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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

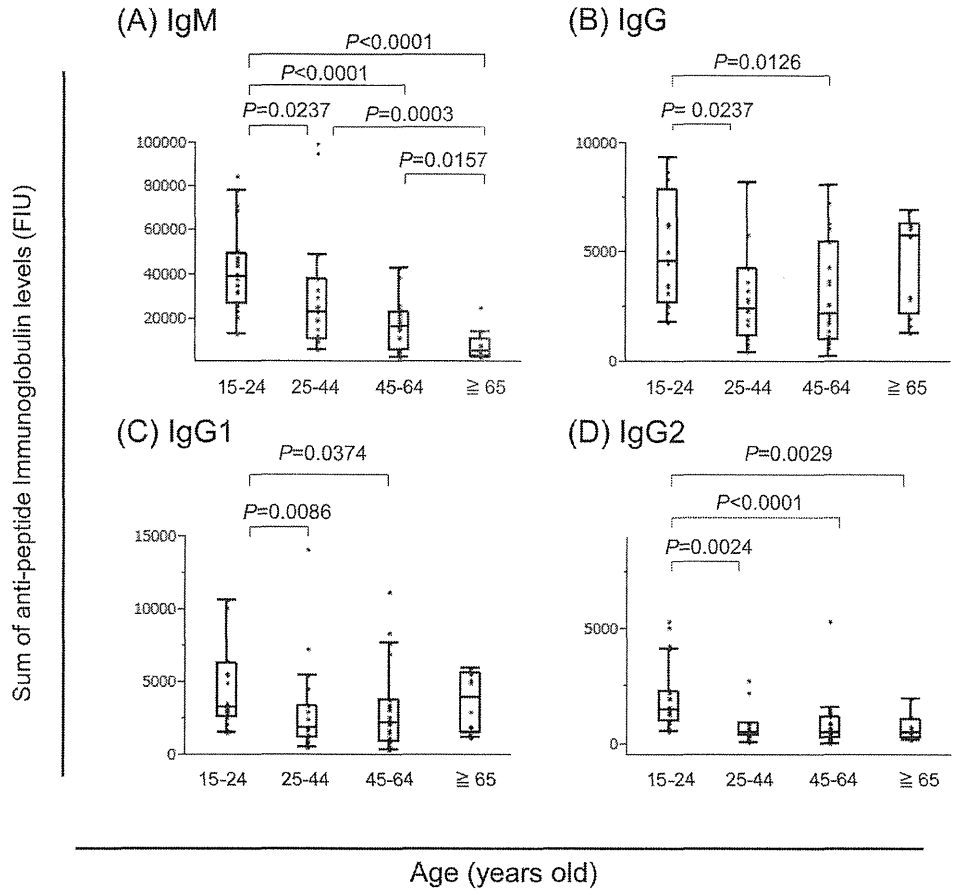

Fig. 1. Detection of immunoglobulins specific to CTL epitope peptides in plasma or sera from healthy donors. Immunoglobulins (IgM, IgG, IgG1, IgG2) specific to each of the 31 CTL epitope peptides were measured by multiplex bead suspension array in plasma or sera from healthy donors. The total sums of 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

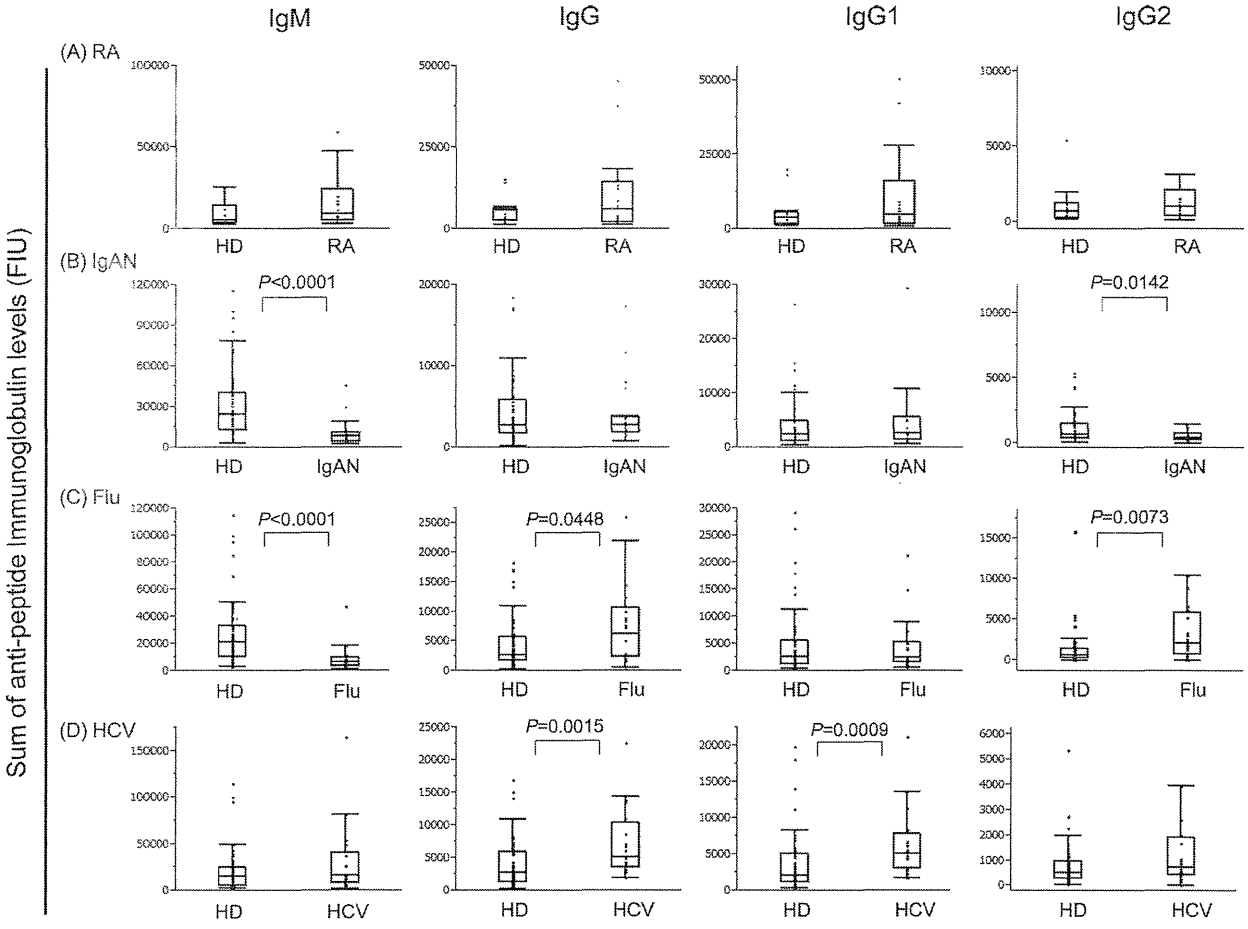

Fig. 2. Comparison of anti-peptide immunoglobulins in plasma or sera between healthy donors and patients with various types of immune-related diseases. Immunoglobulins (IgM, IgG, IgG1, IgG2) specific to each of the 31 CTL epitope peptides were measured by multiplex bead suspension array in plasma or 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.

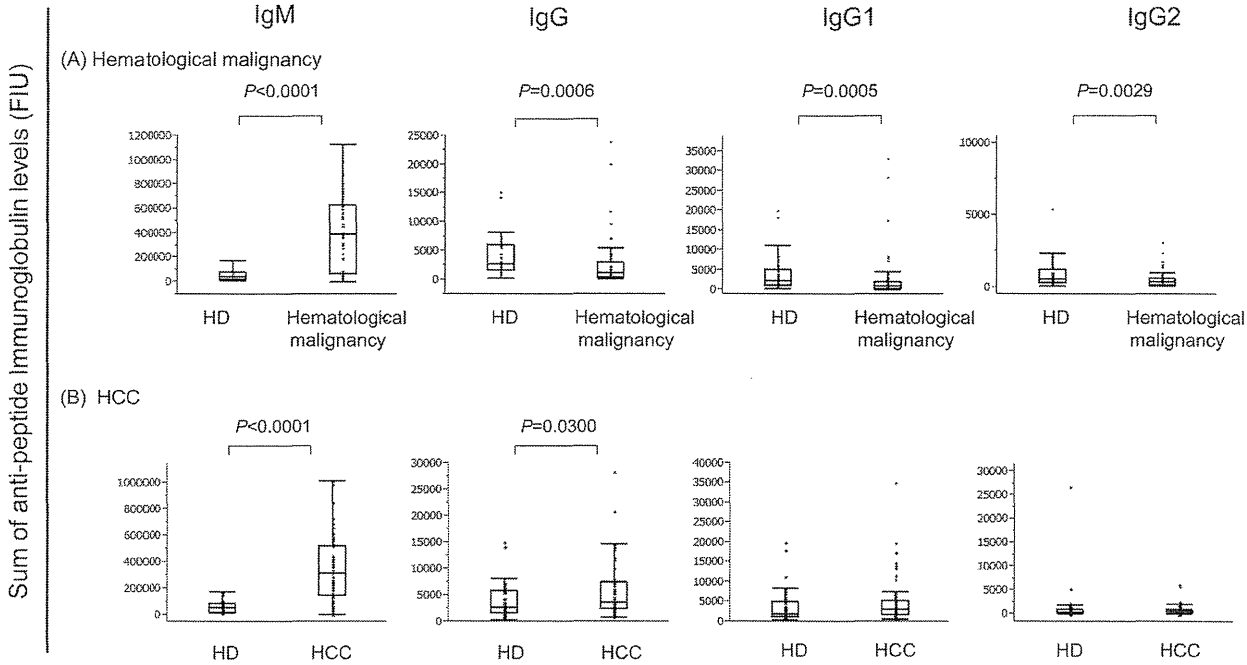

Fig. 3. Comparison of anti-peptide immunoglobulins in plasma or sera between healthy donors and patients with malignant diseases. Immunoglobulins (IgM, IgG, IgG1, IgG2) specific to each of the 31 CTL epitope peptides were measured by multiplex bead suspension array in plasma or sera from patients with 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.

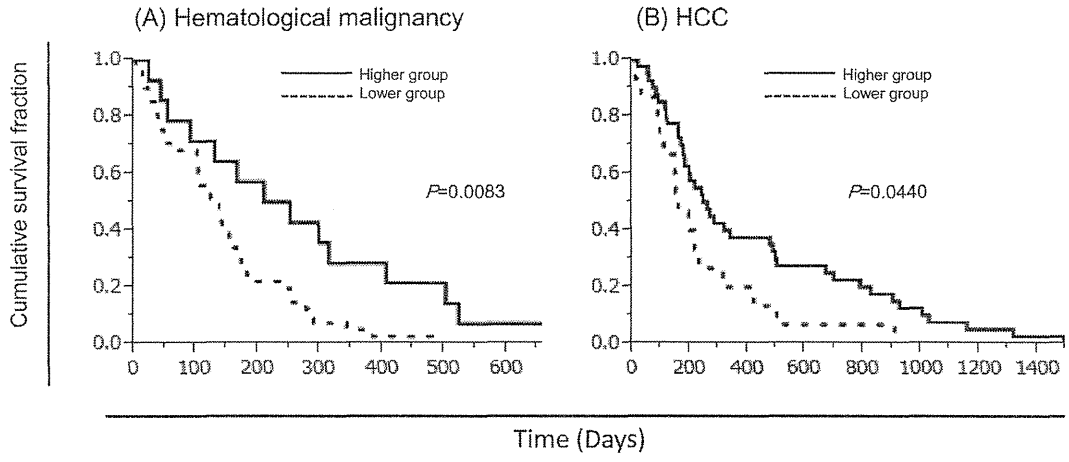

Fig. 4. Correlation between the sums of anti-peptide IgG levels and overall survival in patients with malignant diseases. Patients with hematological malignancies (A) or hepatocellular carcinoma (HCC) (B) were divided into two subgroups by the sums of IgGs specific to each of the 31 CTL epitope peptides. 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

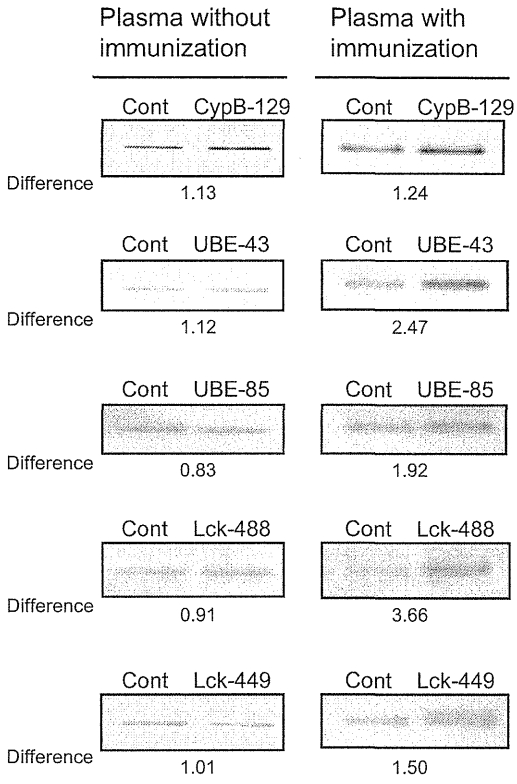

Fig. 5. Detection of IgGs specific to CTL epitope peptides in plasma from cancer patients with or without immunization. IgGs specific to CypB-129, UBE2V-43, UBE2V-85, Lck-488, and Lck-449 peptides were isolated by the peptide-coupled beads from plasma in cancer patients with and without immunization 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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Haptoglobin Proved a Prognostic Biomarker in Peripheral Blood of Patients with Personalized Peptide Vaccinations for Advanced Castration-Resistant Prostate Cancer

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Haptoglobin (Hp) is a well-known acute-phase protein that possibly has influence on tumors through the immune response. This study was conducted to evaluate the correlation between Hp expression and the effect of treatment by cancer peptide vaccines in advanced castration-resistant prostate cancer (CRPC) patients. Hp expression was measured by RT-PCR using peripheral blood mononuclear cells (PBMCs) collected from advanced CRPC patients, who were divided into two groups: long-term survivors and short-term survivors. Before cancer peptide vaccination (pre-vaccination), Hp expression was almost same in the two groups, but after cancer peptide vaccination (post-vaccination), Hp expression was higher in short-term survivors, suggesting that Hp expression in the PBMCs increased in short-term survivors after treatment by cancer peptide vaccines. Our results suggest that Hp expression level in the PBMCs can serve as a prognostic biomarker in treatment by cancer peptide vaccine in advanced CRPC patients.

Key words: haptoglobin; peripheral blood mononuclear cells; castration-resistant prostate cancer; treatment by cancer peptide vaccine

In prostatic cancer, prostatic cells lose their normal growth function and self-propagate in a disorderly manner. Although abnormalities of the gene are considered to be the cause of abnormal cell proliferation, it is not yet understood why normal cells become cancerous. In Japan, prostatic cancer accounts about 3.5% of cancer in males, and its occurrence has been increasing rapidly in recent years. In Europe and North America, about 20% of men are diagnosed as prostatic cancer patients. The incidence of prostatic cancer will probably continue to increase in the near future due to the Westernization of diet in Japan.

Together with the progressive increase in basic knowledge of tumor immunology, the field of cancer vaccines has moved forward dramatically.[1–5] In therapy by cancer peptide vaccines, a particular peptide is injected into a cancer patient to heighten the immune activity that suppresses the cancer. This artificially synthesized peptide is prescribed for the patient as a vaccine, and killer T cells that receive the stimulus are activated and proliferate to attack the cancer cells. In clinical tests, this new method has successfully used in prostatic cancer,[6] pancreatic cancer,[7] and so on. However, there is a problem in that the effect of cancer peptide vaccines varies among patients.

We examined the gene expression profiles in PBMCs of advanced CRPC patients receiving peptide vaccination to identify biomarkers to predict a patient's capacity to fight against cancer. PBMCs were obtained from patients with advanced CRPC patients who had survived for more than 900 d (long-term survivors, $n = 17$) or died within 300 d (short-term survivors, $n = 21$) after cancer peptide vaccination. Forty-one up regulated genes in short-term survivors were found to be differentially expressed in pre- and/or post-vaccination in PBMC.[8] Hp, a well-known immune response gene, was one of the 41 up regulated genes.

Hp is an acute-phase reactant produced in response to inflammatory stimuli and to body fluids that can influence innate immunity, and probably even acquired immunity.[9–12] It plays important roles in the host's defense against infection and neoplasms.[13] It is also involved in tumor and infection pathogenesis, in that it modulates the switch orientation of the Th1/Th2 response.[11,14] It is produced in the liver and immunological cells and secreted into serum. Its level changes in the serum in response to infection, inflammation, and various malignant diseases, including lung and bladder cancer,[15,16] leukemia,[17] breast cancer,[18] malignant lymphoma,[19] urogenital tumors,[20] and esophageal squamous cell carcinoma.[21] Of these 41 genes, we especially observed Hp gene. In recent years, this gene is attracted attention in the field of cancer research, but the expression of it in CRPC patients in response to cancer peptide vaccines is unknown. In this study, we analyzed Hp expression by real-time PCR in PBMCs from advanced CRPC patients, and examined the relationship between Hp gene expression and the effects of cancer peptide vaccination.

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Abbreviations: Hp, haptoglobin; PBMCs, peripheral blood mononuclear cells; CRPC, castration-resistant prostate cancer

Materials and Methods

Patient samples. We did a retrospective analysis of peripheral blood samples from a subset of 164 patients with advanced CRPC. These patients were positive for human leukocyte antigen (HLA)-A24 or HLA-A2 and enrolled in a phase 1, 1-2, or 2 clinical trials for personalized peptide vaccination between February 2001 and April 2008. These studies were approved by the Ethics Review Committees, of participating hospitals in Japan (Kurume University Hospital, Kinki University Hospital, Okayama University Hospital, and Nara Medical University Hospital). The progression of disease (PD) was defined by at least one of the following criteria: i) two consecutive 25% increases in PSA levels at least 2 weeks apart, ii) a greater than 25% increase in bidimensionally measurable soft tissue metastases, or iii) the appearance of new foci on radionuclide bone scans. Other criteria included an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, age 18 years or older, normal hematologic, hepatic, and renal functions, and negative serologic tests for hepatitis B and hepatitis C. Patients with evidence of serious illness, an active secondary malignancy that occurred within 5 years before entry, or autoimmune disease were excluded.[8)]

Among the 164 patients enrolled, those who survived more than 900 d (long-term survivors, n = 17) or who died within 300 d (short-term survivors, n = 21) were selected for examination of Hp expression profiles in PBMCs. There were no significant differences in clinical or pathological features between the two groups. Pre-vaccination PBMCs were available for 38 patients, but post-vaccination PBMCs were obtained after the completion of one cycle of six vaccinations, and were available from only a subset of patients (long-term survivors, n = 16; short-term survivors, n = 17). The number of samples after cancer peptide vaccination decreased due to the one incomplete cycle of six vaccinations, or because the purified mRNA was of poor quality. At 6 weeks post-vaccination, PBMCs were extracted from patients treated with cancer peptide vaccines. Table 1 shows the survival rates for long-term and short-term survivors in days.

RNA isolation from PBMCs. PBMCs were prepared from 20 mL of peripheral blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Uppsala, Sweden). All samples were cryopreserved until RNA extraction. Total RNA was isolated using TRIZOL LS reagent (Invitrogen, Carlsbad, CA), and was purified using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturers' instructions. The quality and integrity of the purified total RNA were confirmed using an Agilent 2100 bioanalyzer (Agilent Technology, Palo Alto, CA) and a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Table 1. Patient Survival Duration after Cancer Vaccine Treatment

Short-term survivors (ID)	Survival duration (d)	Long-term survivors (ID)	Survival duration (d)
4	214	1	901
5	263	3	1,006
7	240	11	1,222
9	234	12	1,120
10	203	44	1,254
27	154	45	1,764
28	97	46	3,264
29	176	47	2,430
31	90	49	1,986
32	142	50	1,482
33	102	51	1,151
34	135	53	1,657
35	178	54	1,351
37	78	55	1,347
38	277	56	1,376
39	278	57	1,089
41	257	60	1,670
42	280		
43	273		
3,019	242		
3,021	186		

Real-time quantitative PCR of Hp mRNA. To measure mRNA expression levels, total RNA was extracted from cultured cells with TRIzol, and 2 μg of total RNA was used for cDNA synthesis by MultiScribe™ reverse transcriptase following the manufacturer's instructions (Applied Biosystems). The Hp and GAPDH transcripts produced by RT-PCR amplification of the cDNA template were quantified using SYBR^R *Premix Ex Taq*™ II (Takara Bio, Ohtsu, Japan). The primers were as follows: Hp forward primer, 5′-TGGCTATGTGGAGCACTCGG-3′, and reverse primer, 5′-TATCC-ACTGCTTCTCATTG-3′. The final reaction for the analysis of Hp expression at cDNA was performed at a 20 μL volume, 0.4 μL from each primer (final concentration: 0.2 μm), 0.4 μL ROX Reference Dye II (Invitrogen, 50×, final concentration 1×), 10 μL 2×SYBR® Premix Ex Taq™ (Clontech, Tokyo, final concentration 1×), 2 μL cDNA sample, and 6.8 μL PCR-grade water. The cycling conditions were 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. All reactions were run in an ABI 7500 Fast system (Applied Biosystems). We performed real-time PCR 3 times.

Results

Hp mRNA expression in pre-vaccination PBMCs

We analyzed Hp expression in pre-vaccination PBMCs from advanced CRPC patients. The average Hp mRNA expression levels in pre-vaccination PBMCs from short-term and long-term survivors were 0.113 and 0.0666 respectively. The average Hp mRNA expression level in short-term survivors was a little higher than those from long-term survivors (Fig. 1), but the variances between patients were very high (long-term survivors' SD = 0.0537, short-term survivors' SD = 0.0858), and hence the difference in Hp mRNA expression between groups was not significant ($p = 0.0537$). This result indicates that the Hp expression level in pre-vaccination PBMCs did not show a statistically significant relation to the effect of cancer peptide vaccination.

Hp mRNA expression in post-vaccination PBMCs

Next we analyzed Hp expression in post-vaccination PBMCs from advanced CRPC patients (Fig. 2). The average expression levels of Hp in post-vaccination PBMCs from short-term and long-term survivors were 0.317 (SD = 0.216) and 0.0674 (SD = 0.0416) respectively. When patients with unusually low Hp mRNA expression levels were excluded, the average value among short-term survivors was 0.336 (SD = 0.208).

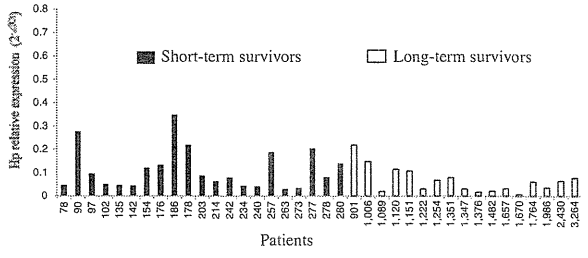

Fig. 1. Hp mRNA Expression in Long-Term Survivors and Short-Term Survivors before Vaccination.

Hp mRNA expression in pre-vaccination PBMCs of the short-term (n = 21) and long-term (n = 17) survivors were analyzed quantitatively by real-time PCR and normalized to the expression level of GAPDH, a housekeeping gene. Relative expression levels were shown as $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{Hp} - Ct_{GAPDH}$). Patients are represented by days of survival (see Table 1).

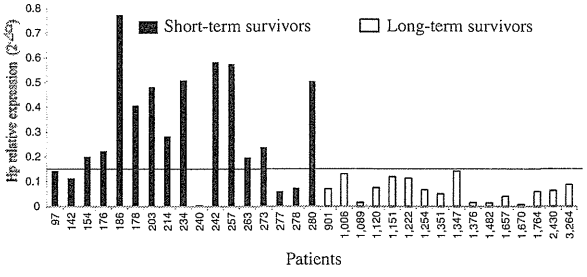

Fig. 2. Hp mRNA Expression in the Long-Term Survivors and Short-Term Survivors after Vaccination.

Hp expression in post-vaccination PBMCs of the short-term (n = 17) and long-term (n = 16) survivors were analyzed quantitatively by real-time PCR and normalized to the expression level of GAPDH, a housekeeping gene. Relative expression levels were shown as $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{Hp} - Ct_{GAPDH}$). The patients are represented by days of survival (see Table 1). A line is drawn at expression level 0.15 to discriminate the patients into two groups, short-term and long-term survivors.

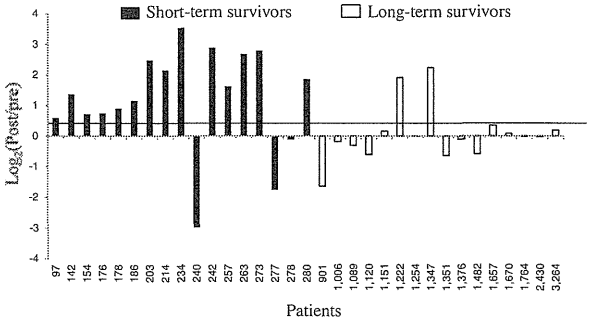

Fig. 3. Effect of Cancer Vaccination Treatment on Changes in the Hp mRNA Expression Level in PBMCs.

The ratio of Hp expression prior to and after cancer peptide vaccination were calculated, and are shown as $\log_2$ (post/pre). A line is drawn at post/pre ratio 1.3 ($\log_2$ (post/pre) = 0.379).

Table 2. Prediction of Effect of Cancer Vaccine Treatment

Diagnostic standard (threshold)	Accuracy (%)	
	Short-term survivors	Long-term survivors
Hp expression in post-vaccination (0.15)	12/17 (71%)	16/16 (100%)
post/pre ratio (1.3)	14/17 (82%)	14/16 (88%)

This indicates that the Hp expression level was significantly higher in post-vaccination PBMCs from short-term survivors than in those from long-term survivors ($p = 0.000284$). Our experimental results are consistent with those of a report that serum Hp was significantly elevated in late-stage epithelial ovarian cancer as compared with that from benign tumors.[22] These data suggests that Hp expression in PBMC following cancer peptide vaccination is involved in advanced CRPC development, and that their expression levels can be used to predict the extent of the effect of cancer peptide vaccination.

Effect of cancer peptide vaccination on Hp mRNA expression levels in PBMCs

Hp mRNA expression levels in PBMCs after cancer peptide vaccination differed significantly between short-term and long-term survivors. Hence, we calculated the extent of change in Hp mRNA expression level after cancer peptide vaccination. Figure 3 shows the ratio of Hp mRNA expression prior to and after cancer peptide vaccination. The ratio was apparently higher in short-term survivors than in long-term survivors ($p = 0.0221$), suggesting a strong correlation between the changes in the Hp mRNA expression level after cancer peptide vaccination and the progress of advanced CRPC. These results provide strong evidence that PBMCs of short-term survivors are capable of expressing Hp in response to cancer peptide vaccination as compared with long-term survivors.

Prediction of the effects of cancer vaccine therapy

Hp expression level might be an effective biomarker to predict the clinical endpoint following cancer peptide vaccination. There are two possible forms for such prediction: the Hp mRNA expression level in post-vaccination PBMCs, and the ratio of Hp mRNA expression level change (post/pre) in PBMCs. In the case of Hp mRNA expression level in post-vaccination PBMCs, the optimal threshold level of expression was 1.5 $2^{-\Delta Ct}$ (Figs. 1 and 2), and this identified short-term and long-term survivors with 71% (12/17) and 100% (16/16) accuracy respectively. On the other hand, the optimal threshold ratio of Hp mRNA expression level change was 1.3-fold, and it identified short-term and long-term survivors with 82% (14/17) and 88% (14/16) accuracy respectively (Table 2).

In sum, we propose that the Hp expression level in PBMCs should be measured post-vaccination, because this can identify long-term survivors well, and because it can improve and extend the lives of advanced CRPC patients who respond well to cancer peptide vaccination.

Discussion

The identification of biomarkers to predict the clinical response to treatment is a challenging but important issue in the effort to individualize therapy. There were no substantial differences in clinical or pathological features of the patients enrolled in the current study.

We examined the expression and clinical relevance of Hp in advanced CRPC patients. The expression levels of Hp in pre-vaccination PBMCs did not show any statistically significant difference as between long-term and short-term survivors, but Hp mRNA expression in post-vaccination PBMCs was significantly elevated in the short-term survivors but not in long-term survivors. We found that post/pre ratio in Hp mRNA expression in short-term survivors was clearly higher than that in long-term survivors. Our experimental results are consistent with a report of significantly elevated serum Hp in late-stage epithelial ovarian cancer, as compared with that in benign tumors.[22] It is also known that patients with cancer show high concentrations of Hp protein. Since Hp inhibits the phytohemagglutinin (PHA)-induced blastogenesis of lymphocytes,[23] this inhibition can be expected to contribute to the protection of tumor against immune attacks.

The differences in expression levels of 16 acute inflammation response genes (APCS, B4GALT1, CD14, CEBPB, CLCF1, CXCL2, IL1A, IL1B, IL1RN, IL6,

IL8, OSM, SIGIRR, STAT5B, TNF, and VNN1), as defined in Ingenuity Pathway Analysis software, as between long-term and short-term survivors, in post-vaccination PBMCs from advanced CRPC patients were examined by *t*-test.[8] Among 16 genes examined, only two (SIGIRR and VNN1) showed a difference in expression level between long-term and short-term survivors of a *p*-value of less than 0.01. This suggests that the elevation in Hp expression after vaccination was not a general inflammation response, but rather a specific response to vaccine treatment. Thus Hp expression level in PBMCs might have been a specific prognostic biomarker of peptide vaccine treatment.

Hp in particular and acute phase proteins more generally are especially interesting parameters for studying immunological lability. Hp circulates in the blood at low but taxon-specific concentrations. Concentrations can increase rapidly in response to acute infection, inflammation, or trauma.[24,25] During infection, target cells secrete primary pro-inflammatory cytokines IL-1β and TNF-α, which send signals to adjacent vascular cells to initiate the activation of endothelial cells and the recruitment of neutrophils. Activated neutrophils are the first line of defense.[26] They generate ROS and recruit other defense cells. Hp is synthesized during granulocyte differentiation and is stored for release when neutrophils are activated.[27]

Hp might exert immunomodulatory effects with suppression of lymphocyte function.[23,28] It has a direct inhibitory effect on T cells. Different subsets of T-helper lymphocytes, termed T-helper 1 (Th1) and T-helper 2 (Th2) cells, are responsible for the induction and regulation of cellular and humoral responses respectively. This suggests that disturbances in the Th1–Th2 balance are responsible for the development or severity of various immunological diseases.[29] Alterations of the ratio of Th1 to Th2 cells are important determinants of susceptibility to viral and parasitic infections, allergies, antitumor responses, and autoimmunity. Hp is perhaps an important endogenous regulator of immune reactions and a crucial molecule in the establishment of the Th1–Th2 balance. It suppresses T-cell proliferation and inhibits T-helper cytokine release, and thus Th2 cytokine release is far more suppressed than Th1 cytokine release. Changes in the Th1–Th2 balance have a major effect on susceptibility to infections. When the Hp expression level increases, it becomes impossible to attack a cancer cell without activating a T-lymph cell.[30]

Recent investigations have revealed that Hp is a natural inhibitor of collagen degradation and is locally expressed by fibroblasts in arterial walls.[31] This glycoprotein thus plays an important role in cell migration and arterial restructuring. Moreover, collagen turnover is an important feature of many physiological processes such as growth and wound healing, and enhanced collagen degradation is causally related to severe tissue destruction and malfunction, which are often encountered in pathological processes such as arthritis and cancer.[32]

To our knowledge, the tight correlation between inflammation and induction of Hp expression by cytokines, known to be critical in regulating the immune system, has led to the hypothesis that Hp has a regulatory role in immune function after cancer vaccine treatment. Our findings appear to be promising for the further development and application of cancer peptide vaccination. In our research, to identify promising prognostic biomarkers, we examined Hp gene expression profiles in PBMCs from 38 advanced CRPC patients before and after cancer peptide vaccination. The most striking feature of the differentially expressed Hp gene is that the Hp expression level was significantly higher in post-vaccinated PBMCs from short-term survivors than in those from long-term survivors. The importance of Hp in PBMCs suggests that a role for cancer progression after treatment by cancer peptide vaccines, and provides evidence that the Hp expression level is a drug target for advanced CRPC patients after treatment by cancer peptide vaccine.

Our retrospective analysis provides evidence that the Hp expression level can be a useful prognostic indicator in patients with advanced CRPC. Overall, our data suggest that lower Hp expression is associated with better survival outcome, but more studies are needed to confirm these findings.

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EGFR T790M Mutation as a Possible Target for Immunotherapy; Identification of HLA-A*0201-Restricted T Cell Epitopes Derived from the EGFR T790M Mutation

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Abstract

Treatment with epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib, has achieved high clinical response rates in patients with non–small cell lung cancers (NSCLCs). However, over time, most tumors develop acquired resistance to EGFR-TKIs, which is associated with the secondary EGFR T790M resistance mutation in about half the cases. Currently there are no effective treatment options for patients with this resistance mutation. Here we identified two novel HLA-A*0201 (A2)-restricted T cell epitopes containing the mutated methionine residue of the EGFR T790M mutation, T790M-5 (MQLMPFGCLL) and T790M-7 (LIMQLMPFGCL), as potential targets for EGFR-TKI-resistant patients. When peripheral blood cells were repeatedly stimulated *in vitro* with these two peptides and assessed by antigen-specific IFN-γ secretion, T cell lines responsive to T790M-5 and T790M-7 were established in 5 of 6 (83%) and 3 of 6 (50%) healthy donors, respectively. Additionally, the T790M-5- and T790M-7-specific T cell lines displayed an MHC class I-restricted reactivity against NSCLC cell lines expressing both HLA-A2 and the T790M mutation. Interestingly, the NSCLC patients with antigen-specific T cell responses to these epitopes showed a significantly less frequency of EGFR-T790M mutation than those without them [1 of 7 (14%) vs 9 of 15 (60%); chi-squared test, $p = 0.0449$], indicating the negative correlation between the immune responses to the EGFR-T790M-derived epitopes and the presence of EGFR-T790M mutation in NSCLC patients. This finding could possibly be explained by the hypothesis that immune responses to the mutated neo-antigens derived from T790M might prevent the emergence of tumor cell variants with the T790M resistance mutation in NSCLC patients during EGFR-TKI treatment. Together, our results suggest that the identified T cell epitopes might provide a novel immunotherapeutic approach for prevention and/or treatment of EGFR-TKI resistance with the secondary EGFR T790M resistance mutation in NSCLC patients.

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Introduction

Lung cancer is one of the most aggressive malignancies, but recently significant progress has been made in the therapeutic strategy against this disease [1–4]. In particular, epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib, have been developed as a novel treatment option for patients with non–small cell lung cancers (NSCLCs) that possess somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) gene [5–7]. Prospective clinical trials of EGFR-TKI treatment in NSCLC patients with activating EGFR mutations, such as delE746-A750 (exon 19) and L858R (exon 21), have demonstrated high clinical response rates of approximately 80% [8–10]. Nevertheless, over time (median of 6–12 months), most tumors develop acquired resistance to EGFR-TKIs. Intense research into these NSCLCs has identified the secondary T790M mutation, which occurs in around 50% of patients with acquired resistance to EGFR-TKIs and is reported to negate the hypersensitivity of activating EGFR mutations [11–15]. However, there have been no effective treatment options for NSCLC patients with this secondary T790M resistance mutation.

In recent years, the field of cancer immunology has moved forward dramatically due to the identification of numerous tumor-associated antigens [16–18]. Notably, various approaches for therapeutic cancer immunotherapies have been developed and clinically examined, including cancer vaccines using

tumor-associated proteins or peptides. Although the early-phase clinical trials demonstrated the feasibility and good toxicity profile of immunotherapeutic approaches, most of the late-phase randomized trials, with a few exceptions, failed to show beneficial therapeutic effects in patients compared to existing treatments [19,20]. Such unexpected results might be attributed, at least in part, to the type of vaccine antigens employed for cancer immunotherapies. Currently, most vaccine antigens are derived from non-mutated self-antigens [21], which cannot be expected to show high immunogenicity due to the central and/or peripheral tolerance mechanism. In contrast, tumor-specific neo-antigens containing mutated amino acid sequences could be immunogenic, since they might be recognized as foreign by the host immune system [22,23]. In particular, vaccine antigens derived from "driver mutations" might be an ideal target for immunotherapy, since they would rarely be lost from tumor cells via escape from immunological pressure [24]. Although there have been some reports demonstrating the feasibility of immunotherapies targeting mutated antigens [25–27], only a limited number of mutated antigens have so far been identified as potential targets for immunotherapies [24]. In NSCLCs, several T cell epitopes derived from mutated antigens were reported [28–32], but there have been no reports on the tumor-specific neo-antigens derived from EGFR driver mutations.

In the current study, we identified HLA-A*0201 (A2)-restricted antigenic T cell epitopes containing the mutated methionine residue of the EGFR T790M resistance mutation. Given their strong immunogenicity for human T cells, the identified T cell epitopes could provide a novel and promising immunotherapeutic approach for prevention and/or treatment of the secondary EGFR T790M mutation in NSCLC patients treated with EGFR-TKIs.

Materials and Methods

Peptides and cell lines

The peptides containing the wild-type (threonine) or mutated (methionine) residue at the position 790 of EGFR and control HLA-A2-restricted peptides, influenza $M1_{58-66}$ (Flu-M1, GILGFVFTL) and HIV-derived epitope (SLYNTVATL), were provided by Thermo Fisher Scientific GmbH (Bremen, Germany) at purities of higher than 90%. T2 cells and NSCLC cell lines, NCI-H1975 (HLA-$A2^-$ $T790M^+$) and HCC827 (HLA-$A2^-$ $T790M^-$), were obtained from the American Type Culture Collection (ATCC; Manassas, VA). PC9 (HLA-$A2^+$ $T790M^-$), PC9/ZD (HLA-$A2^+$ $T790M^+$), 11–18 (HLA-$A2^+$ $T790M^-$), and YM-21 (HLA-$A2^+$ $T790M^-$) were obtained as described previously [33–36]. PC9/ZD cells were established as a gefitinib-resistant clone from PC9 cells [33], and were shown to harbor the T790M mutation of EGFR [34]. NCI-H1975-A2 cells were established by stably transfecting HLA-A2-negative NCI-H1975 cells with the plasmid carrying HLA-A*0201 cDNA (pCMV-HLA-A*0201) (data not shown). These cell lines were maintained in RPMI 1640 medium (Life Technologies, Rockville, MD) supplemented with 10% heat inactivated fetal calf serum (FCS) (MP Biologicals, Solon, OH), 100 μg/ml streptomycin (Life Technologies), and 100 IU/ml penicillin (Life Technologies). Expression of HLA-A2 on their cell surface was examined by flow cytometry with anti-HLA-A2 mAb (BB7.2; BD Biosciences, San Jose, CA).

Blood samples from NSCLC patients

Blood samples were obtained from 6 healthy donors and 22 NSCLC patients treated with EGFR-TKI, gefitinib or erlotinib, between March 2012 and February 2013, at Kurume University Hospital (Kurume, Fukuoka, Japan) or Sendai Kousei Hospital (Sendai, Miyagi, Japan). This study was approved by the Institutional Review Board of Kurume University and Sendai Kousei Hospital, and conforms to the provisions of the Declaration of Helsinki. All of the patients had the EGFR gene mutations in exon 19 (delE746-A750) or exon 21 (L858R), and had received or were receiving gefitinib or erlotinib for treatment against advanced diseases at the blood sampling. For analysis of EGFR gene mutations in exon 19 (delE746-A750) or exon 21 (L858R), the peptic nucleic acid-locked nucleic acid (PNA-LNA) polymerase chain reaction (PCR) clamp method was adopted, using protocols described previously [37].

The EGFR T790M mutation was examined in cell-free DNA obtained from plasma of the patients, since no biopsy specimens for DNA analysis could be obtained because of difficult accessibility of tumors during or after EGFR-TKI-treatment. Heparinized whole blood from patients was centrifuged for 10 min at 400 g. After centrifuging for 10 min at 2000 g again, plasma was transferred to a new tube and stored at −80°C until use. Cell-free DNA was isolated from 200 μl of thawed plasma and eluted in 60 μl extraction buffer by using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany). The EGFR T790M mutation was detected by the droplet digital PCR (ddPCR) system (QX100™ Droplet Digital™ PCR system; Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions [38]. Briefly, the PCR reaction mixture was assembled from a 2X ddPCR Supermix for Probes (Bio-Rad), primers and FAM-labeled probe, and cell-free DNA (8 μl) in a final volume of 20 μl. The primers and FAM-labeled probes for T790M mutation (EGFR mutant allele assay, Hs00000106_mu) and reference (EGFR reference assay, Hs00000173_rf) were purchased from Applied Biosystems (Taqman Mutation Detection Reference Assays, Life Technologies). The droplets were generated from the prepared PCR reaction mixture and droplet generator oil by using QX100 Droplet Generator (Bio-Rad). Thermal cycling conditions were 95°C X 10 min (1 cycle), 95°C X 30s and 65°C X 60s (40 cycles), 98°C X 10 min (1 cycle), and 4°C hold by C1000 Touch Thermal Cycler (Bio-Rad). Analysis of the ddPCR data was performed with QuantaSoft analysis software that accompanies QX100 Droplet Reader (Bio-Rad). A diluted DNA from NCI-H1975 ($T790M^+$) and T2 cells ($T790M^-$) was used as a positive and negative control for this assay, respectively. Samples were prepared in duplicate for each experiment, and repeated twice to confirm reproducibility.

Clinical responses to EGFR-TKIs were evaluated by computed tomography (CT) according to the Response Evaluation Criteria in Solid Tumors (RECIST). Responses were confirmed at least every 4 weeks (for partial response) or every 6 weeks (for stable disease). Among the 22 NSCLC patients, 8 patients were sensitive to EGFR-TKIs at the time of the blood sample collection. In contrast, 14 patients showed primary (n = 2) or acquired (n = 12) resistance to EGFR-TKIs at the blood sampling.

Prediction of HLA-A2-binding peptides containing the mutated methionine residue of the EGFR T790M mutation

Recent benchmarking has shown that NetMHC 3.2 (http://www.cbs.dtu.dk/services/NetMHC) and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind) are among the best preforming systems for predicting HLA-A2 binding peptides [39]. Thus NetMHC 3.2 and BIMAS were employed to predict 9-mer or 10-mer HLA-A2 binding peptides from the EGFR-T790M. For prediction of 11-mer HLA-A2 binding peptides, only NetMHC 3.2 was used because BIMAS does not perform predictions on

11-mer peptides. The top ranking peptides by either or both of these prediction servers were selected for further evaluation.

HLA class I stabilization assay

The actual binding of predicted peptides to HLA-A2 molecules was evaluated by an MHC class I stabilization assay with TAP-deficient RMA-S cells stably transfected with the HLA-A*0201 gene (RMA-S/A2), according to the previously reported method with some modifications [40]. Briefly, RMA-S/A2 cells were cultured for 18 hours at 26°C in 1 ml of RPMI 1640 medium in the presence of synthetic peptides (10 μg/ml) and β2-microglobulin (2 μg/ml; Cortex Biochem, San Leandro, CA). After being washed, the cells were cultured for 3 hours at 37°C, and then stained with anti-HLA-A2 mAb (BB7.2), followed by flow cytometry (FACSCanto II; BD Biosciences). Data were analyzed by using the Diva software package (BD Biosciences). The binding capability of each peptide to HLA-A2 molecules was evaluated by the increase in mean fluorescence intensity (MFI) of the HLA-A2 expression, as follows: MFI increase (%) = (MFI with a given peptide – MFI without peptides)/(MFI without peptides) X 100. The HLA-A2-restricted influenza $M1_{58-66}$ epitope (Flu-M1) was used as a positive control.

Generation of antigen-specific T cells

Peptide-specific T cell lines were generated according to the previously reported method with slight modifications [41]. In brief, peripheral blood was obtained from 6 HLA-$A2^+$ healthy donors and 22 HLA-$A2^+$ NSCLC patients after written informed consent. HLA-A2 positivity was confirmed by flow cytometry with anti-HLA-A2 mAb (BB7.2). Peripheral blood mononuclear cells (PBMCs) (1×10^5 cells per well) purified by Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density centrifugation were incubated with 10 μg/ml of each peptide in 96 round well plates (Nunc, Roskilde, Denmark) in 200 μl of the culture medium containing 45% RPMI 1640, 45% AIM-V medium (Life Technologies), 10% FCS, IL-2 (20 IU/mL; AbD Serotec, Kidlington, UK), and 0.1 mM MEM nonessential amino-acid solution (Life Technologies). At every 3 or 4 days, half of the culture medium was removed and replaced by new medium containing the corresponding peptide (10 μg/ml) and IL-2 (20 IU/mL). After 14 days, the cells were used for interferon (IFN)-γ ELSPOT or cytotoxicity assays.

Immune assays

For IFN-γ ELISPOT assay (MBL, Nagoya, Japan), the peptide-stimulated PBMCs (2×10^4 cells/well) were cultured in duplicate for 18 hours at 37°C with T2 cells (1×10^4 cells/well) loaded with or without the indicated doses of specific or control peptides in a 96-well ELISPOT plate (MultiScreen HTS; Millipore, Bedford, MA) coated with anti-human IFN-γ mAb. After the plates were washed, biotin-conjugated anti-human IFN-γ mAb, streptavidin-ALP, and BCIP/NBT substrate were added to develop the spots, in accordance with the manufacturer's instructions. The numbers of spots were then counted by an ELISPOT reader (CTL-ImmunoSpot S5 Series; Cellular Technology, Ltd., Shaker Heights, OH). The peptide-stimulated PBMCs were also tested for their reactivity against tumor cell lines by IFN-γ ELISPOT assay. In some cases, $CD8^+$ T cells were isolated using a CD8 Negative Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) from the peptide-stimulated PBMCs. To examine the HLA class I restriction, 10 μg/ml of anti-HLA class I mAb (W6/32: mouse IgG2a) was added into wells at the initiation of the culture. As an isotype control, anti-HLA-DR mAb (L243: mouse IgG2a) was used.

Peptide-stimulated PBMCs were also tested for cytotoxicity against tumor cell lines by a standard 6-hour ^{51}Cr-release assay (PerkinElmer, Waltham, MA) using a MicroBeta2 LumiJET microplate counter (PerkinElmer). Effector cells and ^{51}Cr-labelled cells (2000 cells/well) were cultured in duplicate in 96 round well plates at the indicated effector/target ratios. The spontaneous release and maximal release were determined by the target cells cultured in medium without or with 1% Triton X-100 (Wako Pure Chemical Industries, Osaka, Japan), respectively. The specific lysis was calculated as follows: specific lysis (%) = [(test release – spontaneous release)/(maximal release - spontaneous release)] X 100. The means of duplicate samples were used for calculation.

Statistical Analysis

The difference in proportion of the presence of EGFR-T790M mutation between NSCLC patients with and without EGFR-T790M-specific immune responses was statistically evaluated by the chi-squared test (SAS 9.3; SAS Institute Inc., Cary, NC).

Results

Prediction of HLA-A2-binding peptides containing the mutated methionine residue of the EGFR T790M mutation

The amino acid sequences (9 to 11-mer) were predicted to bind HLA-A2 molecule by NetMHC 3.2 and/or BIMAS servers. Eight peptides (10- or 11-mer) containing the mutated methionine residue at the position 790 of EGFR (T790M), which showed better scores by either or both of these prediction servers, were selected for further evaluation (Table 1). But 9-mer peptides did not show good scores by the prediction servers.

HLA-A2-binding capability of the EGFR-T790M-derived peptides

The HLA-A2-binding capability of the selected peptides was confirmed by cell surface HLA class I stabilization assay with the TAP-deficient cell line RMA-S, which stably expressed the HLA-A*0201 gene (RMA-S/A2). As illustrated in Table 1, three of the 8 selected peptides showed substantial binding to HLA-A2. Notably, the binding affinity of T790M-5 (MQLMPFGCLL) to HLA-A2 was much stronger than that of T790M-7 (LIMQLMPFGCL) or T790M-8 (IMQLMPFGCLL). The WT-5 peptide containing the wild-type threonine residue at position 790 of EGFR, which corresponded to T790M-5, showed high binding capability to HLA-A2, whereas the WT-7 peptide corresponding to T790M-7 did not bind to HLA-A2.

Immunogenicity of the EGFR-T790M-derived HLA-A2-binding peptides in T cells from HLA-$A2^+$ normal donors

To examine the immunogenicity of the EGFR-T790M-derived HLA-A2-binding peptides, PBMCs from 6 different HLA-$A2^+$ healthy donors were repeatedly stimulated with the synthetic peptides, T790M-5, T790M-7, or T790M-8. As shown in Figure 1, after repeated stimulation, T cell lines secreting IFN-γ in response to T790M-5 and T790M-7 could be established in 5 of 6 (83%) and 3 of 6 (50%) healthy donors, respectively. However, none of the 6 healthy donors showed antigen-specific T cell responses to T790M-8.

The T cell lines established after repeated stimulation of PBMCs with T790M-5 or T790M-7 were then examined for their reactivity against NSCLC cell lines, including NCI-H1975 (HLA-$A2^-$ $T790M^+$), NCI-H1975-A2 (HLA-$A2^+$ $T790M^+$), and HCC827 (HLA-$A2^-$ $T790M^-$), by IFN-γ ELISPOT assay. As

Table 1. HLA-A2-binding capability of the predicted EGFR T790M-derived peptides.

Peptide name	Amino acid sequence	NetMHC 3.2 ANN IC50 (nM)	BIMAS score	HLA-binding capability (%)[a]
T790M-1	VQLIMQLMPF	3705	0.109	0
T790M-2	QLIMQLMPFG	4054	0.943	0
T790M-3	LIMQLMPFGC	837	24.921	0
T790M-4	IMQLMPFGCL	925	6.478	0
T790M-5	MQLMPFGCLL	977	51.770	207.5
T790M-6	LTSTVQLIMQL	4187	NA[c]	0
T790M-7	LIMQLMPFGCL	530	NA	19.1
T790M-8	IMQLMPFGCLL	118	NA	57.0
WT-5[b]	TQLMPFGCLL	2578	30.453	158.2
WT-7[b]	LITQLMPFGCL	5063	NA	0
Flu-M1	GILGFVFTL	18	550.927	234.4

[a]HLA-A2-binding capability was estimated by the increase in mean fluorescence intensity (MFI) as determined by flow cytometry after staining of RMA-S/A2 cells with anti-HLA-A2 mAb, as follows: MFI increase (%) = (MFI with a given peptide – MFI without peptides)/(MFI without peptides) X 100. The experiments were repeated three times and a representative result is shown.
[b]WT-5 and WT-7 peptides have wild-type sequences, corresponding to the T790M-5 and T790M-7 peptides, respectively.
[c]NA, not available.
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shown in Figure 2A, the T cells lines established by T790M-5 or T790M-7 stimulation showed apparent IFN-γ production in response to NCI-H1975-A2, but not to HLA-A2-negative parental NCI-H1975. In addition, they showed no responses against an HLA-A2-negative, EGFR-T790M-negative cell line, HCC827. The IFN-γ secretion from T790M-5- and T790M-7-stimulated T cells in response to NCI-H1975-A2 was significantly inhibited by incubation with anti-HLA class I mAb (clone W6/32), but not by

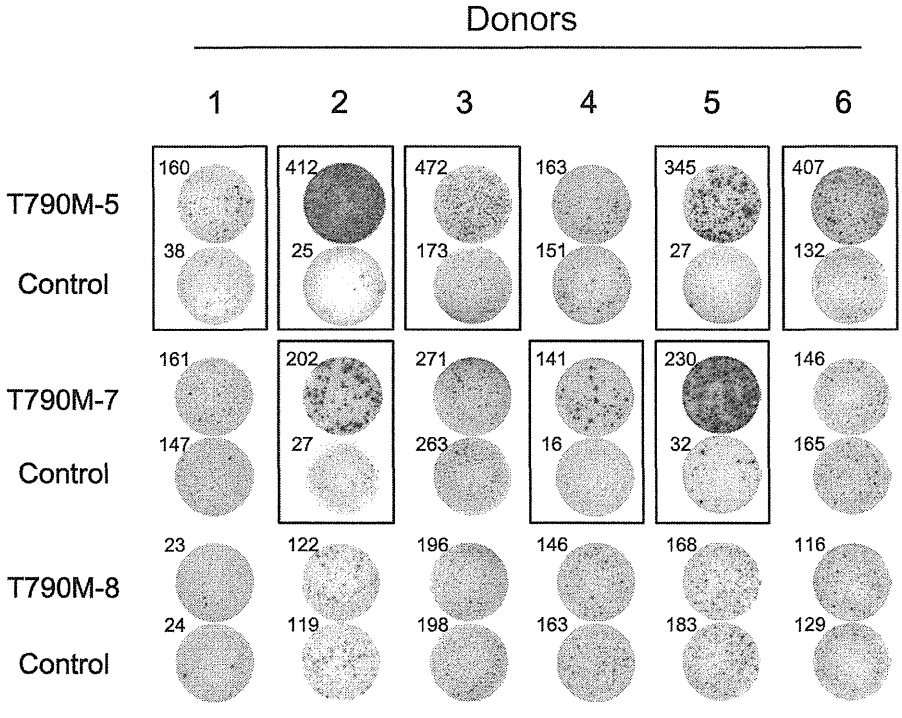

Figure 1. Immunogenicity of T790M-derived peptides in PBMCs from HLA-A2$^+$ healthy donors. The immunogenicity of T790M-deived peptides, T790M-5, T790M-7, and T790M-8, was examined with PBMCs from 6 different HLA-A2$^+$ healthy donors. PBMCs were stimulated 5 times with T790M-5, T790M-7, or T790M-8 peptides (10 μg/ml) every 3 or 4 days. The stimulated PBMCs (2×10^4 cells/well) were examined for reactivity against T2 cells (1×10^4 cells/well) pulsed with the corresponding peptides or control HIV peptide (10 μg/ml) by IFN-γ ELISPOT assay. The assays were carried out in duplicate wells, and representative wells in each donor are shown. The numbers of spots are shown for each well. Positive antigen-specific T cell responses are marked by closed boxes.
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