radiofrequency ablation (RFA). The efficacy of RFA treatment was evaluated using dynamic CT or dynamic MRI within a few days after treatment. Complete ablation of HCC was defined as non-enhancement of the lesion relative to the surrounding liver parenchyma. Patients received additional sessions of an ablative therapy until the treatment was judged as complete.

Statistical Analysis

Differences in the proportions of patients with elevated levels of AFP, AFP-L3, and DCP before and after treatment were determined by Fisher's exact test. Recurrence-free survival of patients with HCC was determined by the Kaplan–Meier method. Logrank test was used to test for equality of recurrence-free survival between the groups. Multivariate analysis of prognostic factors among the clinical features was performed using the Cox stepwise proportional hazards model. The factors included in multivariate analysis were patient age (years), gender (female/male), HBsAg (negative/positive), anti-HCV (negative/positive), alcohol abuse (negative/positive), Child-Pugh class (A/B,C), platelet count ($\times 10^4/\mu l$), tumor size (cm), number of tumors (single/multiple), treatment method (hepatic resection/RFA), pretreatment AFP (ng/ml) (<20/≥20), pretreatment AFP-L3 (%) (<10/≥10), pretreatment DCP (mAU/ml) (<40/≥40), post-treatment AFP (ng/ml) (<20/≥20), post-treatment AFP-L3 (%) (<10/≥10), and post-treatment DCP (mAU/ml) (<40/≥40). Statistical analyses were performed with the SPSS version 17.0 software package (SPSS Japan Inc., Tokyo, Japan). Differences at $p < 0.05$ were considered to be statistically significant.

Results

Clinical Features of Patients

Table 1 summarizes the demographics, etiology of liver disease, hepatic functional reserve ranked by the Child-Pugh classification, platelet count, serum concentration of AFP (ng/ml), AFP-L3 (%), DCP (mAU/ml) tumor size, number of tumors, and treatment modality for the study patients.

This population comprised 136 females and 278 males with a median age of 71 (range 33–89) years. The majority of patients had a viral etiology for their liver disease, anti-HCV being positive in 287 (69.3 %) and HBsAg being positive in 53 (12.8 %). Most patients (86.2 %) were Child-Pugh class A. The median tumor size was 1.9 (range, 0.6–5.0) cm and multiple tumors were present in 17.4 % of the patients. When the cut-off values were set at 20 ng/ml for AFP, 10 % for AFP-L3 and 40 mAU/ml for DCP, the positivity rates for AFP, AFP-L3 and DCP were 40.1, 25.4, and 33.3 %, respectively.

Table 1 Clinical features of patients ($n = 414$)

Age (years) [median (range)]	71 (33–89)
Gender (female/male)	136/278
Etiology	
HBsAg negative/positive	361/53
Anti-HCV negative/positive	127/287
Alcohol abuse negative/positive	396/18
Child-Pugh classification	
A/B/C	357/37/4
Platelet ($10^4/\mu l$) [median (range)]	12.2 (3.6–59.2)
AFP (ng/ml) [median (range)]	11.1 (0.3–14,597)
<20 ng/ml/≥20 ng/ml	248/166
AFP-L3 (%) <10 %/≥10 %	309/105
DCP (mAU/ml) [median (range)]	23 (5–33,283)
<40 mAU/ml/≥40 mAU/ml	273/136
Tumor size (cm) [median (range)]	1.9 (0.6–5.0)
Tumor number (single/multiple)	342/72
Therapeutic modalities	
Hepatic resection/RFA	228/186

Range or percent are shown in *parentheses*

HCC hepatocellular carcinoma, *HBsAg* hepatitis B surface antigen, *HCV* hepatitis C virus, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin, *RFA* Radiofrequency ablation

Impact of AFP, AFP-L3, and DCP on Recurrence-Free Survival of Patients with HCC

Among the 414 patients enrolled in this study, HCC recurrence was observed in 236, and the remaining 178 showed no HCC recurrence within the study period. Recurrence-free survival rates of patients with HCC were evaluated in terms of serum AFP, AFP-L3, and DCP levels before and after treatment. Comparison of the three tumor markers measured before treatment showed that patients with an elevated AFP-L3 level had a significantly lower recurrence-free survival rate than patients without such an elevation of the AFP-L3 level ($p = 0.024$) (Fig. 1). In contrast, there was no significant difference in recurrence-free survival between the groups with and without elevation of the AFP and DCP levels before treatment (AFP, $p = 0.171$; DCP, $p = 0.208$) (Fig. 1). The recurrence-free survival rate of patients with an elevation of AFP, AFP-L3, and DCP measured after treatment were significantly lower than that of patients without such elevation (AFP, $p = 0.009$; AFP-L3 and DCP, $p = 0.001$) (Fig. 2).

In addition, we evaluated the recurrence-free survival rates of patients according to AFP-L3 status measured 9 months after treatment. In patients who had no

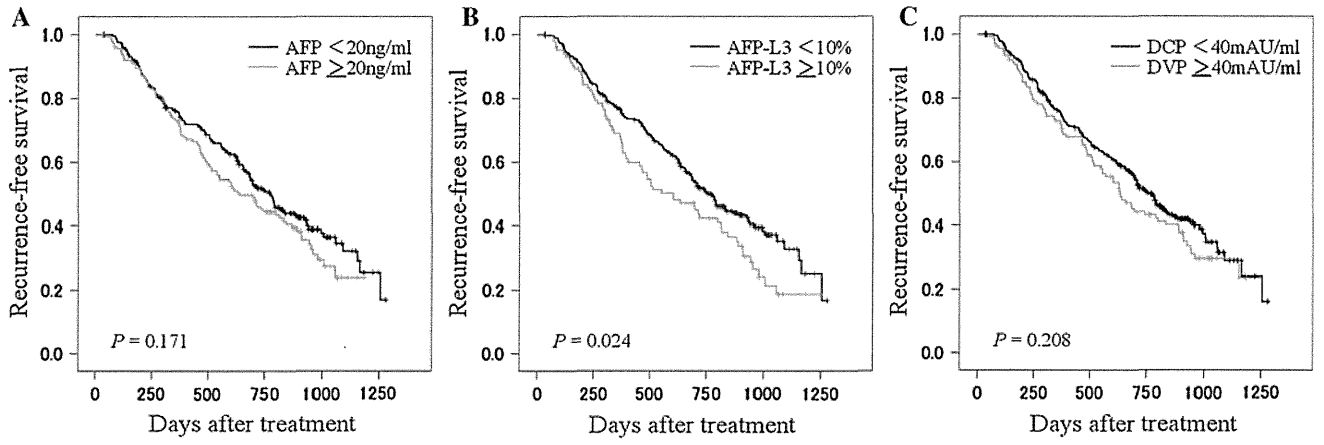

Fig. 1 Comparison of recurrence-free survival rates between patients with and without elevation of the AFP, AFP-L3, and DCP levels before treatment. Recurrence-free survival rates according to AFP (**a**), AFP-L3 (**b**), and DCP (**c**)

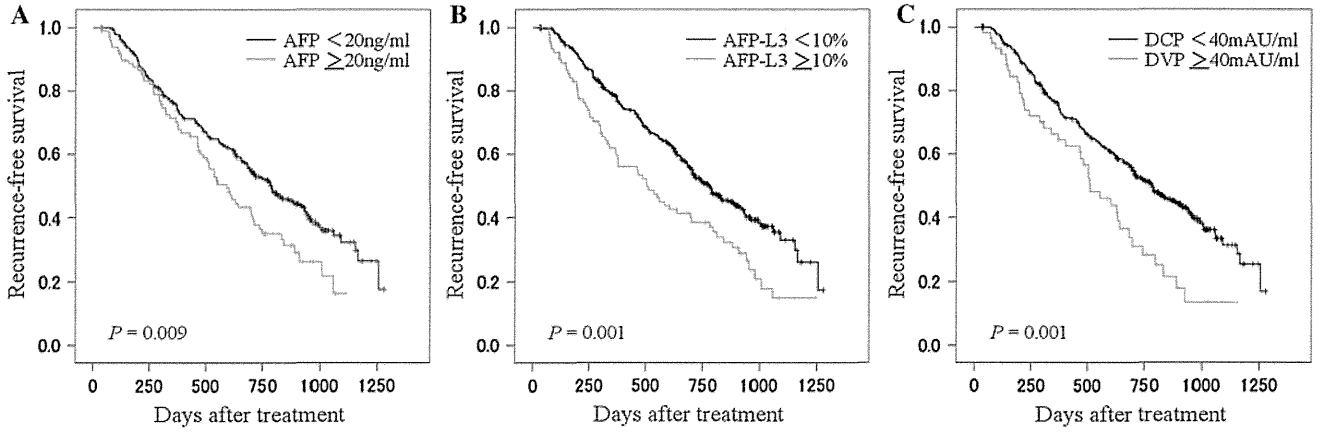

Fig. 2 Comparison of recurrence-free survival rates between patients with and without elevation of the AFP, AFP-L3, and DCP levels at one month after treatment. Recurrence-free survival rates according to AFP (**a**), AFP-L3 (**b**), and DCP (**c**)

recurrence for at least 9 months, a significant difference was also observed between those with and without elevation of the AFP-L3 level ($p = 0.003$) (Fig. 3).

Multivariate analysis using the Cox stepwise proportional hazard model revealed that post-treatment AFP-L3 status ($p = 0.002$) and post-treatment DCP status ($p = 0.004$) were significant independent factors predictive of recurrence-free survival in patients with HCC (Table 2).

Relationship Between HCC Recurrence and Change in Positivity Rates for Serum AFP, AFP-L3, and DCP Before and After Treatment

We evaluated the changes in positivity rates for serum tumor markers before and after treatment in 308 of the 414 patients who were enrolled in this study. These 308 patients comprised 193 who suffered recurrence within 2 years and 115 in whom no recurrence was observed for more than 2 years. Positivity rates for serum AFP, AFP-L3, and DCP were investigated in relation to HCC recurrence, and the results are shown in Table 3. Regardless of HCC recurrence, the proportions of patients showing elevation of the AFP and DCP levels (AFP, ≥20 ng/ml; DCP, ≥40 mAU/ml) for 1 month after curative treatment were significantly lower in comparison to the situation before treatment (AFP, $p = 0.004$ for patients with HCC recurrence and $p = 0.001$ for patients without HCC recurrence; DCP, $p < 0.001$ for both patients with and without HCC recurrence). On the other hand, there was no significant decrease in the proportion of patients showing an elevated AFP-L3 level 1 month after treatment in comparison to the situation before treatment in both of the groups with and without HCC recurrence. Among the 193 patients in the HCC recurrence group, 15 (26.8 %) of 56 patients with an elevated AFP-L3 level (≥10 %) before treatment became negative for AFP-L3 (<10 %) after treatment. In the recurrence-free group, eight (38.1 %) of 21 patients with an elevated AFP-L3 level before treatment became negative for AFP-L3 after treatment. In addition, we investigated the long-term changes

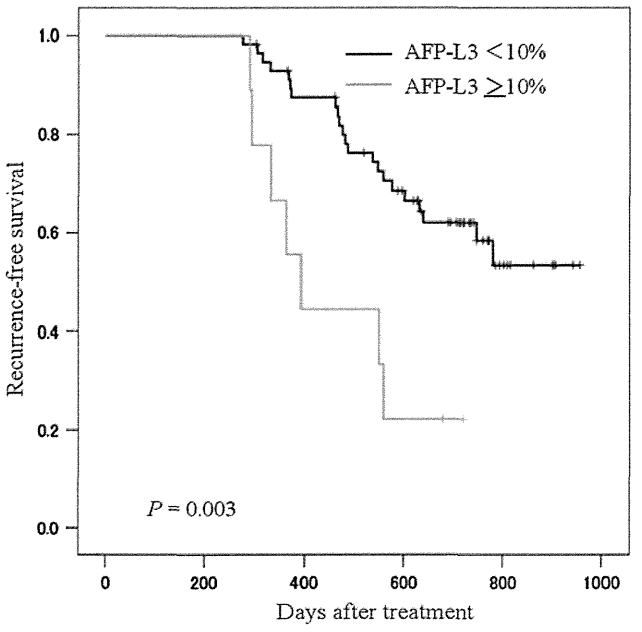

Fig. 3 Comparison of recurrence-free survival rates between patients with and without elevation of the AFP-L3 level at 9 months after treatment

Table 2 Multivariate analysis of factors associated with HCC recurrence after curative treatment

Variables	Hazard ratio (95 % CI)	*p* value
Post-treatment AFP-L3		
<10 %	1	0.002
≥10 %	1.592 (1.183–2.144)	
Post-treatment DCP		
<40 mAU/ml	1	0.004
≥40 mAU/ml	1.687 (1.187–2.398)	

Hazard ratio and *p* value were calculated using Cox's stepwise proportional hazard model

HCC hepatocellular carcinoma, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin, *CI* confidence interval

in the level of AFP-L3 after treatment. Among patients who suffered recurrence of HCC after 9 months or later of treatments, the proportion of those who were still AFP-L3-positive 6 and 9 months after treatment was 28.6 % (4/14) and 24.1 % (7/29), respectively, being similar to the proportion of such patients before treatment (29.2 %; 69/236). On the other hand, in the group who remained recurrence-free for more than 2 years, no patient was AFP-L3-positive at 6 and 9 months after treatment (6 months; 0 %, 0/5: 9 months; 0 %, 0/18).

Discussion

Many studies have shown that AFP-L3 status is a specific marker for HCC, and that the level of AFP-L3 predicts the malignant potential of HCC, and thus the expected outcome after treatment [10–15]. Recently, μTAS AFP-L3 has been newly developed as an automated immunoassay for AFP-L3. A number of investigators, including our group, have shown that the μTAS AFP-L3 is more sensitive for discriminating HCC from benign liver diseases than the conventional LiBASys AFP-L3, particularly in subgroups with lower AFP concentrations and early stage HCC [19–21]. In addition, we have previously reported that μTAS AFP-L3 status is a statistically significant independent prognostic indicator of long-term survival in patients with HCC [19]. On the other hand, there has been little information about the levels of μTAS AFP, AFP-L3, and DCP measured after curative treatment of HCC.

In the present study, we demonstrated that there was a significant difference in recurrence-free survival between groups with and without elevation of the AFP-L3 level before and after treatment. Multivariate analysis revealed that AFP-L3 status measured 1 month after treatment was a significant independent predictor of HCC recurrence after curative treatment. On the other hand, we also found that, irrespective of HCC recurrence, there was no significant decrease in the proportion of patients showing an elevated AFP-L3 level 1 month after treatment in comparison to the situation before treatment. In our present examination of long-term changes in the level of AFP-L3 after treatment, the proportion of patients with an elevated level of AFP-L3 at 6 and 9 months after treatment was decreased relative to that before treatment in the recurrence-free group. By contrast, the proportion of patients who were still AFP-L3-positive after treatment was similar to that before treatment in the HCC recurrence group. Taken together, these results suggest that an elevated AFP-L3 level before treatment is a predictor of HCC recurrence, and that sustained elevation of AFP-L3 after treatment should be considered to indicate HCC recurrence.

The decrease in the total concentration of AFP within 1 month after treatment, in terms of half-life, is reported to be 4–6 days [22, 23]. However, the percentage decrease in the level of AFP-L3 basically does not change, and the lower limit for quantitation of μTAS AFP-L3 is quite low (0.3 ng/ml). We suggest that the minimal change in AFP-L3 positivity in some patients without HCC recurrences within the first month after treatment was attributable to serum AFP that had been produced by HCC just before treatment. Therefore, repeated combination measurement of μTAS AFP, AFP-L3, and DCP should be performed for surveillance of HCC recurrence after curative treatment.

Recently, Yamamoto et al. [24] have investigated the relationship between HCC recurrence and changes in the levels of three tumor markers—AFP, AFP-L3, and DCP—measured using the LiBASys assay. They reported that, among these markers, AFP-L3 positivity after treatment

Table 3 Serum AFP, AFP-L3, and DCP before and 1 month after treatment

Patient group and tumor markers	Before treatment	1 month after treatment	*p* value
Patients with HCC recurrence within 2 years ($n = 193$)			
AFP			
Patient number (≥20 ng/ml/<20 ng/ml)	83/110	55/138	0.004
Percent of patients with AFP ≥20 ng/ml	43.0 %	28.5 %	
AFP-L3			
Patient number (≥10 %/<10 %)	56/137	52/141	0.734
Percent of patients with AFP-L3 ≥ 10 %	29.0 %	26.9 %	
DCP			
Patient number (≥40 mAU/ml/<40 mAU/ml)	69/123	35/155	<0.001
Percent of patients with DCP ≥ 40mAU/ml	34.3 %	18.4 %	
Patients without HCC recurrence for more than 2 years ($n = 115$)			
AFP			
Patient number (≥20 ng/ml/<20 ng/ml)	43/72	20/95	0.001
Percent of patients with AFP ≥ 20 ng/ml	37.4 %	17.4 %	
AFP-L3			
Patient number (≥10 %/<10 %)	21/94	17/98	0.595
Percent of patients with AFP-L3 ≥10 %	18.3 %	14.8 %	
DCP			
Patient number (≥40 mAU/ml/<40 mAU/ml)	31/83	6/109	<0.001
Percent of patients with DCP ≥40 mAU/ml	27.0 %	5.2 %	

p values were calculated by Fisher's exact test

HCC hepatocellular carcinoma, *AFP* alpha-fetoprotein, *DCP* des-gamma-carboxy prothrombin

had the highest risk ratio of 5.0 for HCC recurrence after curative treatment. Kobayashi et al. [25] have investigated the relationship between changes in the serum AFP-L3 level measured by μTAS assay 30–120 days after curative treatment and HCC recurrence. They reported that 29 of 37 patients (78.4 %) with preoperative AFP elevation (>20 ng/ml) showed a decrease in the AFP level to <20 ng/ml, although 16 of 42 patients (38.1 %) with preoperative AFP-L3 elevation (>5 %) showed a decrease in the level to <5 %. On this basis, they concluded that it was rare for AFP-L3 to become negative after treatment. Toyoda et al. [26] also investigated the value of AFP, AFP-L3, and DCP, measured before and 1–2 months after treatment, for prediction of survival and recurrence in patients who had undergone hepatectomy for HCC. They concluded that the combination of tumor markers measured by μTAS assay after hepatectomy had excellent ability to predict postoperative survival and recurrence. Our present results support their findings, although the AFP-L3 cut-off value used in our study was 10 %, unlike their value of 5 %. In our previous study, we suggested that a cut-off value of 7 % was most appropriate for discriminating HCC from benign liver disease using μTAS AFP-L3 [19]. In our present study, however, we chose a cut-off value of 10 % for μTAS AFP-L3 in view of a recently published report by Kanke et al. [27] in which intra-individual biological variation of the μTAS AFP-L3 level was relatively high, at 29.0 %.

Some factors other than AFP-L3 and DCP could potentially have been associated with HCC recurrence. Several studies have implicated obesity and diabetes as risk factors for HCC [28–30]. However, we did not investigate these factors in the present study.

The difficulty in the treatment of HCC is related to the underlying impairment of hepatic functional reserve and high rate of recurrence, even after curative treatment. Therefore, early detection of HCC recurrence after treatment is an important issue for improving the survival of patients with HCC. From this viewpoint, the μTAS assay is an extremely powerful tool for detection of HCC at an early stage. Additionally, clinicians should interpret the values of tumor markers measured by the μTAS assay giving due consideration to their property. In conclusion, the present study has demonstrated that patients with an elevated AFP-L3 level determined before treatment have a high risk of HCC recurrence, and that sustained elevation of AFP-L3 after treatment is a strong predictor of HCC recurrence. In addition, short-term AFP-L3 status is not a reliable indicator of incomplete treatment for HCC. Accordingly, repeated and combined measurement of μTAS AFP, AFP-L3, and DCP should be performed for surveillance of HCC recurrence after curative treatment.

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Conflict of interest None.

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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

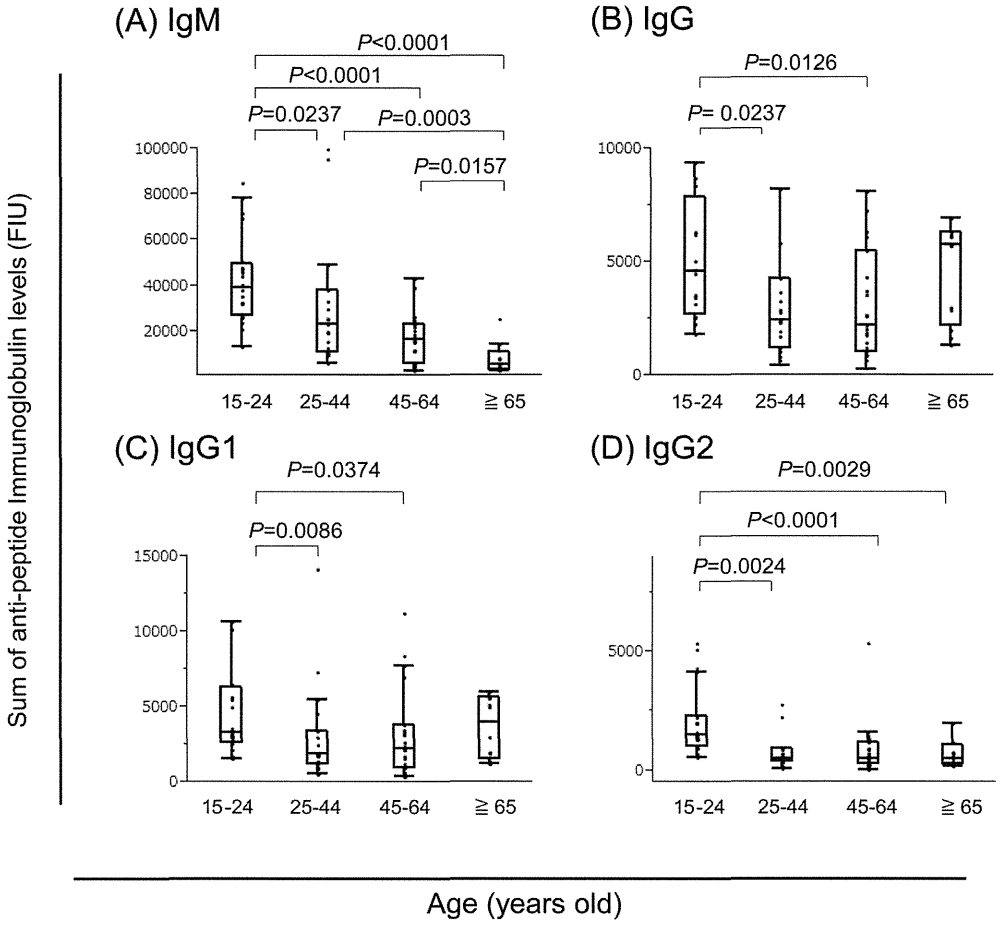

Fig. 1. Detection of immunoglobulins specific to CTL epitope peptides in plasma or sera from healthy donors. Immunoglobulins (IgM, IgG, IgG1, IgG2) specific to each of the 31 CTL epitope peptides were measured by multiplex bead suspension array in plasma or sera from healthy donors. The total sums of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

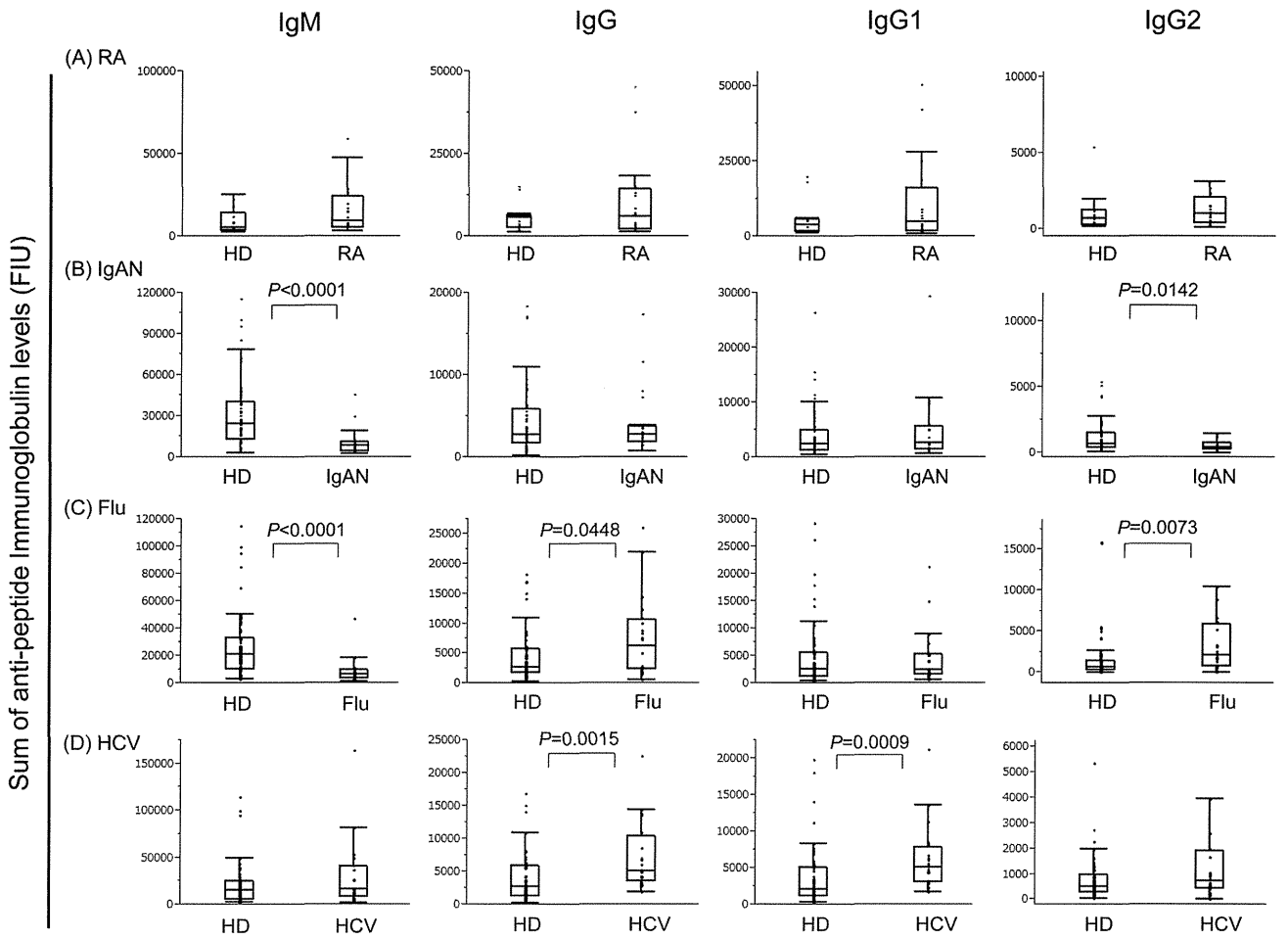

Fig. 2. Comparison of anti-peptide immunoglobulins in plasma or sera between healthy donors and patients with various types of immune-related diseases. Immunoglobulins (IgM, IgG, IgG1, IgG2) specific to each of the 31 CTL epitope peptides were measured by multiplex bead suspension array in plasma or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

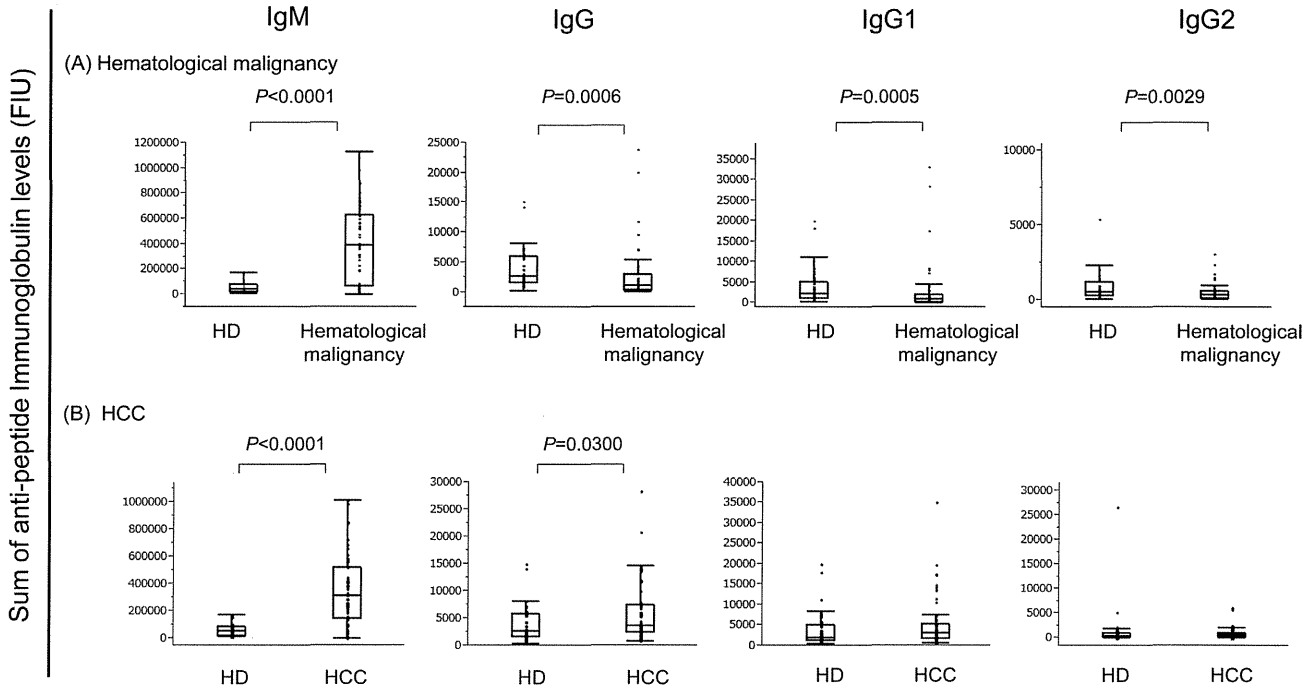

Fig. 3. Comparison of anti-peptide immunoglobulins in plasma or sera between healthy donors and patients with malignant diseases. Immunoglobulins (IgM, IgG, IgG1, IgG2) specific to each of the 31 CTL epitope peptides were measured by multiplex bead suspension array in plasma or sera from patients with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

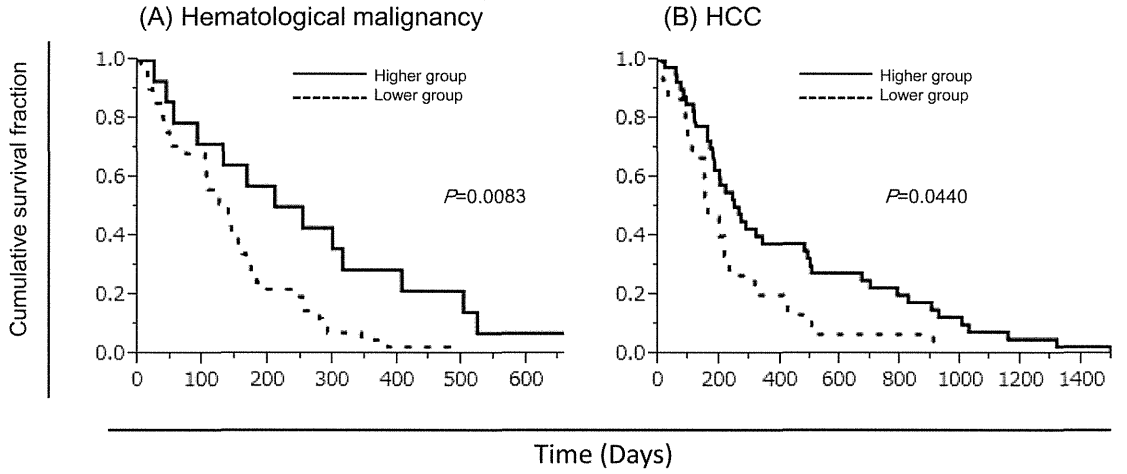

Fig. 4. Correlation between the sums of anti-peptide IgG levels and overall survival in patients with malignant diseases. Patients with hematological malignancies (A) or hepatocellular carcinoma (HCC) (B) were divided into two subgroups by the sums of IgGs specific to each of the 31 CTL epitope peptides.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

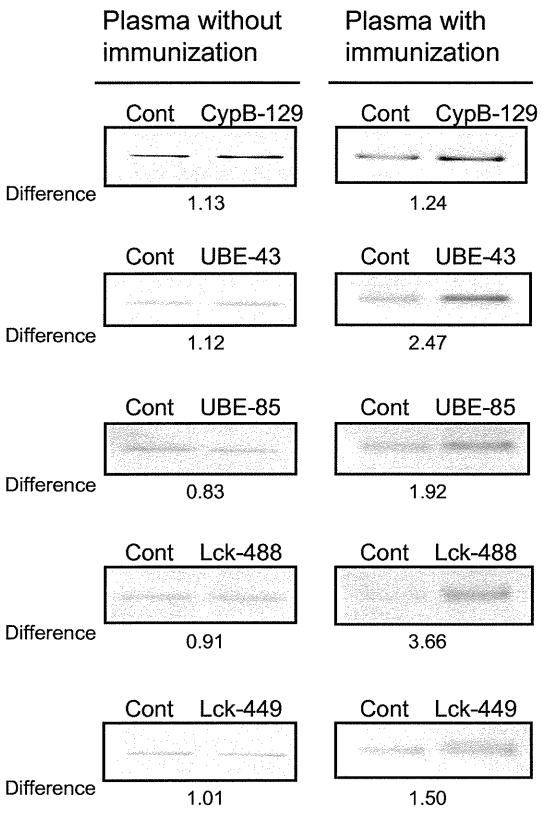

Fig. 5. Detection of IgGs specific to CTL epitope peptides in plasma from cancer patients with or without immunization. IgGs specific to CypB-129, UBE2V-43, UBE2V-85, Lck-488, and Lck-449 peptides were isolated by the peptide-coupled beads from plasma in cancer patients with and without immunization 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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Green tea with high-density catechins improves liver function and fat infiltration in non-alcoholic fatty liver disease (NAFLD) patients: A double-blind placebo-controlled study

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Abstract. Catechins, a major component of green tea extract, have anti-hyperlipidemic effects. The present study investigated the effects of consumption of green tea with high-density catechins in non-alcoholic fatty liver disease (NAFLD) patients. Seventeen patients with NAFLD consumed green tea with high-density catechins, low-density catechins or a placebo for 12 weeks in a randomized double-blind study. Ultrasonography and computed tomography (CT) were performed at baseline and after 12 weeks. Serum alanine aminotransferase (ALT) levels and urine 8-isoprostane were monitored and compared to baseline at 4, 8 and 12 weeks. Body fat was significantly decreased in the high-density catechin group compared with the placebo and low-density catechin groups after 12 weeks of consumption. All the patients in the high-density catechin group showed a significantly improved liver-to-spleen CT attenuation ratio compared with the placebo and low-density catechin groups after 12 weeks of consumption. The high-density catechin group significantly decreased serum ALT levels and reduced urinary 8-isoprostane excretion compared with the placebo and low-density catechin group after 12 weeks of consumption. Based on a reduced proportion of body fat as estimated by bioimpedance measurement, increased liver-to-spleen CT attenuation ratio, decreased serum ALT levels and reduced urinary 8-isoprostane excretion, we concluded that 12 weeks of 700 ml per day of green tea containing >1 g catechin improved liver fat content and inflammation by reducing oxidative stress in patients with NAFLD.

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Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CT, computed tomography; EGCG, epigallocatechin gallate; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis

Key words: fatty liver, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, metabolic syndrome, flavonoid, oxidative stress

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent types of liver diseases. NAFLD prevalence has increased with the change in eating habits, thus identifying effective treatment for NAFLD is a significant public health objective. Lifestyle-related factors such as poor diet, obesity, excessive alcohol intake, diabetes and hyperlipidemia have all been proposed to contribute to NAFLD. In addition to the development of a fatty liver, NAFLD patients may also exhibit inflammation, necrosis and fibrosis of the liver, which are known as non-alcoholic steatohepatitis (NASH) (1). This disease may progress to cirrhosis of the liver and hepatocellular carcinoma (HCC). Lifestyle interventions such as improvement of eating habits or physical activity are commonly recommended for NAFLD and NASH, but no effective medical therapy for these diseases has been established although many medications for the treatment of NAFLD are undergoing clinical trials in the Western countries.

Green tea contains high levels of flavonoids, which have antioxidant properties. Catechin, one of the main flavonoids in green tea, has recently attracted attention for its antitumor and anti-arteriosclerotic effects. Catechins account for ~20% of the flavonoids in green tea leaves. They have been found to decrease oxidative stress (2) and to exert anti-virus (3,4), anti-thrombotic (5), anti-allergenic (6), anticancer (7), anti-hypertensive (8) and anti-hyperglycemic effects (9,10). In addition, results of animal experiments have indicated that catechins affect the lipid metabolism by decreasing triglyceride and total cholesterol levels (11) and enhancing energy utilization (12). However, the effect of the green tea with high-density catechins on humans and its detailed mechanism have yet to be clarified. To the best of our knowledge, this is the first study to examine the effects of green tea containing high-density catechins on NAFLD in humans. We report herein the results of a double-blind, controlled study examining the effects of green tea containing

high-density catechins and a catechin-free green tea-flavored control beverage on liver function and fatty liver status in NAFLD patients. We found that NAFLD patients consuming green tea with high-density catechins for 12 weeks showed improved liver function and reduced liver fat deposition. In addition, we examined the safety of consuming green tea with high-density catechins for NAFLD patients.

Materials and methods

Subject selection. In total, 17 NAFLD patients (7 men and 10 women), aged 20-70 years, were included in this randomized, double-blind, controlled, investigator-initiated trial. The exclusion criteria were the presence of severe acute or chronic diseases (liver, heart or renal failure), infectious or autoimmune liver diseases (positive for hepatitis B surface antigens, anti-hepatitis C virus, anti-nuclear or anti-mitochondrial antibodies), known allergies to compounds of tea or polyphenol-rich food, acute infectious diseases, diseases involving systemic inflammation, participation in another study within the last month, alcohol abuse and the use of concomitant supplements. Green tea adjusted to 1,080 mg/700 ml or 200 mg/700 ml catechin content and green tea-flavored beverage (0 mg/700 ml catechin content) were prepared by Kao Corporation (Tokyo, Japan). The beverages were packaged in 350 ml steel cans with an identical appearance and distributed to patients by courier. An independent investigator performed subject randomization. All the patients provided informed written consent to participate in the study. The study was performed at the Kurume University Hospital and the study protocol conformed to the ethics guidelines of the 1975 Helsinki Declaration, as reflected in prior approval by the institutional Ethics Committee of the Kurume University School of Medicine.

Experimental protocol. Each patient was instructed to refrain from eating flavonoid-rich foods and supplements during the trial and was then instructed to fast for 8 h before undergoing the examinations, which were always performed in the morning. These included clinical examination (height, weight and body fat percentage), blood and urine sampling and routine abdominal ultrasonography. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. The rate of body fat was estimated by bioelectrical impedance analysis performed using InnerScan® (Tanita BC-511, Tanita Corporation, Tokyo, Japan) (13,14). The green tea beverages were consumed for 12 weeks. During this period, patients underwent follow-up examinations in the fourth, eighth and twelfth week. Additional follow-up data were collected four weeks after the end of the green tea consumption. Quantitative abdominal computerized tomography (CT) was performed at the beginning and the end of the study period.

Green teas and placebo (control). Patients were randomized to consume one of the three types of green tea for 12 weeks. Green tea containing 1,080 mg/700 ml or 200 mg/700 ml catechins and green tea-flavored beverage (0 mg/700 ml catechins) were prepared by Kao Corporation (Tokyo, Japan). Patients consumed 700 ml of green tea every day with meals. The tea containing 200 mg catechins per 700 ml was similar in catechin contents to most commercially available green teas. The intake quantity of 700 ml per day is typical of Japanese tea intake. Caffeine content, another component of green tea, was normalized in all three teas to 120 mg per 700 ml.

Quantitative abdominal CT. Abdominal CT was performed to determine the size of each patient's liver and spleen. Findings of many reports have shown that CT liver attenuation corrected for spleen attenuation allows more accurate evaluations of the pathological hepatosteatosis (15-17). Therefore we measured liver attenuation at five sites by CT, one in each hepatic segment from segment II to segment VIII (Couinaud classification), in order to calculate the average liver attenuation. Similarly we also measured spleen attenuation at five sites and calculated the average spleen attenuation. The ratio of liver to spleen attenuation was then calculated and values were compared before and after green tea consumption.

Biomarkers of oxidative stress. Recently, 8-isoprostane (prostaglandin F2α) has attracted attention as an *in vivo* indicator of oxidative stress due to its relative stability among prostaglandin isomers (18,19). Urine 8-isoprostane was measured using an EIA kit (Cayman Chemical Company, MI, United States). This assay was based on competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane-specific rabbit anti-serum binding sites. As the concentration of the 8-isoprostane tracer remained constant while the concentration of 8-isoprostane varied, the amount of 8-isoprostane tracer that was able to bind to the rabbit anti-serum was inversely proportional to the concentration of 8-isoprostane in the well. This rabbit anti-serum-8-isoprostane complex bound to a mouse monoclonal anti-rabbit IgG antibody that was also attached to the well. The plate was washed to remove any unbound reagents and then acetylcholinesterase substrate was added to the well. The product of this enzymatic reaction had a distinct yellow color and was absorbed strongly at 412 nm. The intensity of this color, determined spectrophotometrically, was proportional to the amount of 8-isoprostane tracer bound to the well. Data were corrected for urine creatinine and the urine 8-isoprostane (pg/ml)/creatinine (mg/ml) ratio was expressed as urine 8-isoprostane (pg/mg creatinine).

Statistical analysis. Data are expressed as means ± SD. Associations among the three patient groups for baseline characteristics, liver CT attenuation and urine 8-isoprostane were compared using analysis of variance (ANOVA). Within each group, comparisons were made using the Student's paired t-test. $P<0.05$ was considered statistically significant. Statistical analyses were performed using AIST-ANOVA developed by the National Metrology Institute of Japan (NMIJ) and National Institute of Advanced Industrial Science and Technology (AIST) for statistical analysis.

Results

Subject demographics. Seventeen patients were included in the present study and were randomized to consume either green tea with high- or low-density catechins or a control beverage with no catechins. Clinical and laboratory characteristics of

Table I. Baseline clinical characteristics (n=17).

Group	Placebo (n=5)	Low-density catechins (n=5)	High-density catechins (n=7)	P-value
Age (years)	54.2±8.1	51.4±14.8	47.1±17.2	0.30
Body weight (kg)	74.1±18.3	77.6±13.8	70.7±13.0	0.74
Body fat (%)	36.1±6.2	36.0±4.8	34.3±6.9	0.85
Body mass index (kg/m^2)	30.0±4.4	29.1±1.8	28.0±2.0	0.48

Values are presented as means ± SD. P-value represents the comparison of groups by ANOVA.

Table II. Baseline laboratory characteristics (n = 17).

Group	Placebo (n=5)	Low-density catechins (n=5)	High-density catechins (n=7)	P-value
AST (IU/l)	110±58	104±47	120±57	0.88
ALT (IU/l)	94±50	81±47	101±95	0.89
LDH (IU/l)	225±39	220±32	224±59	0.96
ALP (IU/l)	198±75	246±82	230±63	0.61
γ-GTP (IU/l)	69±54	74±44	81±51	0.33
Ch-E (IU/l)	194±24	201±32	191±42	0.53
Total protein (g/dl)	7.9±1.0	7.5±0.9	7.1±0.7	0.20
Total bilirubin (mg/dl)	0.8±0.4	0.9±0.3	0.9±0.3	0.70
BUN (mg/dl)	10.1±5.0	9.5±3.8	11.0±3.4	0.51
Creatinine (mg/dl)	0.9±0.4	1.0±0.3	1.1±0.2	0.64
Total cholesterol (mg/dl)	161±59	197±33	189±55	0.61
Glucose (mg/dl)	97±18	99±21	94±19	0.18
Hemoglobin (g/dl)	12.8±1.2	14.1±2.4	11.5±1.9	0.56
Erythrocytes ($10^4/\mu$l)	390±41	411±60	405±55	0.21
MCV (fl)	90.3±7.0	89.5±8.1	91.2±9.4	0.87
MCH (pg)	31.4±3.0	30.8±2.5	32.5±2.9	0.90
Thrombocytes ($10^4/\mu$l)	19.5±5.5	17.9±6.8	18.6±4.9	0.84
Leukocytes ($10^3/\mu$l)	6.3±2.1	5.2±2.9	6.2±1.9	0.87

Values are presented as means ± SD. P-value represents the comparison of groups by ANOVA. AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transferase; Ch-E, cholinesterase; BUN, blood urea nitrogen; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin.

the study population are presented in Tables I and II, respectively. There were no significant differences among the three groups for clinical characteristics at baseline, including age, body weight, body fat percentage or BMI (Table I). There were also no significant differences among the three groups in laboratory data at baseline (Table II).

Reduction of body fat percentage in the high-density catechin group. Comparison of the data at baseline and 12 weeks later showed that the largest decrease in body weight occurred in the high-density catechin group. However, there was no significant difference in the percentage change of body weight among the three groups (high-density catechins, -3.8±2.7%; low-density catechins, -0.9±3.5%; placebo, -1.4±3.7%; Fig. 1).

BMI also decreased after 12 weeks of tea consumption, but there was no significant difference among the three groups (high-density catechins, -3.3±1.9%; low-density catechins, -0.5±2.0%; placebo, -1.2±3.9%; Fig. 2).

Body fat percentage decreased significantly from 34.3±6.9% (baseline) to 31.8±6.0% after 12 weeks of high-density catechin tea consumption ($P<0.05$). Body fat percentage decreased significantly more in the high-density catechin group (-7.3±2.2%) than in the placebo (0.9±2.1%) and low-density catechin (-0.6±2.4%) groups after 12 weeks (Fig. 3).

The liver-to-spleen CT attenuation ratio increased from 91.8±4.6% (baseline) to 101.8±4.7% after 12 weeks of consumption of high-density catechin tea. The liver-to-spleen CT attenuation ratio showed greater improvement in all

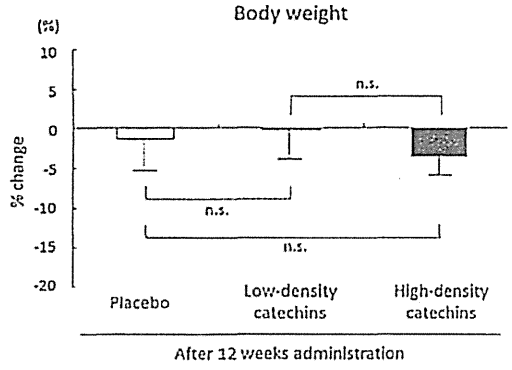

Figure 1. Percentage change of body weight after 12 weeks of catechin consumption. At 12 weeks, the body weight tended to be decreased in the high-density catechin group. However, there was no significant difference among the three groups. n.s., not statistically significant.

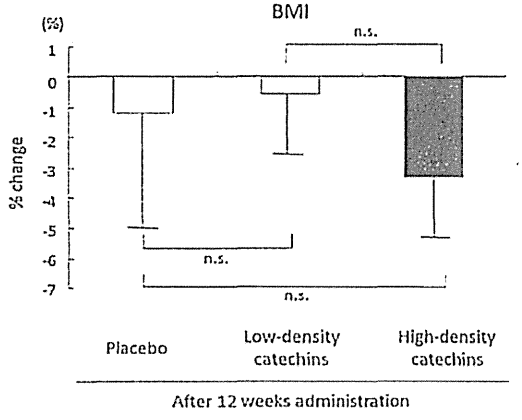

Figure 2. Percentage change of body mass index (BMI) after 12 weeks of catechin consumption. BMI decreased after 12 weeks of catechin consumption, but there was no statistically significant difference among the three groups. BMI, body mass index; n.s., not statistically significant.

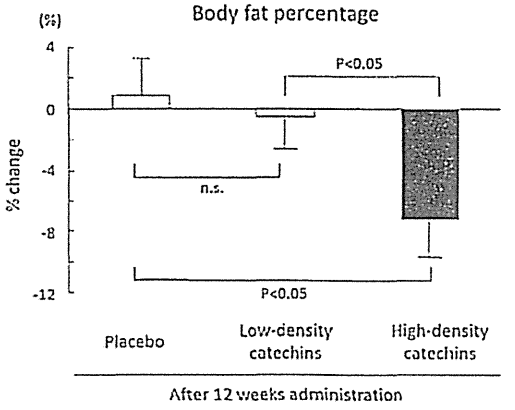

Figure 3. Percentage change of body fat percentage after 12 weeks of catechin consumption. Body fat decreased significantly more in the high-density catechin group than in the placebo and low-density catechin groups after 12 weeks of tea consumption (P<0.05). n.s., not statistically significant.

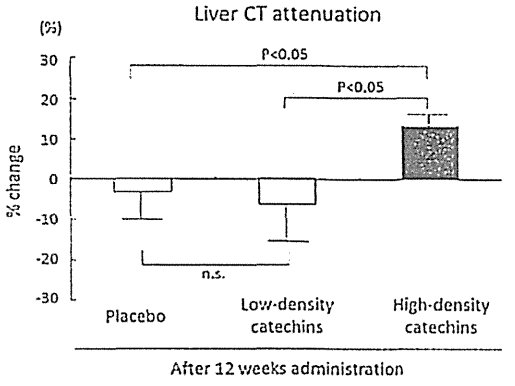

Figure 4. Percentage change of liver-to-spleen CT attenuation ratio after 12 weeks of catechin consumption. The liver-to-spleen CT attenuation ratio was significantly improved in all patients in the high-density catechin group relative to those in the placebo and low-density catechin groups after 12 weeks of tea consumption (P<0.05). n.s., not statistically significant.

patients in the high-density catechin group (11.3±2.8%) than in the placebo (-3.3±8.5%) and low-density catechin (-6.1±12.1%) groups (Fig. 4).

Improvement of serum alanine aminotransferase (ALT) levels by high-density catechin treatment. Serum ALT is an important marker of liver inflammation. Percentage change in serum ALT levels was significantly more negative in the high-density catechin group (-42.1±11.3%) than in the placebo (-3.1±7.8%) and low-density catechin (0.5±5.1%) groups after 12 weeks of consumption of high-density catechin tea (Fig. 5).

Reduction of urinary 8-isoprostane excretion by high-density catechin treatment. Urine 8-isoprostane is a specific marker of oxidative stress. Urine 8-isoprostane excretion was reduced from 249.6±11.6 pg (baseline) to 172.0±9.0 pg after 12 weeks of high-density catechin tea consumption. The percentage change in 8-isoprostane levels was worse in the high-density catechin group (-31.1±9.0%) than in the placebo (-1.7±9.1%) or low-density catechin (2.1±6.1%) groups (Fig. 6).

Discussion

NAFLD is a prevalent disease that is detected by medical examination and ultrasonography. Among NAFLD categories, patients with NASH, which is similar to alcoholic steatohepatitis in terms of pathological findings, have a poor prognosis (20). The lesions most commonly accepted with NASH include steatosis, ballooning degeneration of hepatocyte, mild diffuse lobular mixed acute and chronic inflammation and perivenular and perisinusoidal collagen deposition. Mallory's hyaline, vacuolated nuclei in periportal hepatocytes, lobular lipogranulomas and PAS-diastase-resistant Kupffer cells are also common in NASH. NASH may be an underlying cause of cryptogenic cirrhosis (21,22). The worldwide epidemic of obesity has increased the awareness of NAFLD from that of a curiosity to one of a potentially progressive liver disease that increases the risk of cirrhosis and HCC (23). A report by Marrero *et al* (24) indicated that cryptogenic liver disease is a common etiology of diseases in patients with HCC. NAFLD has been reported to account for

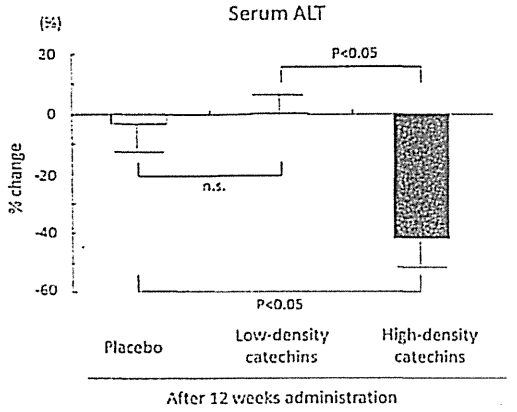

Figure 5. Percentage change of serum ALT values after 12 weeks of catechin consumption. Percentage change of serum ALT values was significantly decreased in the high-density catechin group compared with the placebo and low-density catechin groups after 12 weeks of catechin consumption (P<0.05). n.s., not statistically significant.

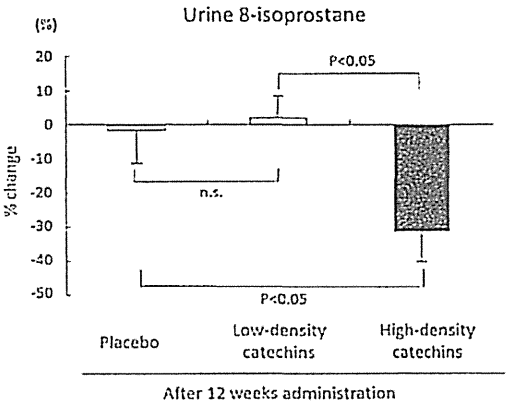

Figure 6. Percentage change of a marker of oxidative stress after 12 weeks of catechin consumption. Percentage change of urine 8-isoprostane was significantly more negative in the high-density catechin group than in the placebo and low-density catechin groups after 12 weeks of catechin consumption (P<0.05). n.s., not statistically significant.

at least 13% of HCC cases (24). Cryptogenic cirrhosis patients were found to have higher plasma levels of glucose, cholesterol and triglyceride, all parameters of insulin resistance (22). Obesity is an independent risk factor for HCC in patients with cryptogenic cirrhosis (25). Not all cases of NAFLD progress to cirrhosis and liver cancer. Early diagnosis and treatment of NAFLD may prevent progression to cirrhosis. As reported above, the improvement of eating habits is necessary to improve hyperlipidemia, insulin resistance and obesity.

As for whether green tea is effective for the improvement of insulin resistance and hyperlipidemia when consumed with a meal, the present study indicates that it is effective for treatment in NAFLD. It is believed that dietary therapy is preferable to medical therapy for the treatment of NAFLD, considering the mechanism of onset, but lifestyle changes can be difficult to implement. Thus, the development of an effective medical therapy is necessary. Epigallocatechin gallate (EGCG), the main catechin in green tea, is believed to reduce liver oxidation stress. The components of NAFLD have not yet been fully elucidated, but the following steps are considered to be the main mechanism. Free fatty acids are absorbed by the liver through the intestinal tract after a meal and are oxidized by mitochondria and peroxisomes. If fatty acid uptake by hepatocytes increases, fatty acid pools in the liver increase and accumulate in the hepatocytes as acylglycerol, increasing the load on hepatic mitochondria. Fatty acids that are not metabolized by mitochondria undergo ω or β oxidation by microsomes or peroxisomes. If a large quantity of fatty acids continues to be deposited in the liver, accumulation of acylglycerol in the hepatocytes induces oxidative stress that may progress to NAFLD (26). It is thought that EGCG reduces oxidative stress in hepatocytes through its potent antioxidant activity. Our study showed that the group consuming 1,080 mg of catechins per day had significantly lower levels of urine 8-isoprostane, a marker of oxidative stress, at the end of the study than at baseline. The low-density catechin and placebo groups did not show decreased oxidative stress, suggesting that it is necessary to consume ~1 g of catechins every day to reduce oxidative stress.

Catechins have inhibition effects on lipase, an enzyme related to glucose and fat absorption. EGCG shows inhibitory activity against lipase at a concentration of 0.349 μM (IC50) (27). In addition, catechins are reported to have inhibitory effects on α-amylase and α-glucosidase (27,28). A clinical study of hypertriacylglycerolaemia showed that the increase of triacylglycerol levels in the plasma after oral administration of butter was blunted by ~29% in response to catechin consumption (29). If fat absorption in the intestinal tract is decreased, liver fatty acid uptake also decreases, which may help prevent the onset of NAFLD.

It has been shown that catechins promote lipid metabolism in the liver (30). Body weight and adiposity were blunted by catechin administration in the obese mouse model C57BL/6J. Increased mRNA expression of acyl-CoA oxidase (ACO), one of the peroxisomal β-oxidizing enzymes and medium-chain acyl-CoA dehydrogenase (MCAD), a mitochondrial β-oxidizing enzyme, was observed in the liver of the catechin administration group. Increased hepatocellular mitochondrial β-oxidation activity promotes the breakdown of fatty acids and it is thought that it acts as a protective mechanism against NAFLD.

Catechins are a natural iron chelator and also serve to influence internal absorption of iron. A controlled study looking at the effects of EGCG on non-heme iron absorption showed that it was decreased by 27% in patients consuming 300 mg EGCG compared with controls consuming placebo (31). Reports on NASH patients showed that elevated iron stores, iron absorption in the liver (32) and serum ALT levels were decreased by bloodletting treatment (33). Restricting iron absorption through catechins may therefore be effective treatment for NAFLD.

NAFLD is a widespread disease and some cases of NAFLD progress to NASH. It is thought that the existence of steatosis and hepatitis is crucial for a diagnosis of NASH, which can be confirmed by a liver biopsy. Liver biopsy is the golden standard for NASH diagnosis. However, many patients without symptoms who present abnormal serum data, suggesting the presence of NAFLD, do not undergo a liver biopsy. For the present study, we used ultrasonography and X-ray CT to monitor NAFLD as these methods are non-invasive and

follow-up data can be collected. Blood biochemistry was used for the determination of hepatitis and steatosis status (34). The present study included only patients who had been diagnosed with NAFLD by a specialist. We instructed some participants to consume tea containing five times as much catechin content as normal tea for 12 weeks and did not observe any negative side effects in this group.

The mechanism of onset of NAFLD and NASH has still not been fully elucidated (35). The mechanism by which ingestion of catechins decreases fat accumulation in the liver has also not been determined. Liver fat was decreased along with an oxidative stress marker in response to the consumption of a high catechin tea. Liver inflammation and blood biochemistry also improved in this group. Findings of this study suggest that catechins are useful for the treatment of NAFLD.

Acknowledgements

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