positive patients. These unpublished results have now been submitted for publication or are under preparation based on results obtained after October 2008. In the sub-analysis, 20 patients who survived more than 900 days (long-term survivors) and 23 patients who died within 300 days (short-term survivors) were selected to compare immune responses from a total of 174 patients with CRPC.

Personalized peptide vaccination and immunological assessment. Personalized peptide vaccination is based on a pre-vaccination measurement of peptide-specific CTL precursors and anti-peptide IgG in the circulation of cancer patients reactive to vaccine candidates, followed by administration of only reactive peptides (up to four peptides) as reported previously.[25-29] Selected peptides were mixed with incomplete Freund's adjuvant (Montanide ISA-51VG; Seppic, Paris, France), and four peptides of 1.5 ml emulsion each at doses of 3 mg/peptide were injected subcutaneously into the regional lymph node area. A total of 77 candidate peptides (32 peptides for HLA-A24-positive cancer patients, 37 for HLA-A2 and 8 for HLA-A3 supertypes) were used in the personalized peptide vaccination. All of these peptides can induce HLA-A24-, A2- and A3-supertype-restricted and tumor-specific CTL activity in PBMCs of cancer patients.[6,13-29,42-44]

Before the first vaccination and 7 days after every sixth vaccination, 30 ml of peripheral blood was obtained and PBMCs were isolated by means of Ficoll-Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using the previously reported culture method.[25-29] Briefly, PBMCs (1 x 10^5 cells/well) were incubated with 10 μM of a peptide in 200 μl of culture medium in u-bottom 96-well microculture plates (Nunc, Roskilde, Denmark). Half of the medium was removed and replaced with a fresh medium containing a corresponding peptide (20 μM) every 3 days. After incubation for 14 days, these cells were harvested and tested for their ability to produce IFNγ in response to CIR-A2402 or T2 cells that were pre-loaded with either a corresponding peptide or HIV peptides (RYL RQQ LLG I for HLA-A24 and LLF GYP VYV for HLA-A2) as a negative control. For HLA-A3 supertype-positive cases, the cells were harvested and tested for their ability to produce IFNγ in response to CIR-A1101, -A31012 or -A3303 cells that were pre-loaded with either a corresponding peptide or an HIV peptide (RLR DLL LIV TR) as a negative control. The level of IFNγ was determined by enzyme-linked immunosorbent assay (ELISA) (limit of sensitivity: 10 pg/ml). All assays were performed in quadruplicate. A two-tailed Student's t-test was employed for the statistical analyses.

The levels of anti-peptide IgG were measured using the Luminex™ system, as previously, reported.[25-29,45] In brief, plasma was incubated with 25 μl of peptide-coupled color-coded beads for 2 h at room temperature on a plate shaker. After incubation, the mixture was washed with a vacuum manifold apparatus and incubated with 100 μl of biotinylated goat anti-human IgG (chain-specific) for 1 h at room temperature. The plate was then washed, followed by the addition of 100 μl of streptavidin-PE to wells and was incubated for 30 min at room temperature on a plate shaker. The bound beads were washed three times followed by the addition of 100 μl of Tween-PBS to each well. Fifty microliters of sample was used for detection with the Luminex™ system.

For evaluation of immune responses during the treatment, peptide-specific CTL precursors among PBMCs and serum levels of peptide-specific antibodies were measured every sixth vaccination. Positive immune responses were defined as either post (sixth vaccination) IgG levels/pre-IgG levels ≥ 2 or post (sixth vaccination) IFNγ levels/pre-IFNγ levels ≥ 2. In addition, in the analysis between long- and short-term survivors, positive immune responses were defined as either post (sixth vaccination) IgG levels/pre-IgG levels ≥ 10 or post (sixth vaccination) IFNγ levels/pre-IFNγ levels ≥ 10.

Adverse events and clinical responses. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. The clinical responses were evaluated on the basis of clinical observations and radiological findings. Patients were assigned a response category according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Statistical methods. Overall survival and 1 and 3 year survival rates were determined by Kaplan-Meier actuarial analysis and the difference between survival curves was assessed by the log-rank test. Cox proportional hazards regression model was used for univariate and multivariate analyses to identify combinations of factors that had a significant impact on survival. All baseline parameters in the survival and proportional hazards regression analysis were analyzed as dichotomous variables using the overall mean values as cut-off levels. All statistical calculations were carried out using the StatView® program (SAS Institute Inc., Cary, NC). A two-sided significance level of 5% was considered statistically significant.

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Disclosure Statement

Although all authors completed the disclosure declaration, the following authors indicated a financial or other interest that is relevant to the subject matter under consideration in this article.

Employment or Leadership Position

Akira Yamada is a part-time executive of Green Peptide Co.; Consultant or Advisory Role: Kyogo Itoh, Green Peptide Co.; Stock Ownership: Kyogo Itoh, Akira Yamada, Green Peptide Co.; Honoraria: none; Research Funding: Kyogo Itoh, Akira Yamada, Green Peptide Co.; Expert Testimony: none; Other Remuneration: none.

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Editorial

Exploring immune therapy for renal cancer

Edited by Yutaka Enomoto, M.D.
Managing Editor

A paradigm shift in cancer immunotherapy

It is now clear that the immune system (both innate and adaptive) not only protects the host from tumor development, but also selects for the formation of tumor cell variants more resistant to immune attack.[1] This implies that clinically-detectable malignancies derive from cancer cells previously "edited" by the host's immunity. It follows that immunotherapy regimens must take into account the fact that the tumor has already found a way to circumvent immune recognition/elimination, including the creation of immune suppressive local tumor environments. It is therefore crucial to develop strategies aimed at overcoming such immunosuppressive mechanisms, as well as enhancing effector T cell responses.

After many disappointments, years of effort in tumor immunology have finally resulted in two important developments in cancer immunotherapy. One is the US Food and Drug Administration (FDA) approval of sipuleucel-T, and the other is the application of anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) antibody therapy. These two innovations, together with the approval of tyrosine kinase inhibitors, will have a huge impact on cancer immunotherapy for the treatment of renal cell carcinoma (RCC) and are likely to cause a paradigm shift in treatment rationales. The emphasis will be increasingly on (i) overcoming immunosuppressive environments rather than mere activation of immune responses; and (ii) a focus on the individual "patient response" rather than "tumor response". I hope readers will get a sense of this paradigm shift in cancer immunotherapy. In this editorial, we have brought together a series of reviews from experts to explore immune therapy for renal cancer.

RCC, like malignant melanoma, appears to be one of the most immune-sensitive cancers occasionally undergoing dramatic spontaneous regressions. This has encouraged a strategy of using immunomodulating therapies more frequently for these cancers than many others. Nearly two decades ago, cytokine-based therapy using high dose interleukin-2 (HD IL-2) was approved by the US FDA, in 1992, and results of its use to treat advanced RCC were already reported in 1995.[2] HD IL-2 treatment has resulted in durable tumor remission in a minority of patients, but with severe adverse effects. Cytokine-based therapy using low-dose IL-2 and/or interferon-α (IFN-α), with reduced side-effects, was standard therapy for RCC for a long time. However, this approach has now been more or less superseded after the development of targeted therapies including vascular endothelial growth factor (VEGF) inhibitors, such as bevacizumab, or multikinase inhibitors, such as sunitinib and sorafenib.[3] In the next chapter, Tomita reviews cytokine therapies in the era of targeted therapy. He points out the difficulties and importance of choosing treatments according to the tumors and characteristics of patients rather than merely following guidelines. He proposes that the combination therapy of cytokines and targeted drugs is the future direction.

After the first identification of a human tumor antigen recognized by $CD8^+$ T cells from a melanoma patient in 1991,[4] many other such targets have been identified in different cancers. However, compared with melanoma, only a few promising tumor antigens have been identified in RCC. This has limited the development of tumor antigen-specific immunotherapies.[5] It is to be expected that if more tumor-specific antigens were to be identified in RCC, tumor antigen-based approaches would become more feasible, as in malignant melanoma, given that RCC can efficiently induce immune responses. To overcome this limitation, Kobayashi developed adoptive immunotherapy using γδ T cells that can recognize tumors in a major histocompatibility antigen (MHC)-independent manner. His group has successfully expanded γδ T cells from RCC patients' peripheral blood mononuclear cells (PBMC) using 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP). The results of a phase I clinical trial include one complete response, five stable disease and five progressive disease.

Tumor antigen-specific immunotherapeutic agents, including peptide-based vaccines, DNA vaccines consisting of genes encoding the target antigen or dendritic cell (DC) vaccines pulsed with tumor antigen peptides and so on, have been developed and are now being tested for treating several different cancers. In a multicenter phase III trial, significantly greater tumor regression after radical nephrectomy was observed in patients who received autologous tumor lysate vaccination than with surgery alone.[6] In a 10-year survival analysis of 1267 RCC patients undergoing radical nephrectomy subsequently treated with autologous tumor cell vaccines, it was shown that this adjuvant treatment resulted in a significantly improved overall survival in pT3-stage RCC patients.[7] Controlled trials using the recent TNM classification and incorporating known risk factors for prognosis are warranted. In this issue, Tatsugami and Naito review the principles of DC-based immunotherapy. Based

on their careful observation that regulatory T cells were decreased by IFN-α therapy, but were increased by IL-2, they used IFN-α as an adjuvant for DC therapy. Suekane *et al.* review peptide-based cancer vaccine. They carried out a phase I trial of so-called personalized peptide vaccine for cytokine-refractory metastatic renal cancer patients. Though the results of clinical trials are not satisfactory, combination therapies with personalized peptide vaccine and molecular target drugs or cytokines are anticipated to achieve a breakthrough in the treatment of RCC.

Although the importance of patient selection and personalized treatment for RCC are shown by other authors, Eto *et al.* review personalized treatment in the immunotherapy for metastatic RCC. They showed that the single nucleotide polymorphism (SNP) in signal transducer and activator 3 (STAT3) were associated with better response to IFN-α. It is expected that the improved patient selection will result in better clinical response.

Although it is not a RCC vaccine at this point, we would like to mention the first such agent to gain FDA approval: sipuleucel-T (Provenge), developed for treating advanced prostate cancers. Sipuleucel-T is based on the individual patient's monocytes, which are incubated with a fusion protein consisting of granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostatic acid phosphatase (PAP). Antigen-specific vaccination with this product induced marked infiltration of effector T cells specific for PAP into the prostate gland, and yielded a statistically significant difference in overall survival between certain immunotherapy groups and the placebo group.[8] After completion of three phase III trials, sipuleucel-T was approved in April 2010 as the first antigen-specific immunotherapy, thus becoming a landmark for the field of cancer immunology and immunotherapy.

Another recent breakthrough in this field is the development of cancer immunotherapy using CTLA-4 antibodies (ipilimumab and tremelimumab). These antibodies inhibit an immunological checkpoint and take the brakes off T cell responses, amplifying the activation of $CD4^+$ and $CD8^+$ effector cells. CTLA-4 blockade has been evaluated in many malignancies, but again, the most mature data are available from melanoma patients. In August 2010, the results of phase III trials of ipilimumab for melanoma were reported.[9] This new treatment boosted immune responses against melanoma and yielded significant survival advantages for treated patients. Ipilimumb has also been tested in several other cancers including RCC and prostate, and objective clinical responses have been reported.[10,11] However, use of this agent was also associated with clinically important immune-related toxicity. Critical issues related to autoimmunity as side-effects still remain; nonetheless, CTLA-4 blockade is likely to be the next promising cancer immunotherapy; FDA announced its approval of ipilimumab for the treatment of advanced metastatic melanoma on 25 March 2011.

For many years, we have focused on how to activate tumor-specific immune responses by inducing and expanding CTL and improving the recognition of tumor antigens to develop cancer immunotherapy. Now we understand more about the immunosuppressive mechanisms acting at the tumor site and the crucial importance of the tumor microenvironment. The phase II clinical trials with ipilimumab proved the concept that overcoming immunosuppressive conditions and breaking immune tolerance are important for developing effective therapies. Many attempts are now being made to counteract the commonly high circulating levels of immunosuppressive factors in cancer patients, including TGF-β, IL-10 and VEGF, regulatory T cells and myeloid-derived suppressor cells (MDSC), as well as the immunological checkpoints mediated by cell surface molecules, such as CTLA-4, PD-1 and others.

In a placebo-controlled randomized phase III trial in which sipuleucel-T was given to patients with metastatic asymptomatic castration-resistant prostate cancer (CRPC), the primary end-point was progression-free survival.[12] Although the primary end-point did not achieve statistical significance ($P = 0.052$), a difference in overall survival was statistically significant ($P = 0.01$, HR = 1.70). In a randomized phase II trial of a poxvirus-based vaccine approach targeting prostate-specific antigen in metastatic CRPC patients, the primary end-point, progression-free survival, was also not met, but again, an overall survival advantage favoring the investigational agent was observed.[13] In phase II and III trials of ipilimumab for the treatment of metastatic melanoma, there was a significant improvement in overall survival among patients.[9] In all these studies, response patterns different from those seen with other therapies, for example, chemotherapy, are beginning to be recognized. Thus, there might be a tumor burden increase at first, even with development of new lesions, but nevertheless, it is later followed by a meaningful clinical response. Therefore, new response criteria, which could be termed "immune-related response criteria", or irRC, have been proposed.[14] Because immunotherapy must induce, facilitate and/or amplify cellular immune responses before it can affect tumor burden or patient survival, adjustments of established end-points to address the different kinetics of immunotherapy compared with cytotoxic agents are required for appropriate investigation of future immunotherapies in clinical trials. We can now feel confident that this paradigm shift in cancer immunotherapy is ushering in an era of targeted biological therapy, which will result in much improvement of the cancer patient's lot.

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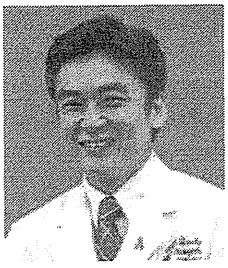
Immunotherapy in targeted therapy era

Reality in the cytokine era



Until 2005, drugs of systemic therapy to metastatic renal cell carcinoma (mRCC), which were shown to be beneficial, were cytokines. Interferon-α (IFN-α) had been proven to have longer survival compared with methylprogesterone or vinblastine in two randomized trials. Interleukin-2 (IL-2) showed a low, but durable, complete response despite severe toxicity when used in a higher dose. It had to be referred to as a "modest response", because their response rates were 10–15%. Once patients had progression under cytokine therapy, they succumbed to disease without exception. However, it is true that a small number of patients survived longer than 5, even 10 years, with cytokine therapy.[15]

Targeted drugs: Their promising results and limitations

The first début of a targeted drug to mRCC was in 2005. Sorafenib, a tyrosin kinase inhibitor (TKI) to inhibit angiogenesis, showed obvious superiority to the placebo in prolongation of progression-free survival (PFS) in patients having regrowth of tumors after cytokine therapy.[16] After targeted drugs consecutively represented promising results including longer PFS, higher response rates were found in different clinical settings. Among them, clinical evidence suggests that sunitinib is a first-line drug to favorable and intermediate Memorial Sloan-Kettering Cancer Center (MSKCC) risk RCC patients.[17] Temsirolimus is considered as a first line drug for RCC patients with poor risk background (more than two).[18] Everolimus is the first choice for patients with disease progression after treatment with TKI.[19] The combination of bevacizumab and IFN-α,[20] and pazopanib is also a treatment option to naive and cytokine-refractory RCC. Thus, building a new road for the cytokine-refractory "impasse" has surely succeeded with targeted drugs, meaning longer survival is promised after initial cytokine therapy. Also, another initial pathway, "sunitinib" leads to longer survival than that of cytokine, and it is also true with "temsirolimus" in poor risk patients.

One of biggest issues that we have is these choice of drugs do not necessarily give the same survival in each patient who has different a background in terms of tumor and patient character. For instance, no tumor character other than clear cell carcinoma is considered in the well-known RCC treatment algorism. Therefore, it cannot be necessarily applied to non-clear histology. In addition, when we talk about the character as its quality, one can ask where the consideration for tumor quantity is in the algorism. It is absolute that patients bearing a higher tumor volume have poorer prognosis. I cannot help but frown when a resident says, irrelevantly to a patient's and tumor character, and simply selected a drug according to the guidelines.

Cytokines revisited

Thus, it is becoming more evident that "mighty" targeted drugs might be an illusion. One of the new movements of cytokines was to explore the possibility of its combination therapy. A promising regime is bevacizumab plus IFN-α, showing longer PFS comparable with sunitinib. In European countries, this combination therapy is one of the major options of first-line treatment for mRCC. We can learn another reality from a recent high-dose IL-2 treatment study, SELECT (ASCO 2011 # 4514), which showed a remarkable response rate and higher complete response (CR) rate than targeted drugs. As for durable CR, immunotherapy might be dominant to obtain a longer cancer-free status than targeted drugs. Presumably, memorized immune cells to attack RCC cells might render a longer clinical CR by continuing the destruction of newly bearded RCC cells from remnant mother cells. Furthermore, the higher response rate shows that the selection of treatment modality based on the experienced physicians' instinct might be justified. However, it is a medical science to clarify practical evidence for prevailing fruit of "the brilliant instinct based on experience".

What is the most beneficial treatment in each patient?

It seems that some patient groups might have vulnerable characteristics to cytokine therapy, but it has not been fully investigated. Gene polymorphism[21] or apotosis-related molecule expression[22] were proven to have a significant correlation to susceptibility to immunotherapy. Clinical demographics that have an implication to response should be accumulated, such as metastasis site, tumor volume, patient age and laboratory findings, because it is easier to apply them to a treatment selection.

A recent important key word has been "order made treatment", meaning properly individualized treatment. To achieve a more sophisticated made-to-order treatment, more intensive and detailed investigation is warranted in a larger number of mRCC patients. It will give us a "breakthrough" for the present situation, and authentic collaboration is definitely needed.

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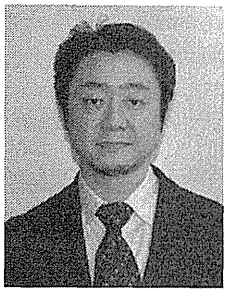
Immunotherapy with dendritic cells for renal cell carcinoma



Immunotherapy plays a significant role in the management of renal cell carcinoma (RCC) patients with metastatic disease and various treatments for RCC, such as cytokine-, antigen- or dendritic cell (DC)-based immunotherapy, have been carried out in multiple clinical trials. Although antitumor immune responses and clinically significant outcomes have been achieved in these trials, the response rate is still low and very few patients show long-term clinical improvement. This review summarizes the principles of antigen-specific immunity and DC-based immunotherapy for RCC.

Antigen-specific immunity and dendritic cells

Tumor antigens are processed by the proteasome within tumor cells and are presented at the surface as peptides on major histocompatibility antigen (MHC) class I molecules. Recognition of these complexes by antigen-specific cytotoxic T lymphocytes (CTL) triggers CTL-mediated cytotoxicity. CTL cannot be activated by direct recognition of antigen on tumor cells; they require activation by antigen-presenting cells (APC), such as dendritic cells (DC), and CD4 helper T cells. The generation of CTL from CD8 T cells requires not only the binding of the T cell receptor on the CD8 T cell with a peptide antigen presented on MHC class I molecules of the APC, but also a costimulatory signal provided by molecules including CD80 and CD86 (Fig. 1). DC act as APC and are able to present foreign antigens to T cells and play a central role in regulating immune responses. They exist in a variety of tissues including lymphoid tissue, non-lymphoid organs and blood. They are able to take up particulate and soluble antigens, and migrate into the lymph nodes. Once in the lymph nodes, DC present antigen to T cells and induce specific immune responses, including the induction of CTL.

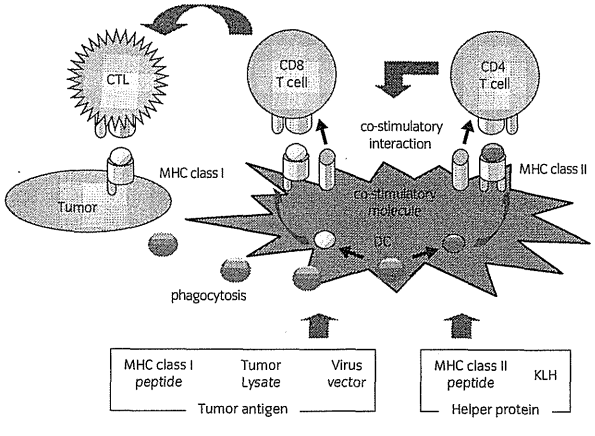


Fig. 1 Induction of antigen-specific tumor immunity via dendritic cells.

Since it was first reported that DC can be generated from peripheral blood cells *in vitro*, immunotherapy with DC has been used to treat cancer patients.[23] DC are generated from both myeloid and lymphoid progenitors derived from the bone marrow. Myeloid progenitors induce mainly cellular immunity. Immature DC can be induced from CD34+ cells, CD14+ mononuclear cells and adherent-mononuclear cells in peripheral blood by culturing them in a medium containing granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. These immature DC are phagocytic, but they do not possess a high ability to present antigen. After exposure to cytokines, such as TNF-α, IL-1, IL6 prostaglandin E2 and Flt-3 ligand, immature DC differentiate into mature DC and show a high degree of antigen-presenting ability, the ability to induce and express costimulatory molecules, and the ability to augment MHC class I and class II molecule expression.

Immunotherapy with DC for renal cell carcinoma

Immunotherapy with DC has relied on a variety of methods for generating DC, different types of antigen and different adjuvants.[24] DC and antigens for vaccination have been derived from autologous (auto) and allogeneic (allo) cells. Although immunotherapy with allo tumor cells tends to induce immune responses against common antigens of the tumor, immune responses against non-self-antigens can also be induced. Auto-DC might have an advantage for host immune systems compared with allo-DC, because the allo-DC might be recognized and attacked as non-self cells by the immune system.

The serological identification of antigen by recombinant cDNA expression cloning (SEREX) method is a serological approach that combines antigen cloning techniques to identify tumor antigens based on IgG antibodies in patient serum and subsequent identification of the tumor antigens from cDNA libraries. Immunotherapy with DC and tumor peptides that were identified using the SEREX procedure was carried out in some experiments. Because most tumor antigens are only weakly immunogenic, adjuvant approaches using cytokines, keyhole limpet hemocyanin (KLH) and MHC class II peptide have been used.[24]

Immunotherapy with DC in combination with cytokines

IL-2 can stimulate the maintenance and proliferation of T cells activated by DC *in vitro*, but the combination of IL-2 with DC therapy was shown to have no advantage in a clinical trial.[25] Recent reports show that IL-2 contributes to

the maintenance of regulatory T (Treg) cells, which play a key role in regulating immune responses, such as those responsible for autoimmune diseases, graft rejection and antitumor immunity. The suppression of DC-activity by Treg cells included their phenotypic maturation, pro-inflammatory cytokines secretion and the resulting ability to present antigens. In our research on RCC patients, Treg cells were decreased after initiation of IFN-α therapy, but were increased after IL-2 therapy.[26]

From the aforementioned results, we used IFN-α as an adjunctive agent for DC therapy, because IFN-α enhances the antitumor effect and activates DC.[27] We showed the safety and efficacy of combination therapy with IFN-α and DC in patients with progressive renal carcinoma after IFN-α and IL-2 therapy.[28] Further examination is required to determine what cytokine will enhance antitumor immunity caused by DC therapy.

Conclusion

DC-based immunotherapy has been evaluated in phase I/II studies that were not randomized and were based on different trial designs. Although these studies showed tumor-specific immune responses, such as delayed-type hypersensitivity reactions (DTH), IFN-γ production or lymphocyte proliferation in response to tumor cells, those responses were surrogate end-points. Significant clinical responses, such as tumor regression, have been seen at a low frequency. The requirements for specialized culture facilities and expertise in DC therapy make it difficult to treat only a small number of cases at a time. Further progress in this field will require larger comparative trials of patients in earlier stages of disease in order to determine the efficacy of this approach.

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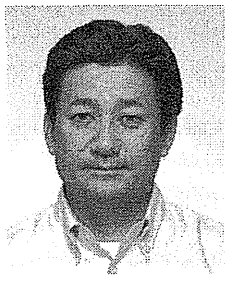
Personalized peptide vaccination for renal cancer patients

Cancer vaccine for renal cancer patients



Of the recent advances in the treatment of metastatic renal cell carcinoma (RCC), immunotherapy remains an important field of investigation. Because RCC is one of the most immunoresponsive cancers in humans, immunotherapy remains a basis of promising treatment strategies. Non-specific stimulations through cytokines, passive specific immunotherapy with antibodies and active specific immunotherapy seem to be suitable options for RCC. The goal of developing curative RCC vaccines is to stimulate the immune system to recognize and to destroy existing tumor cells. RCC vaccines are explored in the metastatic and adjuvant setting.

Therapeutic cancer vaccines are currently under active clinical investigation worldwide.[24] Such therapeutic vaccines can be divided into autologous tumor cell-based vaccines, genetically modified tumor cell-based and dendritic cell (DC)-based vaccines, and peptide-based vaccines. We have been investigating peptide vaccination for metastatic RCC patients, because peptides are non-biological chemicals that can be synthesized on an industrial scale under the current standards of good manufacturing practice. In contrast, cell-based vaccines, such as tumor-cell vaccines or peptide-pulsed DC therapies, have several disadvantages, including limited cell sources for each patient, difficulties in maintaining uniform vaccine quality, labor intensity and high production costs.

Identification of target antigens in specific immunotherapy for renal cancer patients

Several new RCC-associated antigens and derived HLA-class I ligands were recently identified. We previously reported that most target antigens encoding peptides used for vaccination were expressed in cell lines from renal cancer cells.[29] They were SART1, SART2, SART3, MRP3, EZH2, HER2/neu and PTHrP. In those studies, however, prostate-specific antigen (PSA) or prostatic acid phosphatase (PAP) antigen were undetectable in the cell lines. Therefore, we further investigated expression of PSA and PAP antigens in the primary culture of both RCC cells and non-tumorous kidney cells by the reverse transcription polymerase chain reaction method, and found the expression of PAP, but not PSA, in both types of cells. Subsequently, we investigated PSA protein expression in metastatic RCC cells by the immunochemical staining method, and found that PSA antigens were expressed in RCC cells from two of four samples that were surgically harvested from lung metastases. Furthermore, we found that carcinoembryonic antigen, ubiquitin-conjugated enzyme variant Kua and Lck antigens were also expressed in both types of the cells.

What is a personalized vaccination?

We showed that each cancer patient has different sets of activated T cells against cancer antigens, and that these activated T cells are detectable before vaccination. Our approach to developing a cancer vaccine for advanced cancer patients is to carry out six weekly injections of four peptides selected from the patients' own peripheral blood mononuclear cells (PBMC) based on the strength of pre-existing

immunity toward the cancer antigen; we call this a personalized vaccination. We started a phase I clinical study of peptide vaccination with a regimen of conventional prophylactic vaccination; however, the predesigned vaccination induced a weak primary immune response and no clinical response. Personalized vaccinations have induced both a strong secondary immune response and a clinical response.[30]

Biomarkers

There are as yet no definitive biomarkers to predict clinical responses, which hamper the development of cancer vaccines. In a total of 500 advanced cancer patients who received a personalized peptide vaccination, we have shown that the IgG response is superior to cytotoxic T lymphocyte (CTL) response as an immunological biomarker that is predictive of overall survival.[31] These results might provide new insights to better understand biomarkers of cancer vaccine for advanced cancer patients. Application of these results to other types of cancer vaccine using common proteins or common peptides in a non-personalized manner could be worth considering.

Phase I trial of personalized vaccination for renal cancer patients

Only a limited number of clinical studies with peptide-based RCC vaccines have been reported to date. We carried out a phase I trial of personalized peptide vaccination for cytokine-refractory metastatic renal cancer patients.[32] Among 10 patients, there were no major adverse events, although most of the patients developed grade 1 or 2 local dermatological reactions at the vaccine injection sites. There were no hematological, hepatic or neurological toxicities, and performance status remained stable during the vaccine treatment. There was only a slight increase in peptide-specific interferon-γ production in the postvaccination PBMC, despite the higher levels of CTL activity in prevaccination PBMC. In contrast, an increase in peptide-specific IgG of postvaccination (sixth) plasma was observed in the majority of patients. These findings show that humoral responses, but not cellular responses, were markedly boosted by personalized peptide vaccination in cytokine-refractory metastatic RCC patients. These findings might encourage further clinical trials of personalized peptide vaccination. Combination therapy with personalized peptide vaccination and a molecular target drug or cytokines might provide a breakthrough in the treatment of advanced renal cancer.

Conclusions

Despite the limited clinical efficacy of most of the therapeutic vaccines in RCC studies to date and their often small number of patients and non-standardized methodology, there is still interest in the use of vaccines that have much less toxicity than other current therapies for RCC. The discovery of new tumor-associated antigens or immunostimulatory peptides, and increasing insight in basic immunology and molecular biology will certainly lead to the development of more powerful RCC vaccines.

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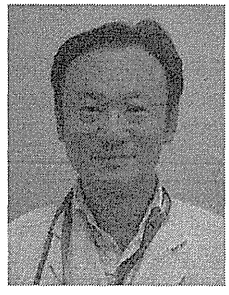
New strategy of adoptive-immunotherapy using γδ T cells



More than a half a century has passed since Foley reported that mice could reject the sarcomas re-inoculated in skin after resection of the same strain of sarcomas. This research showed that the immune system is involved in tumor surveillance. Morgan discovered T cell growth factor in 1976 and Taniguchi identified the involvement of the interleukin (IL)-2 gene in 1983. In the 1980s, Rosenberg developed the use of adoptive immunotherapy of lymphokine-activated killer (LAK) and systemic administration of recombinant IL-2. Contrary to expectations, although initially showing promise, the attempt did not work well in the long term. LAK cells are composed of antigen non-specific T cells and natural killer cells, and have the ability to kill various types of human tumor cells *in vitro*. LAK cells are also efficient at killing such cells *in vivo* and even in mouse models. The cause of eventual failure was not fully understood at that time. Boon reported in 1991 the identification of the human gene *MAGE-1*, which directs the expression of antigen recognized on a melanoma by autologous cytolytic T lymphocytes (CTL). Many researchers worked energetically to discover tumor-associated and/or tumor-rejected antigens, and various approaches were developed involving immunotherapy, using mainly antigen specific αβ T cells as CTL. Active immunization with tumor antigens and also tumor antigen-pulsed dendritic cells (DC) has the same objective of inducing antigen-specific αβ T cells.[33] In the immune system, αβ T cells are so sophisticated that they can not adapt to mutation, loss or downregulation of tumor antigens on the tumor cells, which are the

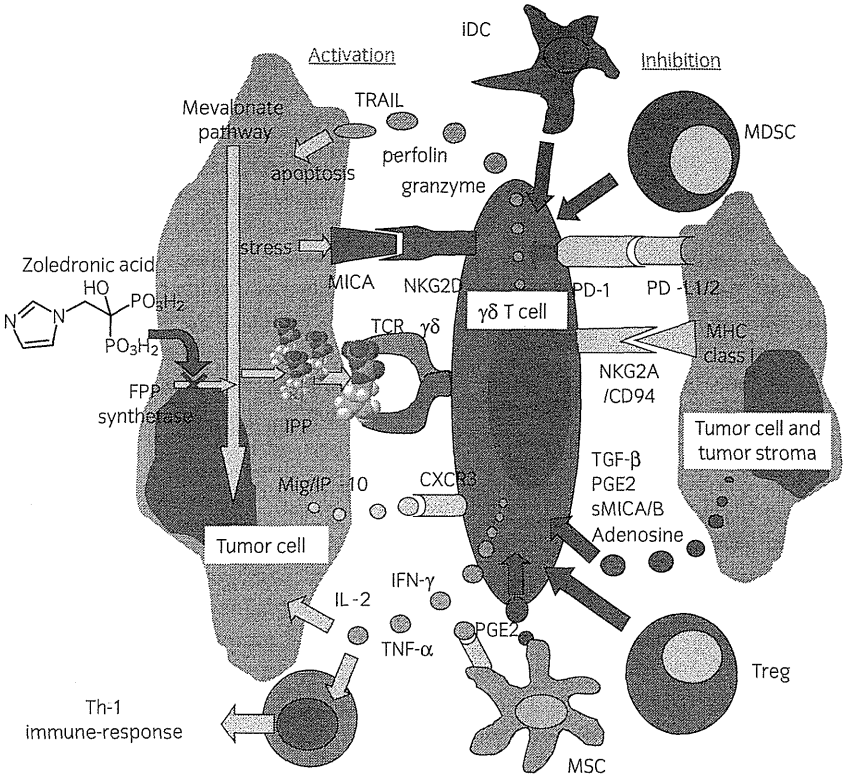


Fig. 2 Activation and inhibition mechanisms of γδ T cells in tumor stroma. γδ T cell recognizes isopentenyl-pyrophosphate accumulated in tumor cell by T cell receptor and major histocompatibility antigen (MHC) class I chain-related A (MICA) on tumor cell by NKG2D, and then γδ T cells show potent cytotoxicity against tumor cells. Tumor stroma contains various kinds of cells to inhibit γδ T cell functions and also tumor cells inhibits γδ T cell functions by interaction of the inhibitory molecules.

major mechanisms of escape from immunotherapy based on αβ T cells.

The T cell receptor (TCR)-γ chain gene was discovered by chance during identification of TCR αβ chain genes, and it was established in around 1986 that γδ T cells were one of the subpopulations of T cells. The behavior of natural ligands of γδ TCR was unclear for quite a while until Tanaka reported in 1995 that γδ T cells recognize isopentenyl-pyrophosphate (IPP) by the TCR.

We have synthesized more than 60 kinds of monoethyl-pyrophosphate derivatives and 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), which show potent stimulation of γδ T cells more than 100-fold that of IPP. We developed a bulk-culture system of γδ T cells using 2M3B1PP and IL-2.[34] γδ T cells induced by 2M3B1PP with potent cytotoxic activity against not only various types of tumor cell lines, but also against autologous renal cell carcinoma (RCC) cells, and we attempted to apply γδ T cells as a new adoptive immunotherapy.[35]

Cytotoxic activity of γδ T cells is induced by recognition of stress-inducible major histocompatibility antigen (MHC) class I chain-related A (MICA) proteins on target tumor cells by natural killer group 2D (NKG2D) of γδ T cells. MHC class I on target tumor cells induced an inhibitory signal through NKG2A/CD94 to γδ T cells at the same time, but cells lacking in MHC class I can be killed by γδ T cells. In contrast, many tumor cells upregulate the mevalonate-pathway. IPP, which is one of the intermediate metabolisms of the pathway, accumulates in tumor cells and γδ T cells, and recognizes IPP of the TCR and shows cytotoxity by release of perfolin, granzyme and TNF-related apoptosis-inducing ligand, resulting in apoptosis of the tumor cells. Also, activated γδ T cells secrete various types of cytokines, such as interferon-γ (IFN-γ), IL-2 and TNF-α, and promote Th-1 type immune reaction in other immune cells. Nitrogen-containing bisphosphonates, such as zoledronic acid (Zol), inhibit farnesyl-pyrophosphate (FPP) synthetase, which is one of the important enzymes in the mevalonate pathway. Inhibition of FPP synthetase resulted in an accumulation of IPP in the tumor cells, which γδ T cells recognizes and then kills the tumor cells with ease (Fig. 2). Assembling these strands of evidence and applying this knowledge of γδ T cells as effector cells has advantages for immunotherapy compared with αβ T cells. γδ T cells make good use of the universal characteristics of tumor cells in surveillance.

We set up a phase I/IIa clinical trial of this new approach of adoptive immunotherapy using autologous γδ T cells induced by 2M3B1PP followed by administration of low-dose IL-2 and Zol. Patients who underwent nephrectomy because of RCC and received IFN-α therapy for recurrent and/or distant metastasis of RCC as a first-line therapy, which resulted in failure, were enrolled. The protocol was reviewed by our Institutional Review Board and

registered to the National Cancer Institute (NCI) as a clinical trial (http://www.cancer.gov/clinicaltrials/TRIC-CTR-GU-05-01). After we had obtained written informed consent, the patients underwent leukopheresis to harvest of peripheral blood mononuclear cells, which were then stimulated with 2M3B1PP and IL-2 for 11 days. All these procedures were carried out in the cell-processing center (CPC) at Tokyo Women's Medical University Hospital. Each patient then received 1.4 million units of IL-2 and 4 mg of Zol. Activated γδ T cells were injected intravenously. IL-2 at 1.4 million units was given for four consecutive days. Each patient received these treatments once a month for a period of 6 months. A total of 11 advanced RCC patients were enrolled in the clinical trial and we obtained the results of one complete response[36], five stable disease and five progressive disease. We found that this approach was safe and well tolerated, and we also observed some clinical responses. Based on these results, we have improved this approach and planned a phase II clinical trial now approved by the Japanese Ministry of Health, Labor and Welfare.

γδ T cells were still enigmatic and their mechanisms of tumor surveillance were not fully understood. Recent reports show the pitfalls of γδ T cells-based immunotherapies.[37] One of the mechanisms of the pitfalls involves the inhibitory factors produced by tumor cells, such as TGF-β, prostaglandin E2, adenosine, soluble NKG2D ligands, galectin-3, HLA-G and indoleamine 2,3 dioxygenase, which weaken potent cytotoxic activity and proliferation of γδ T cells. Other aspects of the mechanisms involve suppressive cells from the tumor microenviroments, such as regulatory T cells (Treg), immature DC (iDC), myeloid-derived suppressive cells (MDSC), mesenchymal stem cells (MSC) and negative costimulators, such as PD-1 (Fig. 2). Almost all these mechanisms are related to tumor microenvironments, an understanding of which is very important in developing not only γδ T cell-based immunotherapy, but also other immunotherapies.

Taking account of these factors that inhibit successful immunotherapies, prevention of recurrence and/or surveillance of micrometastasis might be most effective strategies for carrying out γδ T cell-based immunotherapy.

In light of the latest knowledge about the mechanisms of tumor escape from the immune system, we believe it is possible to resolve and overcome these problems by carrying out well-designed translational research and clinical trials.

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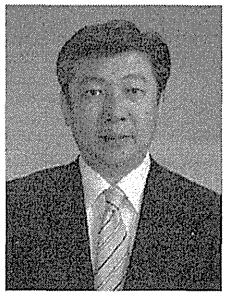
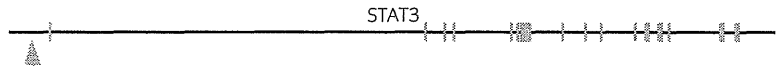
Personalized treatment in the immunotherapy for metastatic renal cell carcinoma



There were an estimated 57 760 new cases and 12 980 deaths expected in 2009 from renal cell carcinoma (RCC) in the USA in 2009.[38] Approximately 30–40% of patients with malignant renal cortical tumors will either present with or later develop metastatic disease. RCC has a poor prognosis when diagnosed in advanced stages. Clear cell carcinomas, which account for 75–85% of renal tumors, are characterized by loss of the von Hippel-Lindau (VHL) tumor-suppressor gene, leading to overexpression of proangiogenic vascular endothelial growth factor (VEGF) and platelet-derived growth factor-β (PDGFβ).[16,39] As a result, the treatment of metastatic RCC (MRCC) in many countries has recently evolved from being predominantly cytokines-based to now being grounded in the use of drugs that target the dysregulated VEGF and PDGFβ pathways.

However, many Japanese urologists still seem to use cytokine therapy for MRCC, especially in patients whose metastases are limited to the lung or lymph nodes. This tendency is based on the good prognosis of Japanese patients with MRCC in the era of cytokine therapy.[15] Considering personalized treatment in the immunotherapy for MRCC, we carried out a retrospective analysis to find out good responders in the interferon-α (IFN-α) treatment for MRCC in Japan. The analysis showed that the single nucleotide polymorphisms (SNP) in signal transducer and activator 3 (STAT3) were most significantly associated with a better response to IFN-α.[21] Linkage disequilibrium mapping showed that the SNP in the 5′ region of STAT3, rs4796793, was the most significant predictor of IFN-α response (odds ratio [OR] = 2.73; 95% confidence interval [CI], 1.38–5.78; Fig. 3).[21] The highest OR was shown in the CC genotype at rs4796793 compared with the GG + GC genotypes (OR = 8.38, 95% CI, 1.63–42.96; Fig. 3).[21] If we would use the CC genotype at rs4796793 as a predictive marker of response to IFN-α therapy in MRCC patients, the positive and negative predictive value would be 52.8% and 88.2%, respectively, on the assumption that response rate of IFN-α is 15% (data not shown). To prove our retrospective SNP result in STAT3, we have been doing a prospective RCC-SNP Ensuring-study for Leading Eligibility of patients in Cytokine Therapy (SELECT) trial since 2007. We hope that we will soon show the final results of the prospective trial on IFN-α.

Interestingly, a similar "SELECT" trial using high-dose (HD) interleukin-2 (IL-2) in patients with MRCC was also recently shown.[40] Their purpose was to pick up good



rs ID	variation	localization	
rs4796793	G/C	5'-upstream	-1697bp from exon1

Allele /Genotype	count NON	RES	freq. NON	RES		Chi-Square	P-value	odds	95% C.I.
G	62	25	0.33	0.57	G vs. C	8.614	0.0033*	2.73	1.38-5.38
C	30	33	0.67	0.43					
GG	18	4	0.39	0.14	GG vs. GC vs. CC	11.110	0.0039*		
GC	26	17	0.57	0.59	GC+CC vs. GG	5.509	0.0189	4.02	1.20-13.48
CC	2	8	0.04	0.28	CC vs.GG+GC	8.312	0.0039*	8.38	1.63-42.96

* P<0.01.

Fig. 3 Association of rs4796793 with interferon-α (IFN-α) response. The upper figure shows the location of rs4796793 in the STAT3 gene. The lower figure shows the significant association between rs4796793 and IFN-α response. χ^2 testing was carried out for the alleles or the genotypes of rs4796793. NON, non-responder; RES, responder (cited from ref.[21]).

responders before they began IL-2 therapy. The response rate (28%) for HD IL-2 in their trial was significantly better than the historical experience, likely as a result of improved patient selection (high incidence of prior nephrectomy, low incidence of non-clear cell carcinoma, etc.).[40] However, in their trial, analysis of tumor-based predictive markers through central pathology review and staining for carbonic anhydrase 9 (CA-9) was unable to improve the selection criteria for HD IL-2.[40] UCLA Survival after Nephrectomy and Immunotherapy (SANI) score[41] only showed the possibility to identify patients who were unlikely to respond to HD ID-2.[40] These results in IL-2 showed the difficulty in prospectively identifying predictive markers in the immunotherapy for MRCC. However, we believe that our trial to discover good responders in IFN-α treatment will surely lead to personalized treatment in the immunotherapy for MRCC in the era of molecular targeted therapy.

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BJUI BJU INTERNATIONAL

A phase I study of personalized peptide vaccination for advanced urothelial carcinoma patients who failed treatment with methotrexate, vinblastine, adriamycin and cisplatin

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Study Type – Therapy (case series)
Level of Evidence 4

What's known on the subject? and What does the study add?
This phase I study showed the safety and boosted immune responses of personalized peptide vaccination for advanced urotherial carcinoma.

This study showed feasibility of personalized peptide vaccination as a new therapeutic modality for advanced urotherial carcinoma patients who failed cisplatin-based chemotherapy.

OBJECTIVE

- To investigate the safety and immune responses of 12 consecutive weeks of once-weekly personalized peptide vaccine (PPV) administration in patients with advanced urothelial carcinoma (UC) for whom therapy with methotrexate, vinblastine, adriamycin and cisplatin (MVAC) has failed.

PATIENTS AND METHODS

- A phase I trial was designed. Ten patients with MVAC-refractory advanced or metastatic UC were treated with weekly personalized peptide vaccine 12 times using positive peptides chosen from 14 and 16 peptides in patients with human leucocyte antigens A24 and A2, respectively.
- Peptide-specific cytotoxic T lymphocyte precursor analysis by interferon-γ production and peptide-reactive immunoglobulin G (IgG) using an enzyme-linked immunosorbent assay was monitored during the treatment.

RESULTS

- The peptide vaccination was safe and well tolerated with no major adverse effects. Increased cytotoxic T lymphocyte response and the anti-peptide IgG titre were revealed by the post-vaccination sera in eight patients.
- Clinical responses were as follows: one complete response, one partial response, two stable disease and six progressive disease.
- Median progression-free survival and overall survival were 3.0 and 8.9 months, respectively. In the four responders, median progression-free survival and overall survival were 21 and 24 months, respectively.

CONCLUSIONS

- This phase I study showed the safety of and boosted immune responses in response to PPV for advanced UC.
- The potential efficacy of 12 consecutive weekly vaccinations with PPV in patients with advanced UC merits further investigation based on these findings.

KEYWORDS

urothelial carcinoma, bladder cancer, peptide vaccine, personalized therapy, phase I clinical trial

INTRODUCTION

The currently available standard chemotherapy for advanced or metastatic urothelial carcinoma (UC) is a cisplatin-based treatment that includes methotrexate, vinblastine, adriamycin and cisplatin (MVAC) or gemcitabine and cisplatin [1–4]. However, there are no established therapeutic modalities for patients with UC who fail with these cisplatin-based therapies. Therefore, new approaches should be taken, and one of them could be specific immunotherapy. Recent advances in tumour immunology have resulted in the identification of a number of antigens and their peptides that are recognized by tumour-reactive and human leucocyte antigen (HLA) class I-restricted

cytotoxic T lymphocytes (CTL) [5]. Cancer vaccines have emerged as a promising therapeutic approach [6]. The efficacy of intravesical BCG in the treatment of superficial disease suggests a role for developing immune recognition strategies to enhance the treatment of UC. The presence of tumour-infiltrating CD8 T cells has been associated with survival in patients with UC [7]. CD8-expressing T cells can also recognize the NY-ESO-1 antigen [8], which occurs in approximately 30–40% of muscle-invasive bladder cancer. A recent clinical trial found that all six of six patients developed antigen-specific immune responses when treated with NY-ESO-1 vaccine [9]. Additional work evaluating the impact of immunomodulating therapy is ongoing, including the use of the anti-cytotoxic T-lymphocyte antigen-4 antibody to overcome inhibitory signals down-regulating T cells [10]. However, their clinical responses have been limited. To overcome this limitation, we devised a new regimen of peptide-based vaccination that consists of measuring pre-existing CTL precursors and IgG reactive to many kinds of vaccine candidates, followed by administration of the positively reactive peptides (personalized peptide vaccination: PPV) [11–14]. A recently conducted randomized clinical trial of PPV for advanced prostate cancer patients showed a favourable clinical response in the vaccinated group [15], whereas most of the other randomized cancer vaccine trials failed to obtain better clinical responses in the vaccine group [16–18]. In this phase I study, we addressed the feasibility of PPV for patients with advanced UC for whom MVAC therapy had failed.

PATIENTS AND METHODS

Eligible patients were included if they were ≥18 years of age with HLA-A24 and/or HLA-A2 status, as determined by commercially available serological tests (SRL, Tokyo, Japan), and were measurable or assessable and histologically proven to have locally advanced (≥T3, N1) or metastatic (M1) UC that included the urinary bladder and upper urinary tract. All patients received surgical treatment or biopsy and MVAC therapy had failed. Previous chemotherapy with radiation therapy for local treatment of the primary lesion was allowed if completed at least 4 weeks before enrolment. Patients were eligible if their disease had progressed at any time after therapy for advanced or metastatic disease or within 12 months of neoadjuvant or adjuvant treatment. Patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 1, adequate bone marrow reserve (white blood cell count ≥3000/μL, lymphocyte count ≥1200/μL, platelets ≥75 000/μL and haemoglobin ≥10 g/dL), hepatic function (serum bilirubin ≤1.5 mg/dL), and renal function (serum creatinine ≤1.5 mg/dL), and an estimated life expectancy of at least 12 weeks. Patients with non-malignant systematic disease that precluded them from receiving therapy, including active infection, autoimmune disease, any clinically significant cardiac arrhythmia, or congestive heart failure were not eligible. Patients also had to be negative for hepatitis B and C antigens. Patients with CNS metastases, second primary malignant lesions, or clinically significant pleural effusions or ascites or who had used any investigational agent 1 month before enrolment were not eligible. The study protocol was approved by the institutional ethical review boards of Kitasato University and Kurume University, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before entering this clinical trial.

The study design was for a non-randomized, open-label, phase I study in patients with advanced or metastatic UC previously treated with MVAC chemotherapy. The treatment was carried out at Kitasato University Hospital and Kurume University Hospital in the outpatients clinic. All immunological analyses were carried out at the Department of Immunology, Kurume University School of Medicine. The peptides used in the present study were prepared by Multiple Peptide Systems (San Diego, CA, USA) under the conditions of Good Manufacturing Practice. The peptide candidates consisted of $SART2_{93-101}$, $SART2_{161-169}$, $SART3_{109-118}$, $Lck_{208-216}$, $Lck_{486-494}$, $Lck_{488-497}$, $MRP3_{503-511}$, $MRP3_{1293-1302}$, $PAP_{213-221}$, $PSA_{248-257}$, $PSMA_{624-624}$, $EZH2_{735-743}$, $EGF\text{-}R_{800-809}$ and $PTH\text{-}rP_{102-111}$ for patients with HLA-A24, and $SART3_{302-310}$, $SART3_{309-317}$, $CypB_{129-138}$, $Lck_{246-254}$, $Lck_{422-430}$, ppMAPkkk$_{294-302}$, ppMAPkkk$_{432-440}$, $WHSC2_{103-111}$, $WHSC2_{141-149}$, $UBE2V_{43-51}$, $UBE2V_{85-93}$, $HNRPL_{140-148}$, $HNRPL_{501-510}$, $EZH2_{569-577}$, $PSCA_{21-30}$ and $EGFR_{479-488}$ for patients with HLA-A2 [8,9,13]. These peptides have the ability to induce HLA-A24-restricted or HLA-A2-restricted and tumour-specific CTL activity in peripheral blood mononuclear cells (PBMCs) of cancer patients, and are frequently expressed in various tumour cell lines [14,15,19]. The peptides were supplied in vials containing 3 mg/mL sterile solution for injection. Three milligrams of peptide with sterile saline was added in a 1:1 volume to the Monotide ISA-51 (Seppic, Paris, France), and then mixed in a Vortex mixer (Fisher, Alameda, CA, USA). The ISA51 is suitable for peptide vaccination because peptides solubilized in water phase are sequestered from peptidase-containing body fluid, and slow release of the peptides from the emulsion provides sustained antigenic stimulation [20]. The resulting emulsion (maximum of four peptides per vaccination) was injected subcutaneously into the femoral area, once a week for 12 weeks. This first cycle of treatment consisted of 12 consecutive weekly vaccinations. The cycle was repeated every 12 weeks for as long as the patients agreed to continue and their condition was considered appropriate for vaccination. Toxicity was evaluated in patients who received at least one vaccination, whereas both immunological and clinical evaluations were conducted in those who received more than six vaccinations. Blood samples for studies of immune responses were obtained on weeks 0, 6 and 12 during cycle 1. Supportive care could include blood transfusion and the administration of anti-emetics and analgesics, as appropriate. Further local therapy, including other chemotherapy regimens or radiation therapy, was allowed in patients with advanced disease after assessment of response to this regimen.

To measure peptide-specific CTL precursors, 30 mL peripheral blood was obtained before and after vaccination, and PBMCs were isolated by Ficoll–Conray density gradient centrifugation. Peptide-specific CTL precursors in PBMCs were detected using a previously reported culture method [21]. Briefly, PBMCs (1×10^5 cells/well) were incubated with 10 μM of a peptide in 200 μL of culture medium in U-bottom-type 96-well microculture plates (Nunc, Roskilde, Denmark). The culture medium consisted of 45% RPMI-1640 medium, 45% AIM-V medium (GIBCO BRL, Grand Island, NY, USA), 10% fetal calf serum, 100 U/mL interleukin-2 and 0.1 μM minimal essential medium non-essential amino acid solution (GIBCO BRL). Half of the medium was removed and replaced with a new medium containing a corresponding peptide (20 μM) every 3 days. After incubation for 14 days, these cells were harvested and tested for their ability to