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Humoral immune responses to CTL epitope peptides from tumor-associated antigens are widely detectable in humans: A new biomarker for overall survival of patients with malignant diseases

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ABSTRACT

Both cellular and humoral immune responses are crucial to induce potent anti-tumor immunity, but most of currently conducted peptide-based cancer vaccines paid attention to cellular responses alone, and none of them are yet approved as a therapeutic modality against cancer patients. We investigated humoral immune responses to CTL epitope peptides derived from tumor-associated antigens in healthy donors and patients with various diseases to facilitate better understanding of their distribution patterns and potential roles. Bead-based multiplex assay, ELISA, and Western blotting were used to measure immunoglobulins reactive to each of 31 different CTL epitope peptides. Importantly, the sums of anti-peptide IgG levels specific to 31 CTL epitope peptides were well correlated with better overall survival (OS) in patients with malignant diseases. Our results suggested that humoral immune responses to CTL epitope peptides were widely detectable in humans. Measurement of immunoglobulins specific to CTL epitope peptides may provide a new biomarker for OS of patients with malignant diseases, although it still remains to be determined whether the correlations between humoral immune responses to epitope peptides and OS are observed only for the CTL epitopes used, or also for other panels of peptides. Quantity of circulating IgG reactive to these peptides was also discussed.

1. Introduction

Peptide-based cancer vaccines have been extensively studied following the discovery of human tumor-associated antigens (TAA) and cytotoxic T lymphocyte (CTL) epitope peptides (Rosenberg et al., 2004; Mellman et al., 2011). However, none of them are yet approved as a therapeutic modality. There might be at least two important hurdles to obtain clinical benefits from the peptide-based cancer therapies currently in practice. One of these hurdles, the negative signaling against CTL activation through check point molecules, such as CTLA-4 and PD-1, was recently overcome by developing blocking antibodies against these molecules (Hodi et al., 2010; Topalian et al., 2012; Brahmer et al., 2012). The second potential hurdle is that no or little humoral immune responses can be induced by the vaccination using most of currently available CTL epitope peptides, although it has been well recognized that both cellular and humoral immune responses are

Abbreviations: TAA, tumor-associated antigen; HD, healthy donor; Flu, influenza virus; HCV, hepatitis C virus; Ig, immunoglobulin; OS, overall survival.

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crucial to induce potent anti-tumor immunity in animal models (Hu et al., 2005; Bequet-Romero et al., 2012; Zeng et al., 2009). In fact, most of currently conducted peptide-based cancer vaccines have paid attention to cellular immune responses alone. To our knowledge, exception is "personalized peptide vaccination" that we have developed (Terasaki et al., 2011), in which CTL epitope candidates for therapeutic cancer vaccines were at first screened based on not only their ability to induce CTL but also reactivity to IgG responses in pre-vaccination samples.

Although humoral immune responses against whole proteins of TAA have been well investigated (Yuan et al., 2011; Toh et al., 2009; Zhang and Tan, 2010), those against CTL epitope peptides derived from TAA, which have been used for therapeutic cancer vaccines, have rarely been studied. We hypothesized that a CTL epitope peptide possessing a B cell epitope could provide more effective clinical benefits than a CTL epitope peptide without it. In fact, we reported potential clinical benefits in advanced glioblastoma multiforme or prostate cancer patients under personalized peptide vaccines using such peptides (Terasaki et al., 2011; Noguchi et al., 2010, 2011a,b; Yajima et al., 2005). In addition, IgG responses were identified as an excellent prognostic marker for predicting overall survival (OS) of the vaccinated patients, although CTL responses also showed a prognostic correlation (Noguchi et al., 2011b; Mine et al., 2004). However, it remains to be fully studied whether anti-peptide immunoglobulins (Igs) are detectable in healthy donors (HD) and patients with various diseases. The current study has addressed this issue to facilitate better understanding of humoral immune responses to CTL epitopes and better designing of cancer vaccine protocols. The results suggest that humoral immune responses are widely detectable in humans and have potential as a new biomarker for overall survival (OS) of patients with malignant diseases.

2. Materials and methods

2.1. Patients and sample collection

Plasma or sera were collected from HD ($n = 74$, 43 ± 20 years old) and from patients with rheumatoid arthritis ($n = 20$, 67 ± 7 years old), IgA nephropathy ($n = 20$, 34 ± 13 years old), influenza virus (Flu) infection ($n = 20$, 34 ± 17 years old), hepatitis C virus (HCV) infection ($n = 20$, 55 ± 8 years old), hematological malignancies ($n = 55$, 61 ± 14 years old; 24 leukemia, 27 lymphoma, and 4 myeloma), or non-HCV hepatocellular carcinoma (HCC, $n = 55$, 60 ± 11 years old; 26 non-B non-C hepatocellular carcinoma, 23 hepatitis B associated hepatocellular carcinoma and six alcoholic hepatic carcinoma) (Supplementary Table 1). HD were categorized into the following four age groups according to the Ministry of Health, Labor and Welfare in Japan; 15–24 ($n = 20$), 25–44 ($n = 19$), 45–64 ($n = 23$), and ≧65 ($n = 12$) years old (Supplementary Table 2). This study was approved by the Kurume University Ethical Committee. After informed consent was obtained from all subjects, blood samples (plasma or sera) were obtained and frozen at −80 °C until use (Noguchi et al., 2011b; Mine et al., 2004).

2.2. Peptides

Thirty-one different peptides employed in the current study were prepared under conditions of Good Manufacturing Practice by Poly Peptide Laboratories (San Diego, CA) or American Peptide Company (Vista, CA), and dissolved in DMSO (Wako, Osaka, Japan). Detailed information on these peptides, including the original protein, peptide position, amino acid sequence, HLA class I A restriction, and references, are given in Supplementary Table 3. Twenty-four of 31 peptides were derived from TAA that were identified by the cDNA expression cloning method, followed by determination of CTL epitopes. The remaining seven peptides including PAP-213, PSA-248, PSMA-624, and PAP-248 were identified by the reverse-immunology method (Kobayashi et al., 2003; Matsueda et al., 2005; Inoue et al., 2001). CTL epitope peptides were determined to be cancer vaccine candidates, based on both their ability to induce CTL activity from peripheral blood mononuclear cells *in vitro* as well as the IgG levels against them in plasma of un-vaccinated cancer patients, and these peptides have been used in clinical trials of personalized peptide vaccine for advanced cancer patients (Terasaki et al., 2011; Noguchi et al., 2010, 2011a,b; Yajima et al., 2005; Mine et al., 2004; Terazaki et al., 2012).

2.3. Measurement of Igs reactive to each of 31 different peptides

The levels of Igs reactive to each of 31 different peptides were measured by multiplex bead suspension array using the Luminex system (Luminex Corp., Austin, TX) as reported previously (Komatsu et al., 2004). In brief, plasma or serum was incubated with 100 μL of peptide-coupled color-coded beads for 1.5 h at 30 °C. To detect IgG or IgM, after washing, the beads were incubated with 100 μL of biotinylated goat anti-human IgG (gamma chain-specific; Vector Laboratories, Burlingame, CA) or biotinylated goat anti-human IgM (mu chain-specific; Vector Laboratories) Abs for 1 h at 30 °C. To detect IgG1, IgG2, IgG3, or IgG4, the beads were incubated with 100 μL of sheep anti-human IgG1, IgG2, IgG3, or IgG4 Abs (Binding Site, Birmingham, UK) for 1 h at 30 °C, followed by washing and incubation with 100 μL biotin-rabbit anti-sheep IgG Ab for 1 h at 30 °C. After washing, the beads were incubated with 100 μL of streptavidin-PE (Life Technologies, Carlsbad, CA) for 30 min at 30 °C, followed by washing and detection of fluorescence intensity unit (FIU) on the beads using the Luminex system (Komatsu et al., 2008). The cut-off values of anti-peptide IgG were set to 10 FIU in 100-time diluted samples, as reported previously (Komatsu et al., 2008). In brief, the calibration curves of FIU were obtained with serially diluted samples. The plasma samples from cancer patients were two times diluted from 160 to 1,310,720. The minimum detectable level of anti-peptide IgGs was 2 FIU when the samples were diluted at 40,960 times as shown by an arrow in Supplementary Fig. 1. However, the levels of anti-peptide IgGs at the minimum detectable range were not reliable since the standard deviations were high. Therefore, we set 10 FIU of the 100-time diluted sample, which was considered to be a reliable value, as a cut-off level as reported previously (Komatsu et al., 2008). There were no significant differences between plasma and serum with regard to the levels of anti-peptide Igs (data not shown).

The specificities of IgG against these peptides were confirmed by competition assay. Plasma was incubated with 100 μL of peptide-coupled color-coded beads and 5 μL of each of the corresponding peptides for 1.5 h at 30 °C. The binding of anti-peptide IgG was detected by same method as described above.

Plasma from frequently vaccinated (12–18 vaccinations) cancer patients who were enrolled in clinical trials of personalized peptide vaccine (data not shown) were used for estimation of anti-peptide IgG levels by other methods, Western blotting and ELISA. To isolate anti-peptide IgGs for Western blotting, plasma was incubated with 100 μL of peptide-coupled color-coded beads for 1.5 h at 30 °C. After washing, the beads were incubated with 6 μL of sample buffer (NuPAGE LDS Sample buffer; Life Technologies, Carlsbad, CA) at 70 °C for 15 min prior to loading onto the SDS–PAGE gel. The separated proteins were transferred to nitrocellulose membrane (Life Technologies, Carlsbad, CA), and IgG gamma chain was detected by using Goat $F(ab')_2$ Fragment anti-human IgG(H+L)-peroxidase (IM0837; Beckman Coulter, Fullerton, CA) Ab and an ECL system (GE Healthcare, Uppsala, Sweden). As a

standard, purified human IgG (R&D Systems, Minneapolis, MN) was used. ImageQuant ver 5.2 (GE Healthcare, Pittsburgh, PA) was used to measure chemi-luminescent signals for quantification.

A human IgG ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) was also used for quantitative analysis of anti-peptide IgGs in plasma from frequently vaccinated patients, which were also used for Western blotting. One hundred-time diluted plasma was incubated with 100 μL of peptide-coupled color-coded beads for 1.5 h at 30 °C. After centrifugation, the supernatant was added to new beads, and incubated again to detect the remaining anti-peptide IgGs in plasma. The beads were washed and incubated with 90 μL of 0.1 M Glysine buffer (pH 2.7) for 5 min at 30 °C, and then 10 μL of 1 M Tri-HCl (pH 9.0) buffer was added to neutralize eluted fraction. After centrifugation, the supernatant was collected for quantitative analysis by ELISA. The optical density was determined using a microplate reader (Infinite® 200; TECAN, Männedorf, Switzerland).

2.4. Statistical analysis

The *t-test and the* Chi-square test were *used* to determine whether there is a significant difference in age or gender. Wilcoxon signed rank test was used to compare Ig levels specific to peptides. The OS in cancer patients was calculated from the date of drawing blood until the date of death or the last date when the patient was known to be alive. Curves for OS were estimated by the Kaplan–Meier method, and the log-rank test was conducted for the comparison of survival curves. A two-sided P value of less than 0.05 was considered to be statistically significant. All statistical analyses were conducted by using the JMP version 9.1 software (SAS Institute Inc., Cary, NC).

3. Results

3.1. Detection of Igs specific to CTL epitope peptides in HD

We first addressed whether Igs reactive to each of 31 different CTL epitope peptides derived from TAA were detectable in plasma or sera from HD ($n = 74$) by the Luminex system. There are no significant differences in Ig levels measured between in sera and in plasma (data not shown). IgM reactive to all but two (Lck-422 and MRP3-503) of the 31 peptides were detected as positive in >50% of HD, since their median values exceeded the cut-off values (10 FIU) (Table 1). Similarly, IgGs reactive to 23 peptides, but not to the remaining eight peptides (Lck-422, ppMAPkkk-432, and the others), were detected as positive in >50% of HD. IgG1, IgG2, IgG3, and IgG4 levels were also detected as positive in >50% of HD in 22, 15, 12, or 0 of 31 peptides, respectively (Table 1).

The specificities of IgG reactive to nine peptides were previously reported (Kobayashi et al., 2003; Matsueda et al., 2005; Harada et al., 2003; Shomura et al., 2004; Ogata et al., 2004; Yao et al., 2004; Minami et al., 2007). The specificities of IgG against the remaining 22 peptides were confirmed in this study by competition assays, in which the binding of anti-peptide IgG was inhibited in the presence of each of the corresponding peptides, and representative results for the 12 peptides are shown in Supplementary Fig. 2.

We next examined the effects of gender and age on the anti-peptide Ig levels. There were no significant differences between males ($n = 41$) and females ($n = 33$) with regard to the levels of IgM, IgG, IgG1, IgG2, IgG3, or IgG4 against any of the 31 peptides, or the sums of the Igs against each of the 31 peptides (data not shown). When the subjects were divided into the following four age groups: 15–24 years old ($n = 20$), 25–44 ($n = 19$), 45–64 ($n = 23$), and ≧65 ($n = 12$) (Supplementary Table 2), there was an age-dependent decrease of IgM levels against each of the 31 peptides (data not shown) or of the total sum of the IgM against each of the 31 peptides (Fig. 1). A similar trend was also observed for the levels of anti-peptide IgG as well as for those of anti-peptide IgG subclasses, including IgG1 and IgG2, whereas IgG and IgG1 levels were somewhat increased in the oldest age bracket (≧65 years old) (Fig. 1).

Table 1
Assessment of immunoglobulins reactive to each of the 31 different CTL epitopes in plasma or sera from healthy donors.

Peptide name	HD (median FIU) [a]					
	IgM	IgG	IgG1	IgG2	IgG3	IgG4
CypB-129	350	20	19	<10	<10	<10
Lck-246	925	42	25	18	<10	<10
Lck-422	<10	<10	<10	<10	<10	<10
ppMAPkkk-432	103	<10	<10	<10	<10	<10
WHSC2-103	4940	114	69	61	16	<10
HNRPL-501	71	<10	<10	<10	<10	<10
UBE2V-43	1696	51	50	10	25	<10
UBE2V-85	73	<10	<10	<10	<10	<10
WHSC2-141	764	49	36	25	<10	<10
HNRPL-140	721	41	41	29	<10	<10
SART3-302	223	38	50	<10	<10	<10
SART3-309	2655	32	34	<10	11	<10
SART2-93	11,500	148	111	66	28	<10
SART3-109	511	<10	<10	<10	<10	<10
Lck-208	654	11	11	<10	<10	<10
PAP-213	2134	48	34	<10	17	<10
PSA-248	2743	50	41	10	12	<10
EGFR-800	2659	58	39	25	<10	<10
MRP3-503	<10	<10	<10	<10	<10	<10
MRP3-1293	1376	48	37	15	12	<10
SART2-161	84	<10	<10	<10	<10	<10
Lck-486	1277	65	49	<10	17	<10
Lck-488	6302	135	111	35	41	<10
PSMA-624	426	23	19	<10	<10	<10
EZH2-735	261	<10	<10	<10	<10	<10
PTHrP-102	1467	13	22	24	<10	<10
SART3-511	5057	70	60	19	17	<10
SART3-734	2302	188	164	74	23	<10
Lck-90	7410	116	90	34	25	<10
Lck-449	590	44	33	<10	<10	<10
PAP-248	420	29	<10	13	<10	<10

[a] IgM, IgG, and IgG subclasses (IgG1, IgG2, IgG3, and IgG4) specific to each of the 31 CTL epitope peptides were measured by the Luminex system in 100-time diluted samples (plasma or sera) from healthy donors (HD). The median values of FIU are shown.

3.2. Detection of Igs specific to CTL epitope peptides in patients with various diseases

We next examined the levels of anti-peptide Igs in patients with various types of immune-related disorders, including autoimmune diseases, immune-complex-related diseases, and acute and chronic infectious diseases (Supplementary Table 1). The total sums of Igs against each of 31 peptides from patients' samples were compared with those from the age- and gender-matched HD samples. We measured anti-peptide Igs in patients with rheumatoid arthritis and IgA nephropathy as an example of autoimmune diseases and immune-complex-related diseases, respectively. There were no significant differences between patients with rheumatoid arthritis and HD with regard to either Ig levels to each peptide or the total sums of them (Fig. 2A). In contrast, the total sums of anti-peptide IgM ($P < 0.0001$) and IgG2 ($P = 0.0142$) in patients with IgA nephropathy were significantly lower than those in HD, respectively (Fig. 2B). In addition, we measured anti-peptide Igs in patients with Flu infection and HCV infection as an example of acute and chronic viral infections, respectively. The total sums of anti-peptide IgM in patients with Flu infection were significantly

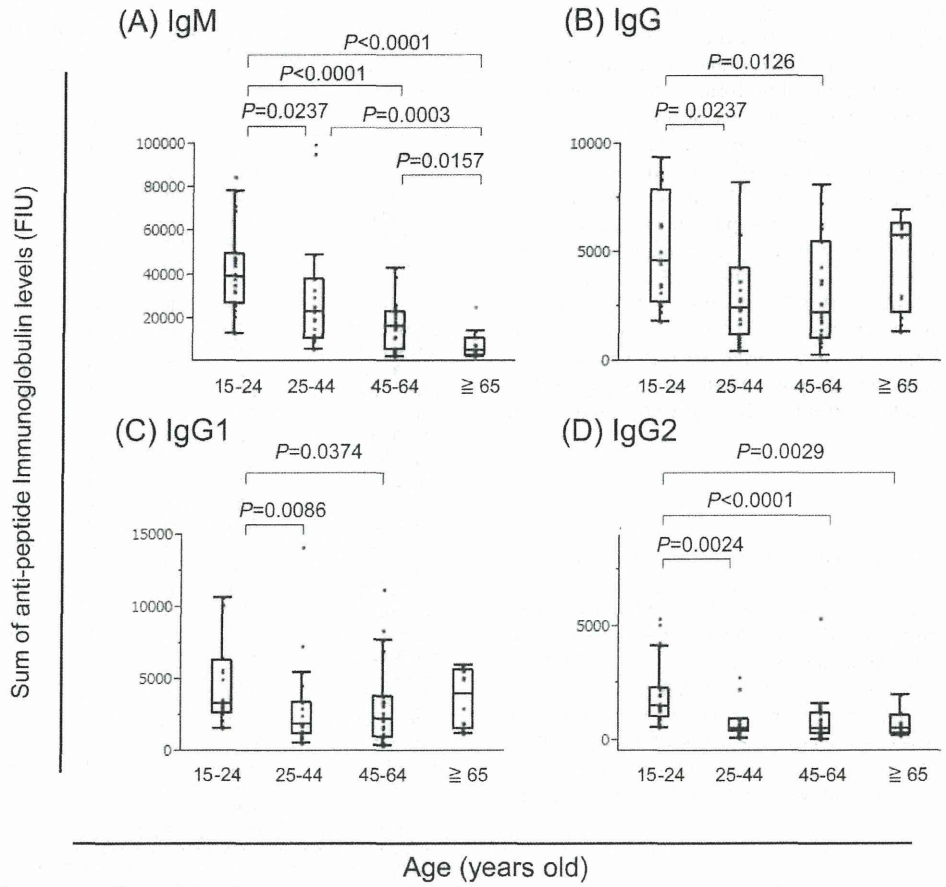

Fig. 1. Detection of immunoglobulins specific to CTL epitope peptides in plasma or sera from healthy donors. Immunoglobulins (IgM, IgG, IgG1, IgG2) specific to each of the 31 CTL epitope peptides were measured by multiplex bead suspension array in plasma or sera from healthy donors. The total sums of the immunoglobulins specific to each peptide were calculated. Healthy donors were categorized into the following four age groups: 15–24 (n = 20), 25–44 (n = 19), 45–64 (n = 23), and ≧65 (n = 12) years old. The differences between each group were evaluated by Wilcoxon test. Only the P values that were statistically significant (P < 0.05) are shown.

lower than those in HD (P < 0.0001), whereas those of anti-peptide IgG and IgG2 were significantly higher than those in HD (P = 0.0448 and P = 0.0073, respectively) (Fig. 2C). In patients with HCV infection, the total sums of anti-peptide IgG and IgG1 were significantly higher, compared to those of HD (P = 0.0015 and P = 0.0009, respectively) (Fig. 2D).

We next examined anti-peptide Ig levels in non-vaccinated cancer patients, and the median values of IgM, IgG, and IgG subclasses (IgG1, IgG2, IgG3, and IgG4) against each of 31 peptides in patients with hematological malignancies and HCC are shown in Table 2. The levels of IgM against most of the 31 peptides were increased in patients with both hematological malignancies and HCC, compared to those of HD. Almost all of IgG, IgG1 and IgG2 levels against each of the 31 peptides were decreased in patients with hematological malignancies, compared to those of HD. In contrast, most of IgG levels against each of 31 peptides, except for one (anti-PAP-248 IgG), were increased in HCC patients, compared to those of HD (Table 2), and similar results were obtained with regard to IgG1.

Subsequently, the total sums of anti-peptide IgM were significantly increased in patients with both hematological malignancies (P < 0.0001) and HCC (P < 0.0001), compared to those of HD (Fig. 3A and B). The total sums of anti-peptide IgG, IgG1, and IgG2 were significantly decreased in patients with hematological malignancies (P = 0.0006, P = 0.0005 and P = 0.0029, respectively) (Fig. 3A), whereas the total sum of anti-peptide IgG was significantly increased in HCC patients, compared to those of HD (P = 0.0300) (Fig. 3B).

3.3. Prognostic significance of anti-peptide Ig levels in patients with malignant diseases

We investigated whether the total sums of anti-peptide Igs against each of 31 different peptides were well correlated with OS in patients with malignancies. When the cut-off values were set to the median values of HD, the total sums of anti-peptide IgG against 31 different peptides was well correlated with better OS in patients with both hematological malignancies (P = 0.0083) and HCC (P = 0.0440) (Fig. 4A and B).

3.4. Quantitative analysis of IgGs specific to CTL epitope peptides

Western blotting was first employed to confirm the reactivity between the peptide-coated beads and anti-peptide IgGs in plasma samples from five different cancer patients, all of whom had received multiple immunizations with CypB-129 (12th vaccinations), UBE2V-43 (18th), UBE2V-85 (18th), Lck-488 (12th), or Lck-449 peptide (12th) as part of clinical trials of personalized peptide vaccines. The levels of anti-peptide IgGs detected by the Luminex system before and after the vaccination period were 161 and 12,717 (79-fold increase), 94 and 2739 (29-fold), 26 and 19,965 (768-fold), 368 and 114,788 (312-fold), or 34 and 5841 (172-fold) FIU/mL of

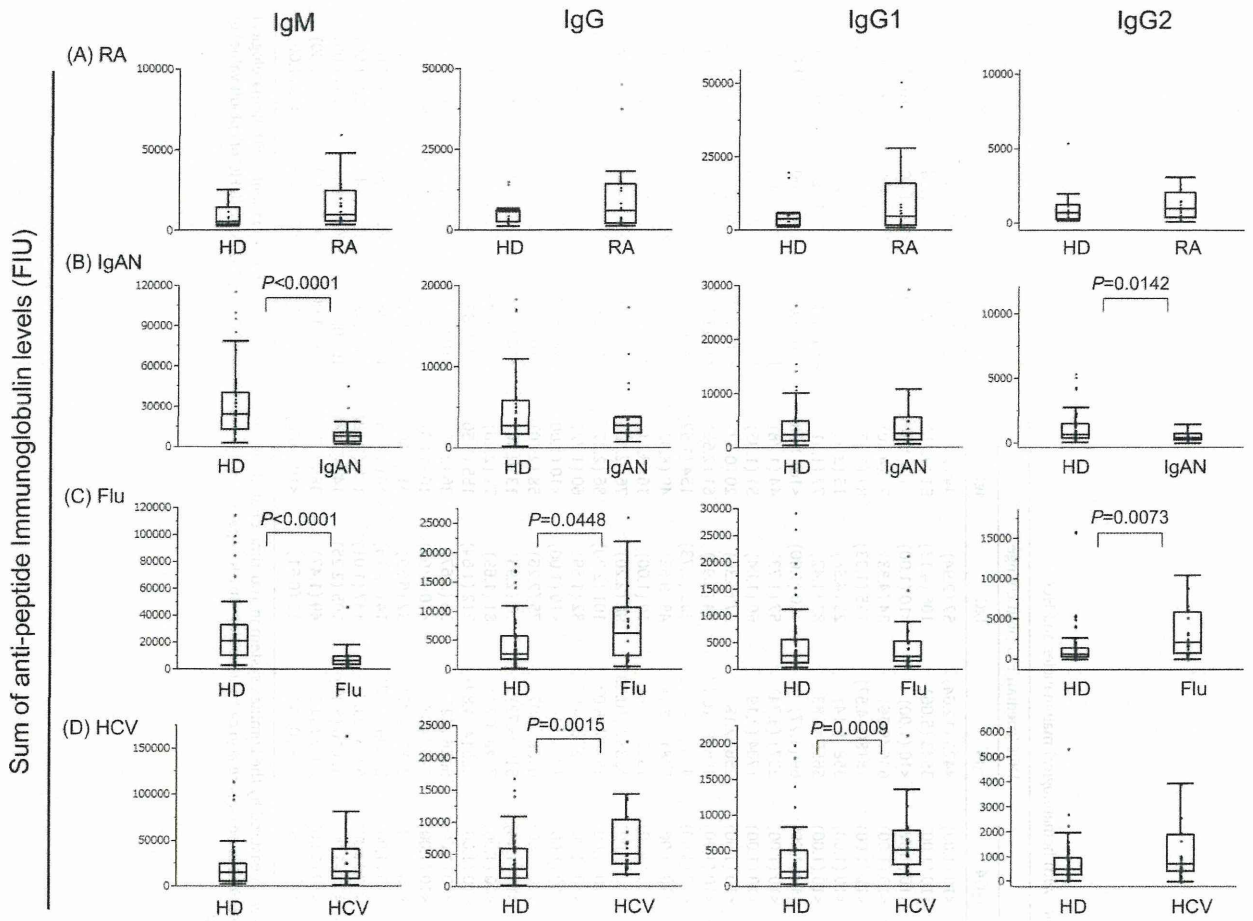

Fig. 2. Comparison of anti-peptide immunoglobulins in plasma or sera between healthy donors and patients with various types of immune-related diseases. Immunoglobulins (IgM, IgG, IgG1, IgG2) specific to each of the 31 CTL epitope peptides were measured by multiplex bead suspension array in plasma or sera from patients with rheumatoid arthritis, IgA nephropathy, influenza virus (Flu) infection, and hepatitis C (HCV) infection and gender- and age-matched healthy donors (HD). The total sums of the immunoglobulins specific to each of the peptides were calculated. (A) rheumatoid arthritis ($n = 20$) vs. gender- and age-matched HD ($n = 15$). (B) IgA nephropathy ($n = 20$) vs. gender- and age-matched HD ($n = 59$). (C) Flu infection ($n = 20$) vs. gender- and age-matched HD ($n = 59$). (D) HCV infection ($n = 20$) vs. gender- and age-matched HD ($n = 48$). The differences between each group were evaluated by Wilcoxon test. Only the P values that were statistically significant ($P < 0.05$) are shown.

100-time diluted plasma, respectively. By Western blotting, IgGs specific to all of these peptides were clearly detected in the post-vaccination samples, but not in the pre-vaccination samples (Fig. 5).

ELISA, a more sensitive quantitative analysis, was then employed to measure the amounts of anti-peptide IgG isolated from the same post-vaccination samples. The samples from HD or cancer patients before vaccinations were not provided for the ELISA primarily because of failure to detect by a mean of Western blotting. As a result, the amounts of IgG specific to CypB-129, UBE2V-43, UBE2V-85, Lck-488, and Lck-449 peptide in the post-vaccination samples were calculated as 146, 35, 21, 52, and 178 ng/mL, respectively.

The amounts of anti-peptide IgG in pre-vaccination plasma of these patients were then estimated by applying the relationship between the FIU levels determined by the Luminex system and the amounts of IgG by ELISA. Accordingly, the amounts of IgG against CypB-129, UBE2V-43, UBE2V-85, Lck-488, or Lck-449 peptide in the pre-vaccination samples could be estimated as 1.85 (146 ng/mL divided by 79-fold), 1.20 (35 ng/mL divided by 29-fold), 0.03 (21 ng/mL divided by 768-fold), 0.17 (52 ng/mL divided by 312-fold), and 1.04 (178 ng/mL divided by 172-fold) ng/mL, respectively. Therefore, the amounts of IgG in the pre-vaccination plasma ranged from 0.03 to 1.85 ng/mL, in which the lowest amount of anti-UBE2V-85 and highest FIU of anti-CypB-129 IgG were 0.03 and 1.85 ng/mL, respectively. As shown in Table 1, the median FIU/mL of anti-peptide IgG against 23 of the 31 peptides in the 100-time diluted plasma of HD ranged from 11 to 188. On the other hand, the FIU/mL values of anti-peptide-IgG against each peptide in the pre-vaccination plasma were ranged from 26 to 368, as shown above. Therefore, the median levels of anti-peptide IgG against 23 of the 31 peptides that were detected as positive in >50% of HD (Table 1), could be estimated to be in the range of 0.01–1 ng/mL.

4. Discussion

We reported in this study that humoral immune responses against the vast majority (29 of 31) of CTL epitope peptides tested were detectable in the circulation of both HD and patients with various diseases. Two exceptions were the response to MRP3-503, a peptide derived from multidrug resistance-associated protein 3 (Yamada et al., 2001), and that to Lck-422, a peptide derived from Lck tyrosine kinase that was expressed on metastatic cancer cells (Imai et al., 2001). However, we reported that humoral responses against these two peptides became detectable in a part of metastatic cancer patients who were resistant to chemotherapies (Noguchi et al., 2010, 2011a,b; Yajima et al., 2005; Mine

Table 2
Assessment of immunoglobulins reactive to each of the 31 different CTL epitopes in plasma or sera from patients with hematological malignancies and HCC.

Peptide name	Hematologic malignancy (median FIU (Fold change))[a]						HCC (median FIU (fold change))[a]					
	IgM	IgG	IgG1	IgG2	IgG3	IgG4	IgM	IgG	IgG1	IgG2	IgG3	IgG4
CypB-129	4347 (13.15)	12 (0.52)	10 (0.51)	<10 (1.00)	<10 (1.00)	<10 (1.00)	4425 (12.64)	57 (2.94)	44 (2.45)	<10 (1.00)	14 (2.77)	<10 (1.00)
Lck-246	1315 (2.02)	<10 (0.20)	10 (0.62)	<10 (0.34)	<10 (1.00)	<10 (1.00)	3543 (5.06)	106 (4.13)	51 (3.15)	19 (3.79)	<10 (1.00)	<10 (1.00)
Lck-422	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)
ppMAPkkk-432	1213 (16.39)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	636 (9.56)	24 (4.83)	22 (4.30)	<10 (1.00)	10 (1.00)	<10 (1.00)
WHSC2-103	17493 (6.61)	23 (0.21)	21 (0.31)	11 (0.23)	<10 (0.36)	<10 (1.00)	18186 (4.57)	135 (1.33)	87 (1.37)	29 (0.64)	16 (1.04)	<10 (1.00)
HNRPL-501	271 (6.95)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	358 (8.14)	23 (4.58)	15 (3.03)	<10 (1.00)	10 (1.00)	<10 (1.00)
UBE2V-43	11901 (8.46)	16 (0.20)	21 (0.32)	<10 (1.00)	13 (0.63)	<10 (1.00)	5655 (3.83)	83 (1.42)	77 (1.51)	<10 (1.00)	25 (1.12)	<10 (1.00)
UBE2V-85	<10 (0.08)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	69 (1.27)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)
WHSC2-141	1572 (2.55)	11 (0.33)	<10 (0.18)	<10 (0.47)	<10 (1.00)	<10 (1.00)	2371 (3.74)	57 (1.73)	44 (1.78)	13 (2.50)	10 (1.00)	<10 (1.00)
HNRPL-140	785 (1.71)	<10 (0.16)	10 (0.33)	<10 (0.25)	<10 (1.00)	<10 (1.00)	1704 (3.19)	60 (1.84)	51 (1.95)	15 (0.80)	<10 (1.00)	<10 (1.00)
SART3-302	215 (3.09)	<10 (0.25)	11 (0.25)	<10 (1.00)	<10 (1.00)	<10 (1.00)	256 (2.18)	27 (1.34)	20 (0.47)	<10 (1.00)	<10 (1.00)	<10 (1.00)
SART3-309	26455 (17.43)	16 (0.51)	13 (0.60)	<10 (1.00)	11 (1.05)	<10 (1.00)	19696 (10.14)	84 (2.90)	61 (2.66)	<10 (1.00)	19 (1.74)	<10 (1.00)
SART2-93	62196 (6.96)	56 (0.35)	35 (0.31)	19 (0.38)	20 (0.71)	<10 (1.00)	45915 (4.12)	253 (1.73)	154 (1.52)	34 (0.72)	29 (1.04)	<10 (1.00)
SART3-109	17244 (111.61)	<10 (1.00)	11 (2.17)	<10 (1.00)	<10 (1.00)	<10 (1.00)	7381 (38.14)	48 (9.63)	40 (8.02)	<10 (1.00)	13 (2.61)	<10 (1.00)
Lck-208	412 (1.20)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	1322 (3.25)	10 (1.00)	16 (3.11)	<10 (1.00)	<10 (1.00)	<10 (1.00)
PAP-213	32182 (52.41)	18 (0.38)	21 (0.60)	<10 (1.00)	15 (0.91)	<10 (1.00)	15387 (10.99)	98 (2.20)	76 (2.42)	<10 (1.00)	25 (1.46)	<10 (1.00)
PSA-248	21095 (13.49)	22 (0.48)	24 (0.61)	<10 (1.00)	<10 (1.00)	<10 (1.00)	19447 (8.02)	101 (2.20)	86 (2.27)	<10 (1.00)	14 (1.33)	<10 (1.00)
EGFR-800	13115 (7.53)	12 (0.21)	12 (0.35)	<10 (0.23)	<10 (1.00)	<10 (1.00)	13276 (6.02)	82 (1.59)	60 (1.72)	12 (0.59)	<10 (1.00)	<10 (1.00)
MRP3-503	145 (29.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	14 (2.8)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)
MRP3-1293	11003 (13.52)	14 (0.38)	15 (0.44)	<10 (1.00)	<10 (0.47)	<10 (1.00)	6306 (5.97)	75 (2.26)	58 (2.10)	<10 (1.00)	13 (1.24)	<10 (1.00)
SART2-161	884 (13.92)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	519 (8.72)	15 (2.93)	13 (2.60)	<10 (1.00)	<10 (1.00)	<10 (1.00)
Lck-486	14036 (14.43)	21 (0.33)	19 (0.44)	<10 (1.00)	18 (1.31)	<10 (1.00)	7148 (6.81)	81 (1.69)	72 (2.26)	<10 (1.00)	25 (1.95)	<10 (1.00)
Lck-488	42491 (8.65)	36 (0.24)	41 (0.35)	11 (0.29)	25 (0.76)	<10 (1.00)	26514 (4.84)	212 (1.64)	155 (1.50)	23 (0.89)	54 (1.62)	<10 (1.00)
PSMA-624	5299 (26.36)	<10 (0.30)	<10 (0.37)	<10 (1.00)	<10 (1.00)	<10 (1.00)	5066 (22.77)	36 (2.67)	36 (2.75)	<10 (1.00)	10 (1.00)	<10 (1.00)
EZH2-735	622 (4.52)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	574 (4.14)	<10 (1.00)	10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)
PTHrP-102	1083 (1.29)	<10 (1.00)	<10 (0.47)	<10 (0.48)	<10 (1.00)	<10 (1.00)	2635 (2.97)	32 (6.39)	41 (3.66)	<10 (0.45)	<10 (1.00)	<10 (1.00)
SART3-511	34670 (13.05)	28 (0.40)	27 (0.50)	<10 (0.42)	14 (0.9)	<10 (1.00)	23299 (7.72)	141 (2.15)	101 (1.85)	11 (1.06)	26 (1.66)	<10 (1.00)
SART3-734	3845 (2.12)	47 (0.36)	51 (0.47)	36 (0.61)	17 (0.73)	<10 (1.00)	5365 (2.87)	147 (1.01)	125 (1.08)	55 (0.92)	19 (0.94)	<10 (1.00)
Lck-90	50172 (11.48)	29 (0.28)	26 (0.36)	<10 (0.19)	14 (0.73)	<10 (1.00)	32010 (5.12)	195 (2.25)	141 (2.04)	19 (0.80)	34 (1.66)	<10 (1.00)
Lck-449	1441 (2.73)	12 (0.31)	<10 (0.17)	<10 (1.00)	<10 (1.00)	<10 (1.00)	2027 (3.65)	49 (1.47)	38 (1.73)	<10 (1.00)	<10 (1.00)	<10 (1.00)
PAP-248	<10 (0.02)	<10 (0.25)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)	271 (0.71)	13 (0.61)	<10 (1.00)	<10 (1.00)	<10 (1.00)	<10 (1.00)

[a] IgM, IgG, and IgG subclasses (IgG1, IgG2, IgG3, and IgG4) specific to each of the 31 CTL epitope peptides were measured by the Luminex system in 100-time diluted samples (plasma or sera) from patients with hematological malignancy or HCC. The median values of FIU are shown. The changes relative to the HD groups (fold increase or fold decrease) are also calculated and shown in parenthesis. Undetectable levels (less than 10 FIU of cut-off value) of anti-peptide antibody titers were tentatively defined as 5 FIU for calculation of fold increase or fold decrease.

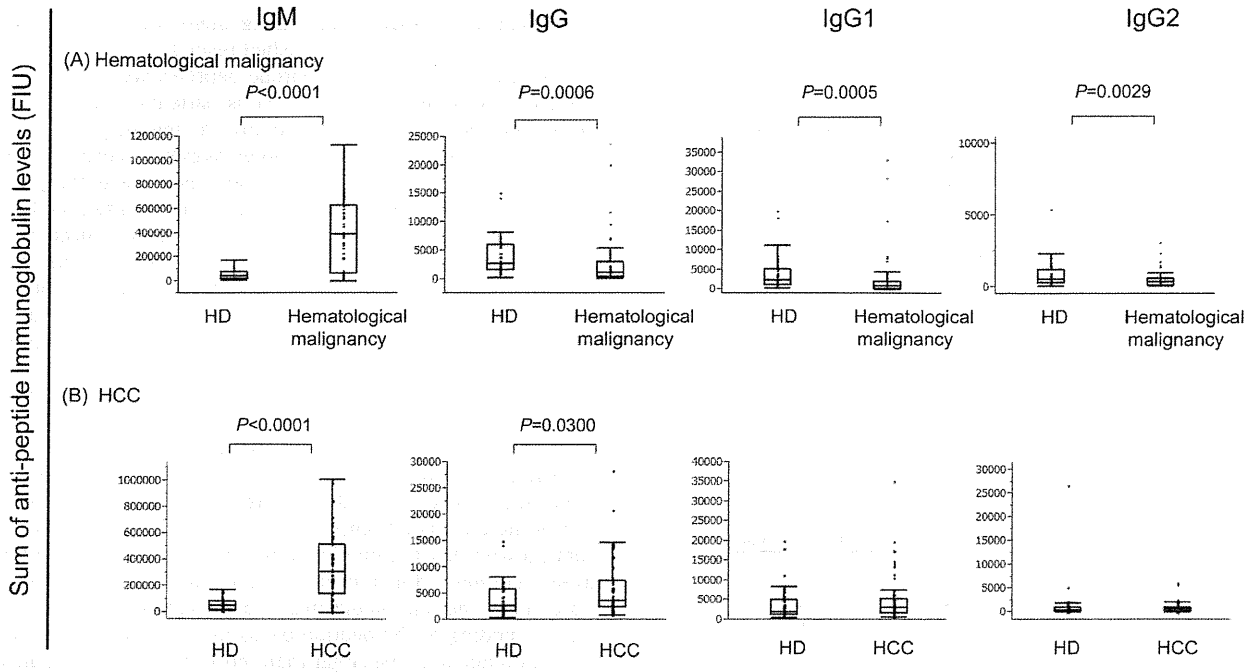

Fig. 3. Comparison of anti-peptide immunoglobulins in plasma or sera between healthy donors and patients with malignant diseases. Immunoglobulins (IgM, IgG, IgG1, IgG2) specific to each of the 31 CTL epitope peptides were measured by multiplex bead suspension array in plasma or sera from patients with hematological malignancies and non-viral hepatocellular carcinoma (HCC) and gender- and age-matched healthy donors (HD). The total sums of the immunoglobulins specific to each of the peptides were calculated. (A) hematological malignancies ($n = 59$) vs. gender- and age-matched HD ($n = 38$). (B) HCC ($n = 55$) vs. gender- and age-matched HD ($n = 52$). The differences between each group were evaluated by Wilcoxon test. Only the P values that were statistically significant ($P < 0.05$) are shown.

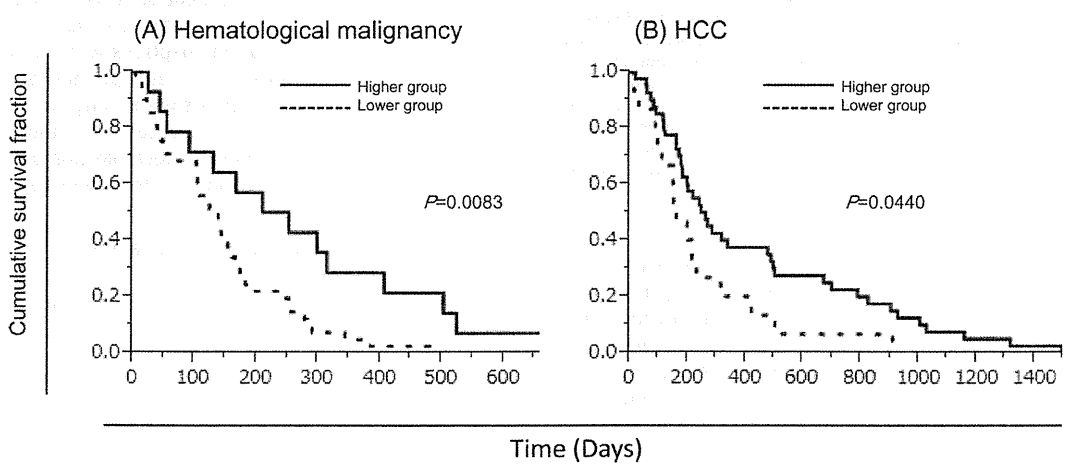

Fig. 4. Correlation between the sums of anti-peptide IgG levels and overall survival in patients with malignant diseases. Patients with hematological malignancies (A) or hepatocellular carcinoma (HCC) (B) were divided into two subgroups by the sums of IgGs specific to each of the 31 CTL epitope peptides. The median values in healthy donors were used as a threshold. Kaplan–Meier curves for overall survival were plotted in the two subgroups. Solid line and dotted line showed the subgroups with higher and lower sums of anti-peptide IgGs, respectively. A log-rank test was used for statistical analysis.

et al., 2004; Kobayashi et al., 2003). These results, along with our previous reports showing that all the 15 TAA tested were preferentially expressed on malignant cells with low levels of expression on normal proliferating cells (see the references citation in the Supplementary Table 3), suggest that both CTL and humoral responses against these CTL epitope peptides are consistently observed in both HD and patients with various diseases.

An age-dependent decrease of anti-peptide IgM responses was observed as far as tested from ages 19 to 91. The similar decrease was also observed on anti-peptide IgG responses, although the IgG levels were somewhat increased at elder ages (≧65 years old). This could be partly explained by age-dependent decline of specific immunity largely due to an atrophic change of the thymus year by year, starting at around 12 years of age.

There were no significant differences in anti-peptide Ig levels between patients with rheumatoid arthritis (RA) and the age- and gender-matched HD samples. This result may suggest that humoral responses against CTL epitope peptides were not affected by the impaired immune responses observed in RA. The total sums of anti-peptide IgM or IgG2 in patients with Flu infection were significantly lower or higher than those in HD, respectively. In patients with HCV infection, however, the total sums of anti-peptide IgG1

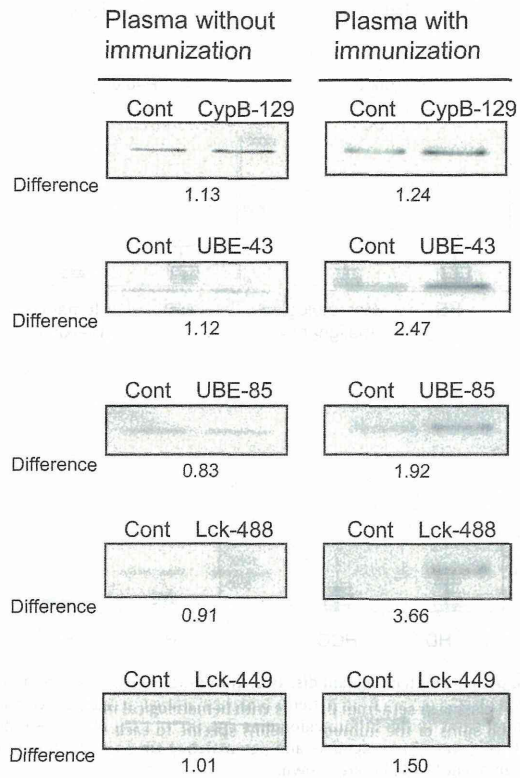

Fig. 5. Detection of IgGs specific to CTL epitope peptides in plasma from cancer patients with or without immunization. IgGs specific to CypB-129, UBE2V-43, UBE2V-85, Lck-488, and Lck-449 peptides were isolated by the peptide-coupled beads from plasma in cancer patients with and without immunization of these peptides, and were detected by Western blotting with anti-human IgG(H + L) antibody. As a control, the beads without coupling with the peptides were used for isolation of the non-specific IgGs. The numbers shown are differences in signal intensities between the peptide-coupled beads and peptide-uncoupled beads in cancer patients with and without immunization: [signal intensity by the peptide-coupled bead]/[signal intensity by the peptide-uncoupled bead].

were significantly higher than those of HD. Th2 cells responsible for IgG2 production or Th1 cells responsible for IgG1 production are reported to be more activated in patients with Flu infection or HCV infection, respectively (Chen et al., 2011; Gordon et al., 2010; Roohvand et al., 2007). Therefore, the results shown above could be partly due to these un-balanced immune responses in these patients.

The levels of IgM or IgG against CTL epitope peptides increased or decreased in patients with hematological malignancies, respectively, compared to those in HD, suggesting the impaired class-switch of Ig as expected. In contrast, the levels of IgG against CTL epitope peptides increased in patients with non-HCV HCC. Augmentation of T cell responses to CTL epitope peptides in cancer patients might be partly responsible for the increment of IgG against them. Large-scale studies on different types of malignancies are now underway.

When the cut-off was set to the median values of Igs from HD, the total sum of anti-peptide IgG against 31 different peptides was significantly correlated with OS in patients with both hematological malignancies and HCC. From a clinical point of view, patients with hematological malignancies holding more anti-peptide IgG than the median value of HD ($n = 15$) survived longer than the other remaining patients ($n = 41$). Similarly, HCC patients holding more than the median value of HD ($n = 40$) survived longer than the other patients ($n = 15$). The same results were observed in pancreatic cancer patients before vaccination, and the patients holding more than the median value among them survived significantly than the other patients (unpublished results).

The IgGs against the CTL epitope peptides were detectable by the Luminex system in HD as well as patients with various diseases, but their levels were too low to measure the absolute amounts of anti-peptide IgGs. Either Western blotting or ELISA with a human IgG ELISA kit was not sensitive enough for quantitative analysis with these samples. We therefore attempted to use plasma from frequently (≧12 times) vaccinated cancer patients for quantitative analysis, and showed that the amounts of anti-peptide IgG in the circulation of these patients ranged from 21 to 178 ng/mL. When such results were employed for approximate quantitative analysis of anti-peptide IgG in HD, the amounts of anti-peptide IgG in circulation of HD could be estimated to be in the range of 0.01–1 ng/mL. This estimation was largely based on the hypothesis that the binding affinities of IgG to certain peptides in HD were similar to those in frequently vaccinated patients. However, the binding affinities in the latter are most likely to be higher than those in HD, due to the somatic hypermutation in B cells of the vaccinated cancer patients. Indeed, our preliminary results showed that the binding affinities of IgG against the immunized peptides after repeated vaccinations were higher than those in pre-vaccination plasma. If so, the amounts of anti-peptide IgG not binding to the peptide beads in samples from non-vaccinated patients might be larger than those in post-vaccination samples, resulting in underestimation of the amounts of anti- peptide IgG in the circulation of HD. Further studies remain to be conducted for more accurate measurement of the amounts of IgG against CTL epitope peptides in HD and non-vaccinated cancer patients by employing more sensitive assays with larger amounts of samples, if available, for enrichment of anti-peptide IgG.

Collectively, the current study showed that humoral immune responses to certain CTL epitope peptides were widely detectable in humans, and the measurement of anti-peptide IgGs may provide a new biomarker for OS of patients with malignant diseases. It further remains to be determined whether the humoral immune responses to peptides are widely detectable not only for the CTL epitopes used, but also for other panels of peptides.

Disclosure of potential conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2013.04.004.

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A phase II study of a personalized peptide vaccination for chemotherapy-resistant advanced pancreatic cancer patients

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Abstract. Pancreatic cancer is one of the most aggressive cancers with a median survival time (MST) of <6 months in chemotherapy-resistant patients. Therefore, the development of novel treatment modalities is needed. In the present study, a phase II study of personalized peptide vaccination (PPV) was conducted, in which vaccine antigens were selected and administered based on the pre-existing IgG responses to 31 different pooled peptides, for 41 chemotherapy-resistant advanced pancreatic cancer patients. No vaccine-related severe adverse events were observed. IgG responses specific to at least one of the vaccine peptides were augmented in 14 of 36 patients (39%) and in 18 of 19 patients (95%) tested after the 5th and 11th vaccination, respectively. MST from the first vaccination was 7.9 months with a 1-year survival rate of 26.8%. Higher serum amyloid A (SAA) and C-reactive protein (CRP) levels in pre-vaccination plasma were unfavorable factors for overall survival (OS). Due to the safety profile and the potential clinical efficacy, the conduction of additional clinical trials of PPV for chemotherapy-resistant advanced pancreatic cancer patients is warranted.

Introduction

Pancreatic cancer, the fourth leading cause of cancer-related mortality worldwide, constitutes one of the most aggressive types of cancer (1). There have been substantial advances in the therapeutic modalities for advanced pancreatic cancer, including carbon beam ion radiotherapy (2), systemic chemotherapies using gemcitabine (GEM), tegaful-gimeracil-oteracil potassium (S-1) (3) and oxaliplatin, irinotecan, fluorouracil, leucovorin (Folfirinox) (4), as well as an EGFR-inhibitor erlotinib (5). However, despite these advances, the median survival time (MST) of advanced pancreatic cancer patients from the first or second line of chemotherapy still remains approximately 7-11 (1-5) or 4-6 months (2,6), respectively. Therefore, the development of novel therapeutic approaches including cancer vaccines is needed.

We previously devised a new regimen of peptide-based vaccination, named personalized peptide vaccination (PPV), in which vaccine antigens were selected from 31 different pooled peptides, and administered based on both HLA-class IA types and levels of peptide-specific IgG responses before vaccination (7-10). In our previous clinical trials, immune responses triggered by PPV were well-associated with overall survival (OS) in advanced pancreatic cancer patients under PPV in combination with GEM as the first-line therapy (7,8). GEM did not inhibit immune responses induced by PPV. Furthermore, the MST of advanced pancreatic cancer patients with positive (n=10) or negative (n=8) immune responses was 15.5 and 6 months, respectively, when non-resectable pancreatic cancer patients were treated with PPV and GEM as the first-line therapy. However, there is no trial of PPV for chemotherapy-resistant advanced pancreatic cancer currently available. Consequently, in the present study, a phase II study of PPV in chemotherapy-resistant advanced pancreatic cancer patients was performed.

Materials and methods

Patients. Patients pathologically and/or clinically diagnosed with pancreatic cancer were eligible for inclusion in the present study, when they had failed at least first-line chemotherapy and showed positive IgG responses to at least 2 of the 31 different vaccine candidate peptides as previously reported (10). Additional inclusion criteria were the following: age between 20 and 80 years, Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, positive status for the HLA-A2, -A24, -A3 supertype (A3, A11, A31 or A33) or -A26, life expectancy of at least 12 weeks, and adequate

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hematologic, hepatic and renal function. Exclusion criteria included pulmonary, cardiac or other systemic diseases, 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 #08167). After a full explanation of the protocol, a written informed consent was obtained from all the patients prior to enrollment.

Clinical protocol. This was an open-label phase II study, in which the main objectives were to evaluate safety and to address whether PPV in combination with additional chemotherapeutic regimens for chemotherapy-resistant pancreatic cancer patients prolongs MST. Thirty-one peptides, the safety and immunological effects of which were reported in previous clinical studies (8-11), were employed for vaccination [12 peptides for HLA-A2, 14 for HLA-A24, 9 for HLA-A3 supertype (A3, A11, A31 or A33) and 4 for HLA-A26]. The peptides were prepared under the conditions of Good Manufacturing Practice (GMP) by PolyPeptide Laboratories (San Diego, CA, USA) and the American Peptide Company (Vista, CA, USA).

The peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, by assessing the titers of IgG specific to each of the 31 different vaccine candidates (10). A maximum of 4 peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, in complex with incomplete Freund's adjuvant (Montanide ISA 51; Seppic, Paris, France) were subcutaneously administered once a week for 6 consecutive weeks.

After the first cycle of 6 vaccinations, up to 4 vaccine peptides were re-selected according to the titers of peptide-specific IgG and administered every 2 weeks. Vaccine peptides were re-selected at every cycle of 6 vaccinations until the discontinuation of PPV. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 3.0. Complete blood counts and serum biochemical tests were performed at every cycle of 6 vaccinations. The clinical responses were evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST) with radiological findings of computed tomography (CT) scanning or magnetic resonance imaging (MRI) before and after vaccinations.

Measurement of laboratory markers. Levels of C-reactive protein (CRP), serum amyloid A (SAA) and IL-6 in plasma were examined by ELISA using kits from R&D Systems (Minneapolis, MN, USA), Invitrogen (Carlsbad, CA, USA) and eBioscience (San Diego, CA, USA), respectively. Bead-based multiplex assays were used to measure cytokines, including IL-4, IL-13, IL-21, IP-10, BAFF and TGF-β with the Luminex 200 system (Luminex, Austin, TX, USA).

Measurement of immunoglobulins (Igs) reactive to each of the 31 different peptides. The levels of Igs reactive to each of the 31 different peptides were measured using the Luminex 200 system as previously reported (9-11). In brief, plasma was incubated with 100 μl of peptide-coupled color-coded beads for 1.5 h at 30°C, followed by washing and incubation with 100 μl of biotinylated goat anti-human IgG (Vector Laboratories, Burlingame, CA, USA). After washing, 100 μl of streptavidin-PE (Invitrogen) was added and incubated for 30 min at 30°C. After washing, the fluorescence on the beads was detected using the Luminex 200 system. The Igs levels were expressed in fluorescence intensity units (FIU) as previously reported (9-11). Peptide-specificity of IgG against each of the 31 peptides was confirmed (unpublished data).

Statistical methods. The Wilcoxon signed-rank test and paired t-test were used to compare differences between pre- and post-vaccination measurements. OS was calculated from the first day of peptide vaccination until the day of death or the last day when the patient was known to be alive. Prognostic factors for OS were evaluated by univariate and multivariate analyses with the Cox proportional hazards regression model. Curves for OS were estimated using the Kaplan-Meier method, and the log-rank test was conducted for the comparison of survival curves. Two-sided P-values of <0.05 were considered to indicate statistically significant differences. All statistical analyses were conducted using the JMP version 10.0.1 software (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. Between November 2008 and March 2011, 41 advanced pancreatic cancer patients who had failed at least first-line chemotherapy were included in the present study. Patient characteristics are listed in Table I. There were 27 male and 14 female subjects with a median age of 61 years (range, 44-78). All patients had advanced stages of cancer (stage IVa, n=7; IVb, n=24; recurrent, n=10). Prior to enrollment, the patients had failed 1 (n=11), 2 (n=24), 3 (n=5) or 4 (n=1) regimen(s) of chemotherapy. The median duration of chemotherapy prior to PPV was 8 months with a range from 1 to 36 months. The performance status at the time of enrollment was grade 0 (n=37) or 1 (n=4). The numbers of vaccine peptides employed at the first cycle of vaccinations were 4 peptides in 33 patients, 3 in 5 patients and 2 in 3 patients. The median number of vaccinations was 10 with a range of 3 to 36. PPV was combined with GEM (n=11), S-1 (n=6), GEM and S-1 (n=8) or other combinations of chemotherapeutic agents including CDDP-based regimens (n=8). PPV alone was administered to 8 patients, since chemotherapy could not be tolerated (n=4) or due to patient refusal (n=4).

Toxicities. A grade 1 or 2 dermatological reaction at the injection site was observed in 39 cases. Anemia (n=15), lymphocytopenia (n=20), thrombocytopenia (n=11), leukocytopenia (n=7), hypoalbuminemia (n=15) and hyperglycemia (n=8) were also frequently observed. Grade 3 adverse events included anemia (n=1), lymphocytopenia (n=1), hypertension (n=1), GGT increase (n=1) and creatinine increase (n=1). According to assessment by the Independent Safety Evaluation Committee in this trial, all the grade 3 adverse events were concluded to be not directly associated with PPV.

Humoral responses to peptides. IgG responses specific to the vaccine peptides in pre- and post-vaccination plasma samples

Table I. Patient characteristics.

Characteristic	Value
Age (years), median (range)	61 (44-78)
Gender, n	
Male	27
Female	14
Disease location, n	
Head	14
Body	15
Limbs	6
Body and limbs	6
Performance status, n	
0	37
1	4
Stage, n	
IVa	7
IVb	24
Recurrent	10
No. of previous regimens, n	
1	11
2	24
3	5
4	1
Duration of previous treatment (months), median (range)	8 (1-36)
No. of vaccinations, median (range)	10 (3-36)
Combined treatment, n	
(-)	8
GEM	11
S-1	6
GEM and S-1	8
Other regimens	8
Treatment response, n	
SD	28
PD	13
Overall survival time (days), median (95% CI)	238 (151-313)

GEM, gemcitabine; S-1, tegaful-gimeracil-oteracil potassium; SD, stable disease; PD, progressive disease; CI, confidence interval.

were analyzed. Post-vaccination plasma samples were available from 36 and 17 patients after the 5th and 11th vaccination, respectively. When peptide-specific IgG titers to at least one of the vaccine peptides in the post-vaccination plasma were >2-fold higher compared to those in the pre-vaccination plasma, antigen-specific humoral responses were considered to be increased. The IgG responses specific to at least one of the vaccine peptides were augmented in 14 of 36 patients (39%) and in 18 of 19 patients (95%) after the 5th and 11th vaccination, respectively (Table II).

Table II. IgG responses to the vaccinated peptides.

		IgG response		
Patient no.	Peptide	Pre	5th	11th
1	ppMAPkkk-432	43	40	na
	WHSC2-103	68	69	na
	HNRPL-501	191	**638**	na
	HNRPL-140	209	189	na
2	SART3-109	226	**1,896**	na
	Lck-422	44	66	na
	CypB-129	23	45	na
	WHSC2-103	322	401	na
3	PSA-248	28	**4,999**	**28,025**
	MRP3-1293	75	70	**3,259**
	SART2-161	37	38	**7,860**
	Lck-486	38	31	**23,697**
4	MRP3-503	57	56	na
	MRP3-1293	79	69	na
	SART2-161	51	53	na
	Lck-486	53	ND	na
5	CypB-129	161	120	**12,717**
	ppMAPkkk-432	368	ND	ND
	UBE2V-43	396	399	**60,508**
	SART3-302	272	235	**11,267**
	HNRPL-501	150	**343**	ND
6	HNRPL-140	13	ND	na
	SART3-302	40	ND	na
7	SART3-109	42	52	na
	SART3-511	27	ND	na
	Lck-90	13	ND	na
	Lck-449	45	ND	na
8	SART2-93	32	18	na
	PAP-213	1,249	1,573	na
	EGF-R-800	40	ND	na
	MRP3-503	98	38	na
	SART3-109	23	11	na
9	Lck-246	376	623	**3,264**
	UBE2V-43	188	ND	**16,549**
	UBE2V-85	294	314	**2,053**
	SART3-302	207	330	**1,929**
	HNRPL-140	ND	**494**	**2,780**
10	HNRPL-501	578	ND	ND
	UBE2V-85	70	ND	14
	SART3-302	36	ND	ND
	SART3-309	18	ND	ND
11	SART3-109	21	ND	**653**
	MRP3-503	69	ND	**14,787**
	PTHrP-102	14	ND	ND
12	SART2-93	164	ND	na
	Lck-208	206	13	na
	Lck-486	245	298	na
	EZH2-735388	503	na	

Table II. Continued.

Patient no.	Peptide	IgG response Pre	5th	11th
12	Lck-422	783	532	na
	HNRPL-140	456	380	na
13	SART3-109	1,475	1,279	na
	Lck-486	1,644	1,833	na
14	SART3-109	2,309	2,136	**6,782**
	MRP3-1293	43	40	**23,180**
	SART2-161	32	27	ND
	Lck-486	1,515	1,234	**267,768**
15	SART3-109	1,500	**5,872**	**180,917**
	SART2-161	31	22	**3,278**
	Lck-486	650	224	**58,780**
	Lck-488	54	37	**21,889**
	SART3-511	99	57	ND
16	SART3-511	1,699	1,503	1,522
	PAP-248	70	69	ND
	Lck-422	180	ND	16
	WHSC2-103	188	ND	**2,629**
	Lck-90	35	45	63
	CypB-129	16	23	20
17	ppMAPkkk-432	83	88	ND
	SART3-109	62	49	ND
	Lck-486	2,176	2,191	**3,523,034**
	PTHrP-102	129	162	135
	SART2-93	47	**100**	59
18	MRP3-1293	103	ND	na
	Lck-486	5,731	10,510	na
	PSMA-624	99	ND	na
	ppMAPkkk-432	126	115	na
	SART3-109	55	50	na
	Lck-488	38	35	na
19	CypB-129	57	53	na
	ppMAPkkk-432	106	90	na
	HNRPL-501	974	934	na
	SART3-302	473	**2,233**	na
	Lck-246	17	**61**	na
20	Lck-246	409	441	**2,349**
	EGF-R-800	83	134	183
	Lck-486	95	72	**37,353**
	EZH2-735	117	ND	**10,454**
	CypB-129	183	192	190
	ppMAPkkk-432	120	185	233
21	PAP-213	48	**98**	na
	Lck-486	20	22	na
22	CypB-129	109	112	**393**
	Lck-246	22	13	**56**
	WHSC2-141	22	ND	15
	SART3-302	631	**1,459**	**5,168**
	Lck-422	14	12	78

Table II. Continued.

Patient no.	Peptide	IgG response Pre	5th	11th
23	PAP-213	13	**123**	**4,179**
	Lck-486	25	**580**	**2,552**
	Lck-449	37	37	43
	WHSC2-103	40	14	**165**
	SART3-511	ND	**289**	173
	PAP-248	ND	**1,200**	63
24	PAP-213	122	122	na
	Lck-449	129	102	na
	CypB-129	186	183	na
	WHSC2-103	69	ND	na
25	PAP-213	16	**2,772**	na
	PSA-248	64	**1,372**	na
	Lck-486	17	**105**	na
26	CypB-129	90	81	105
	Lck-246	20	12	**39**
	SART3-309	12	**374**	**4,738**
	PAP-248	21	ND	ND
27	SART2-93	11	ND	**55**
	SART3-109	156	222	**1,871**
	Lck-486	185	313	**12,511**
	Lck-488	15	12	**3,980**
	PAP-213	ND	**14**	**ND**
28	PAP-213	31	44	**657**
	PSA-248	45	**446**	**15,954**
	EGF-R-800	30	33	**2,926**
	Lck-486	22	23	**11,356**
29	SART2-93	11	11	na
	Lck-486	25	ND	na
	Lck-488	14	16	na
30	CypB-129	246	232	na
	WHSC2-141	317	21	na
	SART3-302	86	**865**	na
	Lck-208	11	**2,016**	na
31	SART2-93	40	37	**478**
	Lck-486	23	32	**2,567**
	Lck-488	31	47	**20,641**
	PTHrP-102	40	46	**523**
32	WHSC2-141	433	398	**20,518**
	PSA-248	29	**2,109**	**13,221**
	MRP3-1293	149	**4,155**	**11,903**
		121		
	Lck-486	121	18,577	
33	SART2-93	22	**51**	60
	SART3-109	14	ND	16
	Lck-486	39	ND	**2,479**
	SART2-161	ND	**76**	59
34	CypB-129	263	239	na
	WHSC2-103	43	ND	na

Table II. Continued.

Patient no.	Peptide	IgG response Pre	5th	11th
34	WHSC2-141	231	125	na
	SART3-734	32	ND	na
35	MRP3-1293	62	ND	na
	Lck-486	85	ND	na
	SART3-734	123	ND	na
	CypB-129	149	93	na
36	SART2-93	13	11	12
	SART3-109	11,200	10,657	10,093
	Lck-488	16	13	**2,017**
	EGF-R-800	ND	11	ND

Underlined peptides indicate the selection of new peptides for the second cycle of PPV. Bold values represent increased IgG responses. na, not applicable; ND, no data.

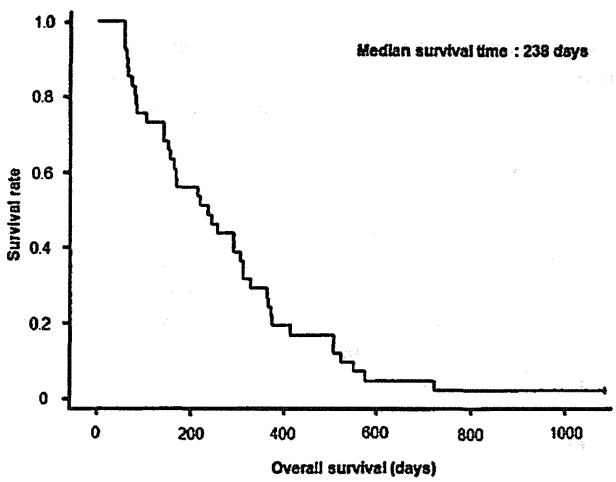

Figure 1. Kaplan-Meier survival analysis of the enrolled patients. The median survival time (MST) of patients who were vaccinated (n=41) was 238 days (7.9 months) and the 1-year survival rate was 26.8%.

Laboratory markers. Two inflammation markers, CRP and SAA, and 7 cytokines including IL-4, IL-6, IL-13, IL-21, IP-10, BAFF and TGF-β, were examined in plasma before and after the 5th vaccination. Since 5 of 41 patients did not complete the first cycle of 6 vaccinations due to rapid disease progression, they were excluded from the marker analysis. However, no significant differences before and after vaccinations were observed in the markers tested (data not shown).

Clinical outcome. No complete response (CR) or partial response (PR) was observed during PPV. Optimum clinical responses after the 6th vaccination or at discontinuation of PPV were observed in 28 cases of stable disease (SD) and 13 cases of progressive disease (PD) (Table I). MST from the first vaccination was 7.9 months (238 days) with a 1-year survival rate of 26.8% (Table I). All the 41 patients, except for 1 patient, had succumbed to the disease at the time of examination. Survival curve is shown in Fig. 1. MST in patients treated with PPV in combination with (n=33) or without (n=8) chemotherapies was 9.6 or 3.1 months, respectively (P=0.0013) (data not shown). When calculated from the initiation of the first-line chemotherapy, MST of all 41 cases was 19.0 months [95% confidence interval (CI), 15.0-25.0 months].

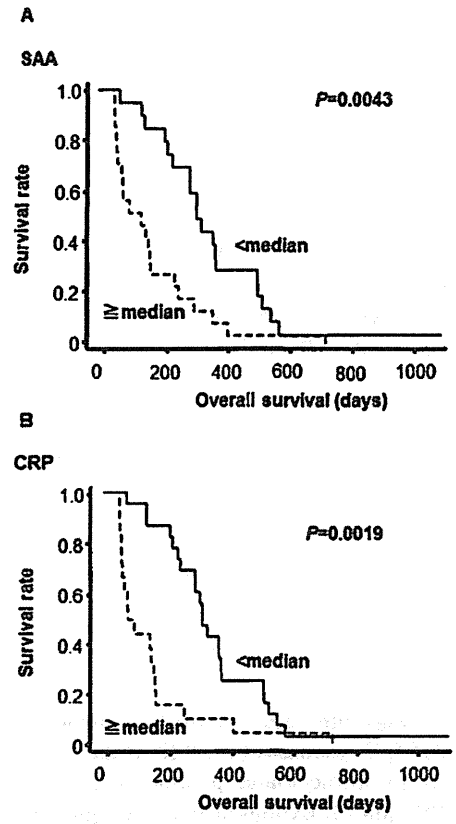

Figure 2. Evaluation of pre-vaccination factors by Kaplan-Meier survival analysis. Patients were divided into two subgroups according to the median value of the (A) SAA and (B) CRP levels before vaccination. Curves for overall survival were estimated by the Kaplan-Meier method, and differences in survival rates were evaluated using the log-rank test. SAA, serum-amyloid A; CRP, C-reactive protein.

Prognostic factors for OS. Pre-vaccination prognostic biomarkers for OS were investigated in 36 patients who completed at least the first cycle of 6 vaccinations. SAA levels in pre-vaccination samples were found to be inversely associated with OS using the univariate Cox proportional hazards model [hazard ratio (HR) per 1 mg/dl increment = 1.10, 95% CI=1.03-1.15, P=0.004] (Table III). CRP levels also showed a significant association (HR per 1 mg/dl increment = 1.68, 95% CI=1.03-2.58, P=0.039). Similar results were obtained using the multivariate Cox proportional hazards model. The patients were allocated into two subgroups according to the median value of SAA or CRP. The survival curves were estimated by the Kaplan-Meier method and differences in survival rates were compared using the log-rank test. The patients with higher SAA (P=0.0043) or CRP levels (P=0.0019) in the pre-vaccination samples exhibited worse prognosis (Fig. 2).

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

	Univariate analysis		Multivariate analysis	
Factor	Hazard ratio (95% CI)	P-value[a]	Hazad ratio (95% CI)	P-value[a]
Age (years)	1.58 (0.40-6.44)	0.52		
Gender (female<male)	0.98 (0.52-1.95)	0.96		
Clinical stage (IVa<recurrent<IVb)	1.18 (0.78-1.80)	0.43		
Duration of previous chemotherapy (months)	0.98 (0.94-1.02)	0.27		
Regimen no. of previous chemotherapy	0.93 (0.59-1.44)	0.75		
Lymphocyte count ($x10^2/mm^3$)	1.00 (1.00-1.00)	0.39		
Hemoglobin (g/dl)	0.93 (0.75-1.16)	0.53		
Albumin (g/dl)	0.58 (0.32-1.10)	0.09		
Creatinine (mg/dl)	1.88 (0.51-5.23)	0.31		
SAA (mg/dl)	1.09 (1.03-1.15)	0.004[b]	1.08 (0.99-1.18)	0.09
CRP (mg/dl)	1.68 (1.03-2.58)	0.039[b]	0.95 (0.41-2.06)	0.91

[a]P-values determined by Cox proportional hazard regression model; [b]significant difference. CI, confidence interval; SAA, serum amyloid A; CRP, C-reactive protein.

In addition, concerning post-vaccination samples, the patients with boosted IgG responses (n=19) [in response to the vaccinated (n=14) or unvaccinated peptides selected for the 2nd cycle of PPV (n=5)] exhibited better prognosis compared to those with no IgG boosting (n=17) (P=0.0485) (data not shown).

Discussion

The MST of 41 chemotherapy-resistant advanced pancreatic cancer patients under PPV was 7.9 months with a 1-year survival rate of 26.8%. Among them, the MST in patients treated with PPV combined with (n=33) or without (n=8) chemotherapies was 9.6 or 3.1 months, respectively (P=0.0013). OS of the patients treated with PPV not combined with chemotherapies was significantly short, suggesting that PPV alone did not provide survival benefits to advanced pancreatic cancer patients. This failure was expected based on the results from our previous study (13). These results suggest that PPV has the potential to improve OS in chemotherapy-resistant advanced pancreatic cancer patients when administered in combination with chemotherapeutic agents.

With regard to post-vaccination biomarkers, several factors, including CTL responses, Th1 responses, delayed-type hypersensitivity (DTH) and autoimmunity, have been reported to be associated with clinical responses in some clinical trials (14,15). We have also shown that an increase in peptide-specific IgG and/or CTL responses after PPV is significantly associated with longer OS (11,12). In contrast to such post-vaccination biomarkers, there are currently no validated pre-vaccination prognostic biomarkers widely used. Therefore, this issue was addressed in the present study. As a result, plasma SAA and CRP levels were inversely correlated with OS. These results were expected based on our previous study on PPV (10). These biomarkers are suggested to be important not only in cancer vaccines, but also in other treatment modalities for advanced pancreatic cancers.

Collectively, due to the safety profile and the potential clinical efficacy of PPV, further clinical trials to determine a protocol suitable for PPV-based therapy in chemotherapy-resistant advanced pancreatic cancer patients are warranted.

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Phase II study of personalized peptide vaccination for refractory bone and soft tissue sarcoma patients

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Refractory bone and soft tissue sarcomas are challenging diseases to treat because of their robustness to chemotherapy. Although cancer vaccines have the potential to become an attractive treatment modality, their progress has been hampered by the presence of many subtypes of sarcomas and different human leukocyte antigen (HLA)-types. We investigated whether personalized peptide vaccination (PPV) would be feasible for the vast majority of sarcoma patients. Twenty refractory bone and soft tissue sarcoma patients with nine different subtypes and 11 different HLA-class IA phenotypes were enrolled in this study. A maximum of four HLA-matched peptides showing higher peptide-specific IgG responses in pre-vaccination plasma were selected from 31 pooled peptide candidates applicable for the HLA-A2, -A3, -A11, -A24, -A26, -A31, and -A33 types, and were subcutaneously administered weekly for 6 weeks and bi-weekly thereafter. Measurement of peptide-specific CTL and IgG responses along with other laboratory analyses were conducted before and after vaccination. No patients were excluded by either sarcoma subtypes or different HLA-types. No severe adverse events associated with PPV were observed in any patients. Peptide-specific immunological boosting was observed in the post-vaccination samples from the majority of patients. Tumor reduction of the lung metastasis and a long stable disease was observed in each case, and the median overall survival time of the 20 cases was 9.6 months. Taken together, PPV could be feasible for the vast majority of refractory sarcoma patients because of the safety and higher rates of immunological responses regardless of the presence of different sarcoma subtypes and various HLA-types. (*Cancer Sci* 2013; 104: 1285–1294)

Refractory bone and soft tissue sarcomas are challenging diseases to treat with an unmet need for effective systemic therapy.[1,2] Several molecularly targeted agents, such as mammalian target of rapamycin (mTOR) inhibitor[3] and antibody to the insulin-like growth factor 1 receptor (IGF-1R),[4] have shown clinical benefits in a subgroup of sarcoma patients with refractory sarcomas, achieving a median survival time (MST) of 7.6–9.2 months. However, new treatment modalities still remain to be developed to improve overall survival (OS) of these patients, and cancer vaccines have been discussed as a promising approach against refractory sarcomas because of the expressions of tumor-associated antigens (TAA) on sarcoma tissues.[1,2,5–9] Nevertheless, there have been few clinical trials of cancer vaccination for refractory sarcoma patients. One of the hurdles could be the fact that there are many sarcoma subtypes along with different human leukocyte antigen (HLA)-types.

We have developed a novel regime of personalized peptide vaccination (PPV), in which vaccine antigens are selected and administered from a pool of 31 different peptide candidates based on the pre-existing IgG responses specific to peptides before vaccination.[10–13] In previous studies, the PPV was feasible for the vast majority of cancer patients with different HLA-types.[10–13] A recently conducted randomized clinical trial of PPV in advanced prostate cancer patients showed a favorable clinical outcome in the vaccinated group.[14] In the present study, we addressed whether PPV treatment would be feasible for refractory bone and soft tissue sarcoma patients with various HLA-types by conducting a small-scale phase II study.

Materials and Methods

TAA and HLA-class I expressions in sarcoma tissues. The expressions of 15 different TAA, from which the vaccine peptides used for PPV were derived, were examined by immunohistochemistry (IHC) in 26 sarcoma tissues (11 leiomyoarcoma, five synovial sarcoma, five malignant fibrous histiocytoma, and five liposarcoma) as previously reported.[15] The expression of HLA-class I was also examined by IHC in the 26 sarcoma tissues by using an anti-HLA-class I antibody (murine monoclonal, clone EMR8-5; Abcam, Cambridge, UK).

Patients. Patients with histological diagnosis of bone and soft tissue sarcoma were eligible for inclusion in the present study. All patients were required to have evaluable recurrent and/or metastatic tumors at the time of entry. Patients whose general condition was tolerable for chemotherapy or radiotherapy were eligible only after the failure of these therapies. Patients, who had poor general conditions intolerable for chemotherapy or radiotherapy, or refused them, were also eligible. All patients were required to show positive IgG responses to at least two of the 31 different vaccine candidate peptides, as reported previously.[10–12] Other inclusion criteria were as follows: age between 20 and 80 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; positive status for the HLA-A2, -A24, -A3 supertypes (A3, A11, A31, or A33), or -A26 types; life expectancy of at least 12 weeks; and adequate hematologic, 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. Patients with a lymphocyte count of <1000/μL were excluded from the study, since we previously reported that pre-vaccination lymphocytopenia (<1000 cells/μL) is an unfavorable factor for OS in cancer patients receiving PPV.[16,17] The protocol was approved by the Kurume University Ethical Committee and registered in the UMIN Clinical Trials Registry

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(UMIN#000002282). All patients were given a full explanation of the protocol and provided their informed consent before enrollment.

Clinical protocol. This was a phase II study to evaluate the safety and immunological responses in refractory bone and soft tissue sarcoma patients under PPV. Thirty-one peptides, whose safety and immunological effects for other types of cancer were confirmed in previously conducted clinical studies,[11–14] were used for vaccination (12 peptides for HLA-A2, 16 peptides for HLA-A24, nine peptides for HLA-A3 supertypes [-A3, -A11, -A31, and -A33], and four peptides for HLA-A26) (Table S1). These peptides were prepared under the condition of Good Manufacturing Practice (GMP) by the PolyPeptide Laboratories (San Diego, CA, USA) and American Peptide Company (Vista, CA, USA). Peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, as assessed by the titers of IgG specific to each of the 31 different vaccine candidates.

A maximum of four peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, were subcutaneously administrated with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France) once a week for six consecutive weeks. After the first cycle of six vaccinations, up to four antigen peptides, which were re-selected according to the titers of peptide-specific IgG at the 6th vaccination, were administered every 2 weeks. During the PPV, patients were allowed to receive combination therapies, such as chemotherapies or radiotherapies. Since the frequency of bone and soft tissue sarcomas had been low, we enrolled the patients treated both with and without combination therapies to facilitate the enrolment. 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 every six vaccinations. The clinical responses were determined by the Response Evaluation Criteria in Solid Tumors (RECIST) in the vaccinated patients. The RECIST-based clinical responses were evaluated every six vaccinations by radiological findings of computed tomography (CT) scan and/or magnetic resonance imaging (MRI), and the

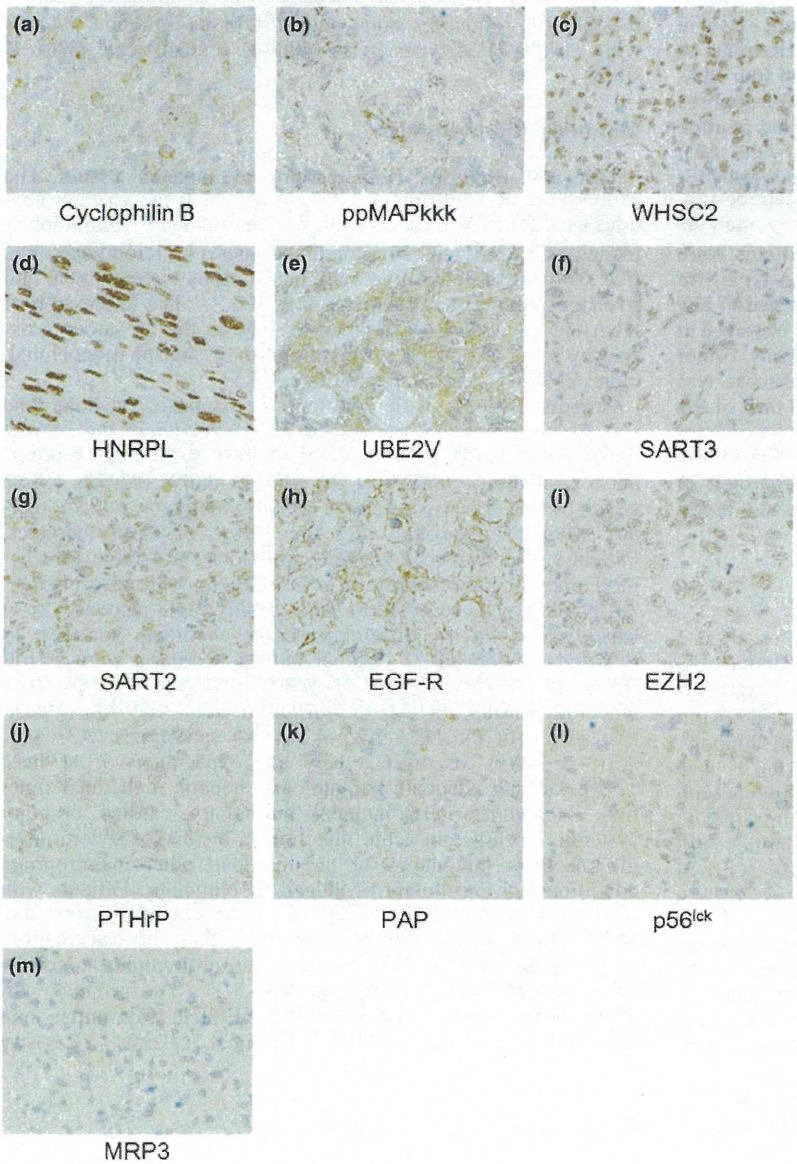

Fig. 1. Expressions of tumor-associated antigens (TAA) in soft tissue sarcoma tissues. Expressions of TAA were examined by immunohistochemistry in soft tissue sarcoma tissues. Thirteen out of the 15 TAA were expressed at different frequencies in soft tissue sarcoma tissues. Representative results are shown (a–m: ×400). The remaining two prostate-related antigens, PSA and PSMA, were not detectable by immunohistochemistry in sarcoma tissues tested (not shown).

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best overall responses during PPV treatment were shown. For the patients who did not complete the first cycle of six vaccinations, the newest radiological findings were evaluated.

Measurement of humoral and cellular immune responses and inflammatory cytokine and markers. Humoral immune responses specific to each of the 31 peptide candidates were determined by peptide-specific IgG levels using the Luminex system (Luminex, Austin, TX, USA), as previously reported.[17] If the titers of peptide-specific IgG to at least one of the vaccinated peptides in the post-vaccination plasma were more than two-fold higher than those in the pre-vaccination plasma, the changes were considered to be significant as reported previously.[11–16] Cellular immune responses specific to the vaccinated peptides were evaluated by interferon (INF)-γ ELISPOT using PBMCs as reported previously.[11–16] As a control, cellular immune responses specific to CEF peptides (MABTECH, Cincinnati, OH, USA), a mixture of virus-derived CTL epitopes, were also examined. Inflammatory cytokine and markers, including, interleukin-6 (IL-6), C-reactive protein (CRP), and serum amyloid A (SAA), in plasma samples were also examined by ELISA as reported previously.[15]

Statistical analysis. The two-sided Wilcoxon test was used to examine differences between pre- and post-vaccination measurements. *P*-values <0.05 were considered to be statistically significant. Progression-free survival (PFS) or OS were calculated from the date of the first vaccination until the date of disease progression or death, respectively, or the last date when the patient was known to be alive. Predictive factors for OS

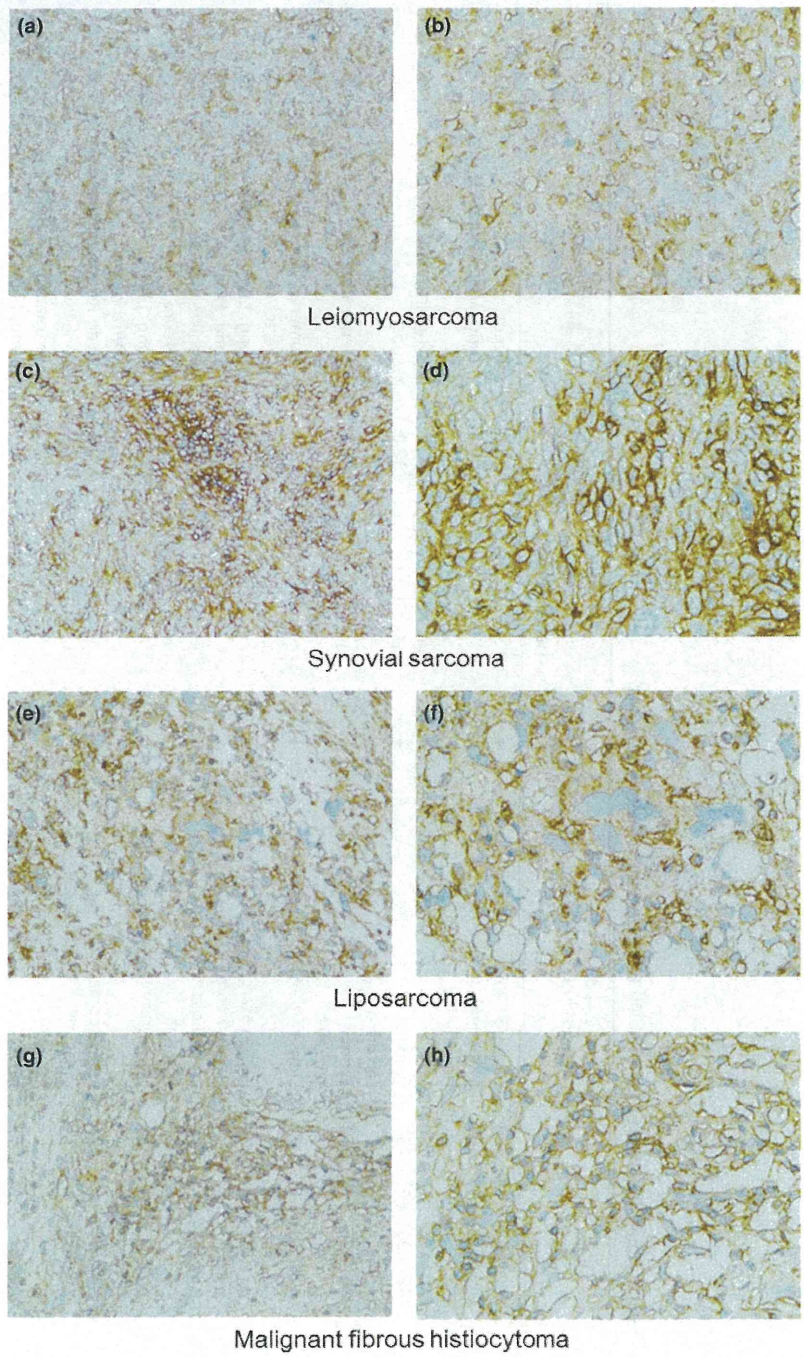

Fig. 2. Expressions of HLA-class I in soft tissue sarcoma tissues. Expressions of HLA-class I were examined by immunohistochemistry in different subtypes of soft tissue sarcoma tissues. (a,b) leiomyosarcoma (a, ×200; b, ×400). (c,d) synovial sarcoma (c, ×200; d, ×400). (e,f) liposarcoma (e, ×200; f, ×400). (g,h) malignant fibrous histocytoma (g, ×200; h, ×400).

Table 1. Characteristics of the enrolled patients with refractory sarcoma

No.	HLA type	Sex	Age	Pathology	Stage	PS	Previous radiotherapy	No. of previous chemotherapy	Periods of chemotherapy	Disease location	No. of vaccination	Combined therapy	Treatment response	PFS	Survival times
1	A2/A24	M	64	Osteosarcoma	rec.	0	+	2	23.0	Lung	12	–	PD	4.8	9.7
2	A2/A11	M	31	Malignant neurinoma	rec.	0	+	1	6.8	Lung, Mediastinal LN	17	–	SD	6.8	7.6
3	A2/A24	M	60	MFH	rec.	0	–	–	–	Lung, Inguinal LN	6	–	PD	2.0	2.3†
4	A2/A24	F	30	Synovial sarcoma	rec.	0	+	4	32.0	Lung	6	Radiotherapy	PD	1.8	4.6
5	A2/A26	M	54	Synovial sarcoma	IV	0	–	1	10.0	Mediastinal LN	6	–	SD	33.0	35.0†
6	A24/A33	F	74	Liposarcoma	rec.	1	+	–	–	Humerus, Thoracic vertebra	14	–	SD	7.4	9.2†
7	A11/A33	F	49	Leiomyosarcoma	IV	0	–	2	3.8	Lung, Liver, Sacrum	15	–	PD	4.5	10.0
8	A33	F	23	Osteosarcoma	rec.	0	–	4	6.9	Lung, Hilar LN	4	–	PD	1.6	2.2
9	A24/A33	M	43	Epithelioid sarcoma	rec.	1	+	–	–	Parasternal LN, Pleura	12	–	SD	7.0	25.0†
10	A24	F	33	Leiomyosarcoma	rec.	0	–	3	17.0	Lung, Liver, Peritoneum	12	GEM + DTX	PD	4.4	9.6
11	A24/A26	F	62	Liposarcoma	rec.	0	–	2	2.7	Liver, Retroperitoneum	4	–	PD	1.5	7.2
12	A24/A26	F	55	Clear cell sarcoma	rec.	1	–	3	4.6	Lung, Intraabdominal LN	6	–	PD	3.5	21.0†
13	A24	M	73	MFH	rec.	0	–	–	–	Lung, Liver	11	–	SD	11.0	11.0
14	A26/A30	F	38	MFH	rec.	1	–	2	2.3	Liver, Retroperitoneum	3	–	PD	1.4	2.5
15	A24	F	75	Leiomyosarcoma	rec.	0	–	–	–	Liver, Lumbar vertebra, Sacrum, Femur	12	–	PD	4.4	6.2
16	A2/A24	M	64	Chondrosarcoma	rec.	0	–	–	–	Lung, Pleura	14	–	SD	5.7	5.7†
17	A2	F	63	Leiomyosarcoma	rec.	0	+	3	24.0	Lung, Subcutaneous tissue	6	GEM	PD	1.8	4.7
18	A2/A11	M	71	Osteosarcoma	rec.	0	–	–	–	Local recurrence, Lung	11	–	PD	3.5	4.1†
19	A2/A26	M	45	Alveolar soft part sarcoma	rec.	0	+	3	65.0	Lung, Brain	10	Radiotherapy CPA	PD	3.5	3.6†
20	A11/A31	M	27	Synovial sarcoma	rec.	0	–	2	8.9	Lung, Liver, Retroperitoneum	6	Sorafenib	PD	1.6	3.2

†Survivor. CPA, cyclophosphamide; DTX, docetaxel; F, female; GEM, gemcitabine; LN, lymph nodes; M, male; MFH, malignant fibrous histocytoma; PS, performance status; PD, progressive disease; PFS, progression-free survival; rec., recurrent status; SD, stable disease.

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