RFA creates a tumor antigen source for the generation of antitumor immunity and enhances host immune responses.[5] Our previous mouse study also showed that RFA induced antitumor immune responses with massive T cell infiltration into a tumor, and the effect was enhanced by an active variant of CC chemokine ligand 3.[6] These studies suggest that additional immunological approaches to RFA may reduce HCC recurrence after treatment. However, in human studies, important data needed to develop a new immunotherapeutic approach have been lacking. First, the types of tumor-associated antigens (TAAs) and the epitopes to which these enhanced immune responses occur have not been fully identified. Second, the proportion of patients with enhanced antitumor immune responses and the effect of antitumor immunity for a patient's prognosis after RFA are still unclear. Third, the factors that affect TAA-specific immune responses and the functions and phenotype of T cells induced by RFA have not been identified.

In the present study, we analyzed immune responses in peripheral blood mononuclear cells (PBMCs) before and after RFA in 69 HCC patients using 11 TAA-derived peptides that we identified previously to be appropriate for analyzing HCC-specific immune responses. This approach offers useful information to develop a new strategy for HCC immunotherapy and improve the prognosis of patients treated by RFA.

Patients and Methods

Patients and Laboratory Testing. In this study, we examined 69 human leukocyte antigen (HLA)-A24–positive HCC patients with RFA. The diagnosis of HCC was histologically confirmed in 11 patients. For the remaining 58 patients, the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT.[7]

RFA was performed with a cool-tip RFA system consisting of an 18-gauge, cooled-tip electrode with a 2- or 3-cm exposed tip (Radionics, Burlington, MA) and radiofrequency generator (CC-1 Cosman Coagulator, Radionics). After local anesthesia, the electrode was inserted through a guide needle under ultrasound guidance. Radiofrequency energy was delivered for 6 to 12 minutes for each session. The energy was increased from 40 watts to 120 watts in a stepwise fashion. During ablation, the electrode was cooled by circulating ice-cooled saline in the electrode lumen to maintain the tip temperature below 20°C. During each treatment, the electrode tip was inserted into the tumor 1-3 times until the target tumor was surrounded by a high-echoic area. Complete necrosis after RFA was confirmed by dynamic computed tomography (CT) or magnetic resonance imaging (MRI). RFA was repeated in some cases until complete necrosis was confirmed. Thirty-nine and 30 patients received RFA 1 and 2-4 times, respectively. After treatments, HCC recurrence was evaluated with dynamic CT or MRI every 3-4 months.

All patients gave written informed consent to participate in the study in accordance with the Helsinki declaration, and this study was approved by the regional ethics committee (Medical Ethics Committee of Kanazawa University).

Blood samples were tested for hepatitis B surface antigen and hepatitis C virus (HCV) antibody using commercial immunoassays (Fuji Rebio, Tokyo, Japan). The patients with HCV antibody were tested for serum HCV RNA by real-time PCR (Roche, Tokyo, Japan), and 49 of 52 patients with HCV antibody were HCV RNA–positive. HLA-based typing of PBMCs from patients and normal blood donors was performed using reverse sequence-specific oligonucleotide analysis with polymerase chain reaction (PCR-RSSO). The serum alpha-fetoprotein (AFP) level was measured via enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan), and the pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer.[8] The severity of liver disease was evaluated according to the criteria of Desmet et al. using biopsy specimens of liver tissue, where F4 was defined as cirrhosis.[9] Fifty-five patients who participated in the present study received liver biopsy with RFA. Another 14 patients received liver biopsy 1-3 years before RFA.

Peptides and Preparation of PBMCs. Eleven peptides that we previously identified as being useful for analysis of immune response in HLA-A24–positive HCC patients were selected.[10-13] Human immunodeficiency virus (HIV) envelope-derived peptide ($HIVenv_{584}$)[14] and cytomegalovirus (CMV) pp65-derived peptide ($CMVpp65_{328}$)[15] were also selected as control peptides. Peptides were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry, and their purities were determined to be >90% by analytical high-performance liquid chromatography. PBMCs were isolated before and 2-4 weeks after HCC treatments as described.[11] In the patients who received RFA 2-4 times, PBMCs were obtained 2-4 weeks after the final treatment. In some patients, PBMCs were also obtained 24 weeks after RFA. PBMCs were resuspended

in Roswell Park Memorial Institute 1640 medium containing 80% fetal calf serum and 10% dimethyl sulfoxide and cryopreserved until use.

Interferon-γ ELISPOT Assay. Interferon-γ (IFN-γ) ELISPOT assays were performed as described.[11] Negative controls consisted of an HIV envelope–derived peptide ($HIVenv_{584}$).[14] Positive controls consisted of 10 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMVpp65-derived peptide ($CMVpp65_{328}$).[15] The colored spots were counted with a KS ELISpot Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of an antigen from the number in its presence. Responses to peptides were considered positive if more than 10 specific spots were detected, which is greater than the mean + 3 SD of the baseline response detected in 11 HLA-A24–positive normal blood donors against TAA-derived peptides, and the number of spots in the presence of an antigen was at least twofold that in its absence. The results of an ELISPOT assay with more than 25 spots in the wells without peptides (control wells) were excluded from the analysis.

IFN-γ ELISPOT assays were also performed using PBMC-depleted $CD4^+$ or $CD8^+$ cells to determine what kind of T cell is responsive to the peptides. In the assay using PBMC-depleted $CD4^+$ or $CD8^+$ cells, the number of cells was adjusted to $3 - 10^5$ cells/well after the depletion. Depletion of $CD4^+$ or $CD8^+$ cells was performed using the MACS separation system with CD4 or CD8 MicroBeads (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions.

Detection of Myeloid-Derived Suppressor Cells. For the detection of myeloid-derived suppressor cells (MDSCs), PBMCs were isolated from 20 randomly selected patients 2-4 weeks after HCC treatment. To determine the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs, two-color fluorescence-activated cell sorting analysis was performed using the following antibodies: anti-CD14 and anti–HLA-DR (Becton Dickinson). Flow cytometry was performed using the FACSAria II system (Becton Dickinson). The frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs was calculated as a percentage of $HLA\text{-}DR^{-/low}$ cells in $CD14^+$ cells.

Tetramer Staining and Flow Cytometry. Peptide $MRP3_{765}$-, AFP_{357}-, AFP_{403}-, and $hTERT_{461}$-specific tetramers were purchased from Medical Biological Laboratories Co., Ltd. (Nagoya, Japan). PBMCs were stained with anti–CD8-APCAb (Becton Dickinson, Tokyo, Japan), anti–CCR7-FITCAb (eBioscience, Tokyo, Japan), anti–CD45RA-PerCP-Cy5.5Ab (eBioscience, Tokyo, Japan), and tetramer-PE for 30 minutes at room temperature. Cells were washed, fixed with 0.5% paraformaldehyde/phosphate-buffered saline, and analyzed using the FACSAria II system.

Table 1. Patient Characteristics (n = 69)

Characteristic	Value
Age, years	67.3 ± 9.4 (69.0)
Sex, male/female	51/18
Platelet count, $\times 10^4/\mu L$	15.9 ± 26.5 (10.9)
Platelet count, $>15 \times 10^4/\leq 15 \times 10^4/\mu L$	19/50
ALT, IU/L	46.7 ± 33.8 (38.0)
ALT, >30/≤30 IU/L	44/25
Prothrombin time, %	78.4 ± 14.6 (77.0)
Prothrombin time, >70%/≤70%	50/19
Albumin, g/dL	3.6 ± 0.5 (3.6)
Albumin, >3.5/≤3.5 g/dL	42/27
Total bilirubin, mg/dL	1.1 ± 0.6 (0.9)
Total bilirubin, >2.0/≤2.0 mg/dL	4/65
AFP, ng/mL	134.7 ± 468.3 (11.0)
AFP, >100/≤100 ng/mL	12/57
HCC differentiation, well/moderate/poor/ND	7/3/1/58
Tumor diameter, >2/≤2 cm	28/41
Tumor multiplicity, multiple/solitary	29/40
Vascular invasion, +/−	1/68
TNM factor	
T1/T2-4	40/29
N0/N1	68/1
M0/M1	69/0
TNM stage, I/II/IIIA/IIIB/IIIC/IV	39/29/0/0/1/0
Histology of nontumor liver, liver cirrhosis/chronic hepatitis	55/14
Liver function, Child-Pugh score A/B/C	52/17/0
Etiology, HCV/HBV/other	52/8/9
Additional treatment,* +/−	14/55

Data are expressed as the mean ± SD (median) or as the number of patients.

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; ND, not determined, TNM, tumor-node-metastasis.

*Transarterial embolization.

Statistical Analysis. Data are expressed as the mean ± SD. The estimated probability of tumor recurrence-free survival was determined using the Kaplan-Meier method. The Mantel-Cox log-rank test was used to compare curves between groups. The prognostic factors for tumor recurrence-free survival were analyzed for statistical significance using the Kaplan-Meier method (univariate) and the Cox proportional hazard model (multivariate). Linear regression lines for the relationship between the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs and the number of TAA-specific T cells were calculated using Pearson's correlation coefficient. A level of $P < 0.05$ was considered significant.

Results

Patient Profile. The clinical profiles of the 69 patients analyzed in the present study are shown in Table 1. HCC was histologically classified as well, moderately, and poorly differentiated in 7, 3, and 1 cases,

Table 2. Peptides and Response Frequency

Peptide Name	Amino Acid Sequence	Number of Specific Spots in Normal Donors (mean ± SD)	Frequency of T Cell Response		P*
			Before RFA	After RFA	
$SART2_{899}$	SYTRLFLIL	1.0 ± 1.4	0/69 (0.0%)	14/69 (20.3%)	<0.001
$SART3_{109}$	VYDYNCHVDL	2.1 ± 1.9	7/69 (10.1%)	20/69 (29.0%)	0.009
$MRP3_{503}$	LYAWEPSFL	0.2 ± 0.5	3/69 (4.3%)	17/69 (24.6%)	0.001
$MRP3_{692}$	AYVPQQAWI	1.5 ± 2.1	4/68 (5.9%)	8/69 (11.6%)	0.366
$MRP3_{765}$	VYSDADIFL	0.9 ± 1.0	3/69 (4.3%)	17/69 (24.6%)	0.001
AFP_{357}	EYSRRHPQL	1.8 ± 2.0	3/68 (4.4%)	14/68 (20.6%)	0.008
AFP_{403}	KYIQESQAL	1.1 ± 1.5	9/66 (13.6%)	24/68 (35.3%)	0.005
AFP_{434}	AYTKKAPQL	0.8 ± 1.1	7/68 (10.3%)	14/68 (20.6%)	0.153
$hTERT_{167}$	AYQVCGPPL	0.8 ± 1.1	9/65 (13.8%)	15/68 (22.1%)	0.263
$hTERT_{324}$	VYAETKHFL	0.5 ± 0.7	6/62 (9.7%)	9/68 (13.2%)	0.591
$hTERT_{461}$	VYGFVRACL	0.7 ± 1.2	11/64 (17.2%)	23/69 (33.3%)	0.046
HIV env_{584}	RYLRDQQLL	1.3 ± 2.0	1/63 (1.6%)	2/68 (2.9%)	>0.999
CMV $pp65_{328}$	QYDPVAALF	13.3 ± 15.7	43/68 (63.2%)	39/67 (58.2%)	0.599

*Analysis via chi-squared test.

respectively. In the other cases, HCC was diagnosed on the basis of typical CT findings and elevated AFP levels. In terms of size and number, the tumor was classified as large (>2 cm) in 28 cases, small (≤2 cm) in 41 cases, multiple in 29 cases, and solitary in 40 cases. Vascular invasion was noted in one patient. Using tumor-node-metastasis staging of the Union Internationale Contre Le Cancer (UICC) system (6th edition),[16] patients were classified as having stage I (n = 39), II (n = 29), IIIA (n = 0), IIIB (n = 0), IIIC (n = 1), or IV (n = 0) tumors.

Detection of TAA-Specific T Cells Before and After RFA. Detection of TAA-specific T cells was performed by direct *ex vivo* analysis (IFN-γ ELISPOT assay). Positive T cell responses against each TAA-derived peptide were observed in 0 to 11 (0.0%-17.2%) patients before RFA (Table 2). The same responses against HIV- and CMV-derived peptides were observed in 1 (1.6%) and 43 (62.3%) patients, respectively. After HCC treatments with RFA, positive T cell responses against TAA-, HIV- and CMV-derived peptide were observed in 8-24 (11.6%-35.3%), 2 (2.9%), and 39 (58.2%) patients, respectively. The increase of the frequency of TAA-specific T cells after RFA observed in 7 of 11 peptides ($SART2_{899}$, $SART3_{109}$, $MRP3_{503}$, $MRP3_{765}$, AFP_{357}, AFP_{403}, and $hTERT_{461}$) was statistically significant (Table 2).

The magnitude of TAA-specific T cell responses determined by the frequency of T cells and the proportion of the patients who showed a significant increase of TAA-specific T cells are shown in Fig. 1. When the T cell responses against a single peptide with more than or equal to 10 specific spots and two-fold increase were defined as significant, a significant increase was observed in 4-16 (6.5%-24.6%) patients for each TAA-derived peptide and in 24 (39.3%) patients for total of TAA-derived peptides. On the other hand, the numbers of patients who showed a significant increase against HIV- and CMV-derived peptide were 1 (1.6%) and 8 (11.9%), respectively. The number of patients who showed a significant increase against at least one TAA-derived peptide after RFA was 43 (62.3%).

To determine what kind of T cell is responsive to the peptides, TAA-derived peptide-specific IFN-γ–producing T cells were also analyzed by ELISPOT assay using PBMC-depleted $CD4^+$ or $CD8^+$ cells. The assay showed that IFN-γ–producing T cells against the peptides ($SART2_{899}$, $SART3_{109}$, $MRP3_{503}$, $MRP3_{692}$, $MRP3_{765}$, AFP_{357}, AFP_{403}, AFP_{434}, $hTERT_{167}$, $hTERT_{324}$, and $hTERT_{461}$) mainly consisted of $CD8^+$ cells (Supporting Fig. 1).

Effect of Increase of TAA-Specific T Cells After RFA for the Prognosis of Patients. To examine the effect of increase of TAA-specific T cells after RFA for the prognosis of patients, we analyzed the relationship between the number of TAA-specific T cells and HCC recurrence-free survival after RFA. First, we divided the patients into two groups with high (above median) and low (below median) specific spots detected via ELISPOT assay. In the analysis, we found that a high number of TAA-specific T cells after HCC treatment correlated significantly with the length of HCC recurrence-free survival ($P = 0.044$) (Fig. 2A). The difference between the groups was emphasized when 50 spots were defined as highly specific spots ($P = 0.006$) (Fig. 2B). On the other hand, there was no correlation between the number of TAA-specific T cells before HCC treatment and the length of HCC recurrence-free survival ($P = 0.758$) (Fig. 2C). Furthermore, the magnitude of enhancement of TAA-specific immune responses did not correlate significantly with the length of HCC recurrence-free survival ($P = 0.267$) (Fig. 2D).

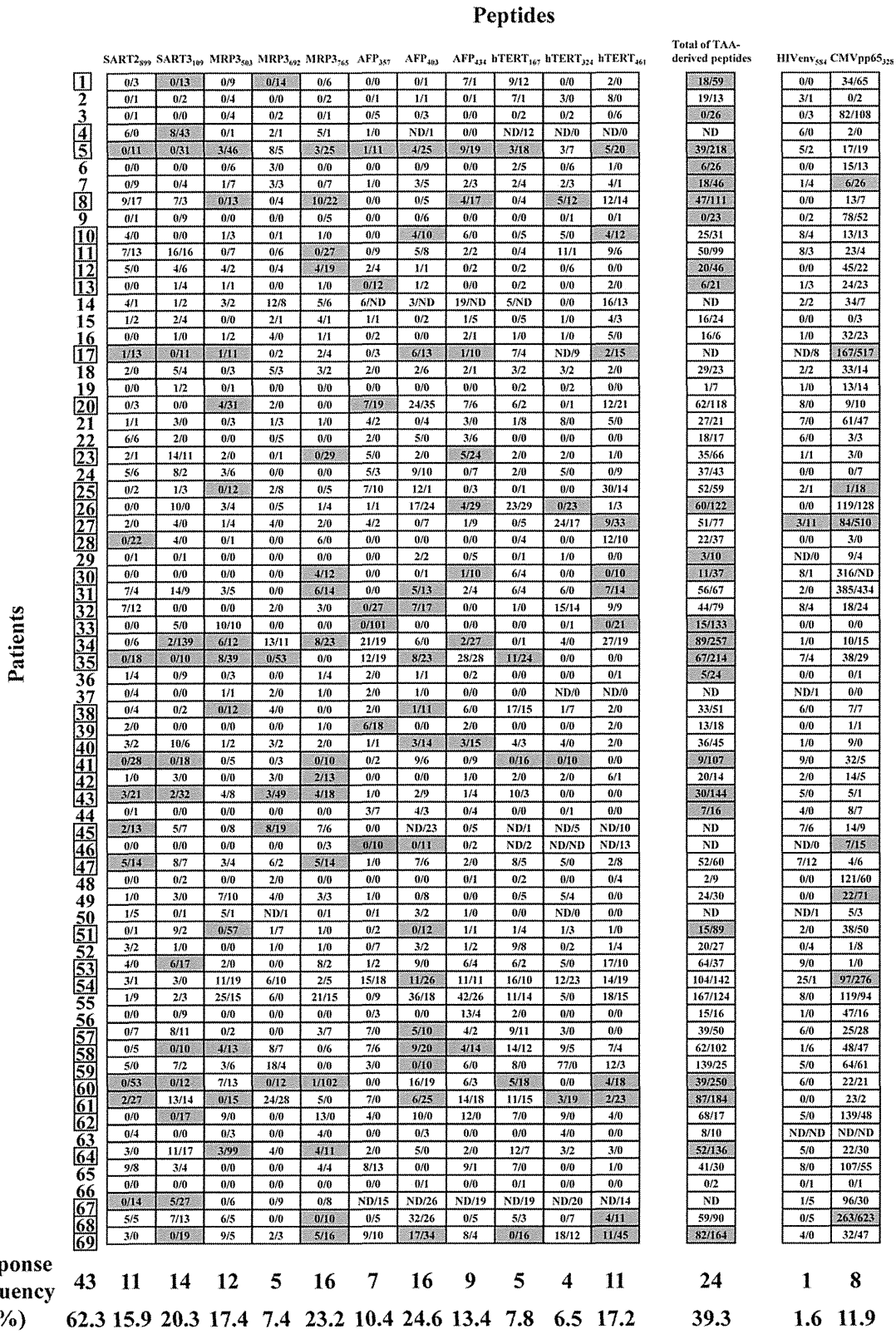


Fig. 1. Enhancement of TAA-derived peptide-specific T cell responses after RFA. The magnitude of TAA-specific T cell responses determined by the frequency of T cells responsive to each peptide before (the number of left side) and after (the number of right side) RFA and proportion of the patients with a significant increase are shown. Results with a significant increase are shown in gray boxes. The box numbers show the patients with a significant increase in TAA-specific T cell responses. The T cell responses were examined by IFN-γ ELISPOT assay. The results of ELISPOT assay are shown as a specific spot, which was determined by subtracting the number of spots in the absence of an antigen from the number in its presence. The increase was considered significant if more than or equal to 10 specific spots per 300,000 PBMCs were detected and if the number of spots after RFA was at least two-fold that before RFA. The patient characteristics are described in Table 1 and the peptide sequences are listed in Table 2.

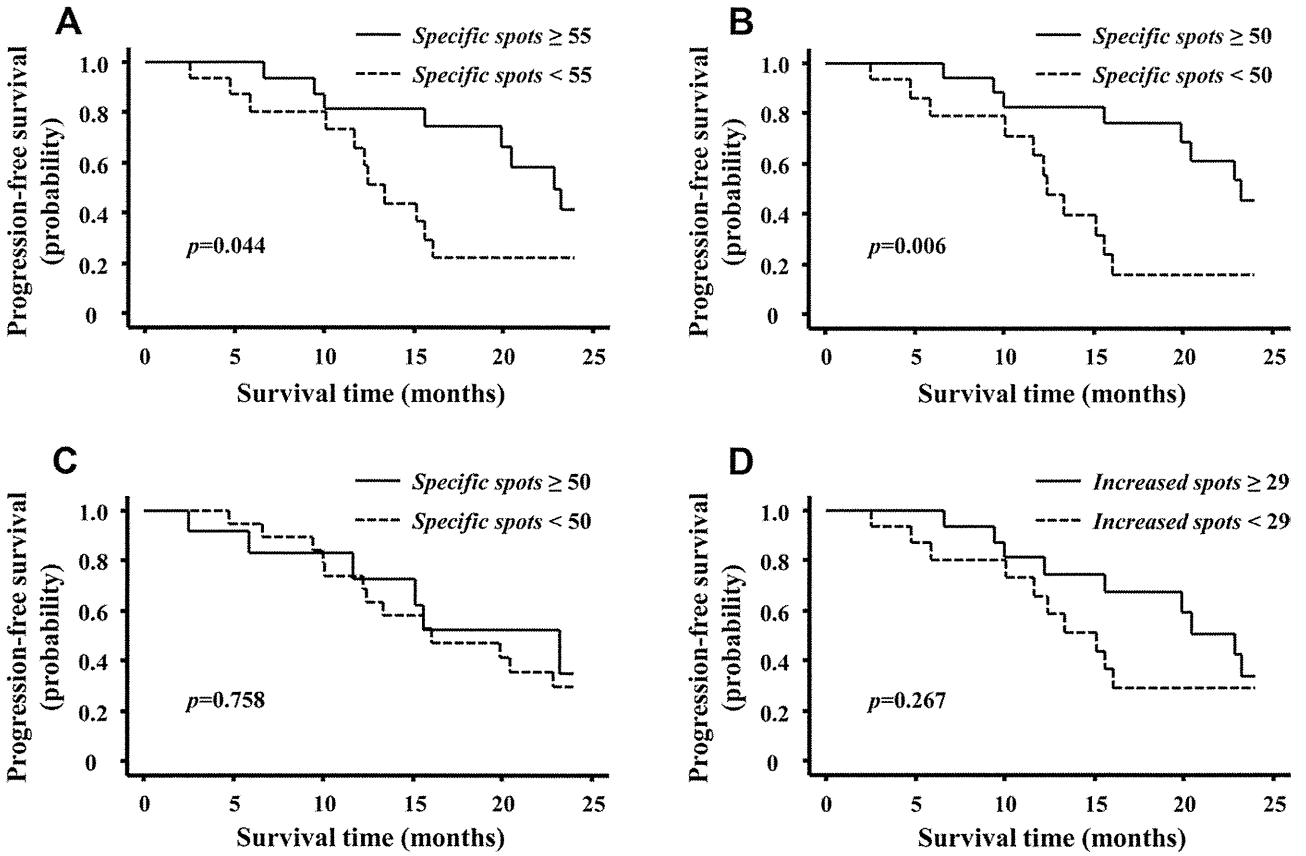


Fig. 2. Kaplan-Meier curves of HCC recurrence-free survival. (A) Kaplan-Meier curves indicating the relationship between month after RFA and HCC recurrence-free survival rate were grouped by the median number of TAA-specific T cells detected by IFN-γ ELISPOT assay after RFA. (B) The difference between the groups was emphasized when 50 spots were defined as highly specific spots. (C) Kaplan-Meier curves indicating the relationship between month after RFA and HCC recurrence-free survival rate were grouped by the number of TAA-specific T cells detected by IFN-γ ELISPOT assay before RFA. (D) Kaplan-Meier curves indicating the relationship between month after RFA and HCC recurrence-free survival rate were grouped by the median increased number of TAA-specific T cells after RFA.

When univariate analysis of prognostic factors for HCC recurrence-free survival was performed, γ-glutamyltransferase (<30), AFP (<400), Okuda stage,[1] and number of TAA-specific T cells after RFA (≥50) were detected as factors that decrease HCC recurrence rate after RFA (Table 3). When multivariate analysis including these three factors was performed, only the number of TAA-specific T cells after RFA (≥50) was found to be a factor that decreases HCC recurrence rate after RFA.

Analysis of the Factors that Affect the Number of TAA-Specific T Cells After RFA. To identify the factors that affect the number of TAA-specific T cells after RFA, we analyzed clinical parameters of patients and the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs after HCC treatment. We could not find any clinical parameters correlated with the number of TAA-specific T cells after RFA.

The frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs after RFA showed various levels and depended on the patient (Fig. 3A,B). The frequency decreased significantly after RFA ($P = 0.022$) except in three patients (Fig. 3B) and correlated inversely with the number of TAA-specific T cells after RFA, but not with that of CMV-specific T cells (Fig. 3C).

Phenotypic Analysis of TAA-Specific T Cells Before and After RFA. Next, we examined the naïve/effector/memory phenotype of increased TAA-specific T cells after RFA using a tetramer assay. The memory phenotype was investigated by the criterion of CD45RA/CCR7 expression.[17] In tetramer analysis, the frequency of TAA-derived peptide-specific $CD8^+$ T cells before RFA was 0.00%-0.03% of $CD8^+$ cells (Fig. 4A). On the other hand, the frequency was increased after RFA in 10/12 (83.3%) patients, and the range was 0.00%-0.10% of $CD8^+$ cells. The frequency of $CD45RA^-/CCR7^+$ (central memory), $CD45RA^-/CCR7^-$ (effector memory), and $CD45RA^+/CCR7^-$ (effector) T cells in tetramer-positive cells depended on the patients, and the ratio of these cells changed after RFA (Fig. 4B). The frequency of tetramer-positive cells with $CD45RA^-/CCR7^+$ and $CD45RA^-/CCR7^-$ in $CD8^+$ cells was increased in 6/7 (85.7%) and

Table 3. Univariate and Multivariate Analysis of Prognostic Factors for Tumor-Free Survival

Variable	P		HR (95% CI)
	Univariate Analysis	Multivariate Analysis	
Age, <65/≥65 years	0.582		
Platelet count, <10/≥10 × 10^4/μL	0.570		
AST, <40≥40 IU/L	0.298		
ALT, <40≥40 IU/L	0.628		
γ-GTP, <30≥30 IU/L	0.010	0.223	2.408 (0.586-9.898)
Albumin, <3.5≥3.5 g/dL	0.588		
Total bilirubin, <1≥1 mg/dL	0.386		
Prothrombin time, <60%/≥60%	0.282		
DCP, <100/≥100 mAU/mL	0.630		
AFP, <400/≥400 ng/mL	0.008	0.056	0.216 (0.045-1.039)
L3, <10%/≥10%	0.100		
Child-Pugh score, A/B	0.260		
Tumor diameter, <2/≥2 cm	0.706		
Tumor multiplicity, solitary/multiple	0.686		
Okuda stage, 1/2	0.043	0.103	5.828 (0.698-48.630)
BCLC stage, A/BC	0.190		
CLIP score, 0,1/2,3	0.703		
HCV antibody, −/+	0.080		
No. of TAA-specific T cells before RFA, <50/≥50*	0.740		
Number of TAA-specific T cells after RFA, <50/≥50*	0.006	0.024	4.054 (1.203-13.664)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCLC, Barcelona Clinic Liver Cancer; CLIP, Cancer of the Liver Italian Program; DCP, des- γ-carboxy prothrombin γ-GTP, γ-guanosine triphosphate.

*Number of TAA-specific T cells before and after RFA was calculated per 3 × 10^5 PBMCs.

6/7 (85.7%) patients, respectively, whose samples were available for the assay before and after RFA. Interestingly, the tetramer-positive cells with $CD45RA^-/CCR7^+$ were newly induced after RFA in 5/7 (71.4%) patients.

Kinetics of TAA-Specific T Cells Induced by RFA. Although the number of TAA-specific T cells was a predictive factor of a decrease of HCC recurrence rate after RFA (as shown in Fig. 2A), more than 50% of the patients with a high number of TAA-specific T cells showed HCC recurrence for 25 months after treatment. To identify the relationship between TAA-specific T cell responses and HCC recurrence more precisely, we examined the kinetics of TAA-specific T cells in 16 patients whose PBMCs were available for analysis at 24 weeks after RFA. The frequencies of TAA-derived peptide-specific T cells decreased in most of the peptides and patients at 24 weeks after RFA (Fig. 5). In the analysis of the total of each type of TAA-derived peptide-specific T cells, the frequency decreased in 14/16 (87.5%) patients analyzed, and most of them showed fewer than 50 specific spots per 3 × 10^5 PBMCs, with the exception of one patient. In contrast, the frequencies of CMV-derived peptide-specific T cells were maintained in most of the patients.

Discussion

In recent years, HCC-specific TAAs and their T cell epitopes have been identified, which has made analysis of immunological status in HCC patients possible and shown that TAA-specific T cell responses can be detected in peripheral blood.[11,18-20] The immunological analysis of HCC patients with RFA using 11 TAA-derived peptides in this study showed that the enhancement of TAA-specific T cell responses occurred in 62.3% of patients, the antigens and their epitope to which enhanced T cell responses occurred were diverse, and some of them were newly induced. The mechanism of enhancement of tumor-specific immune response by RFA is still unclear. den Brok et al.[5] showed that RFA created an antigen source for antitumor immunity by destruction of tumor cells using a mouse tumor model. The antigens used in this study have been reported to be located in the cell membrane (MRP3), cytoplasm (SART2 and AFP), and nucleus (hTERT and SART3).[21-24] The diversity of the target proteins of enhanced T cells suggests that the central mechanism of enhancement of tumor-specific immune response by RFA is due to tumor cell destruction, which supports the results mentioned previously.[5]

In the present study, we also showed that the number of TAA-specific T cells after RFA was associated with the HCC recurrence-free survival of patients. The univariate and multivariate analyses clearly showed it was a predictive factor for HCC recurrence after RFA. These results suggest that TAA-specific T cells induced by RFA contribute to protection from HCC recurrence, and additional immunological approaches should be applied to enhance the protective effect after treatment.

To understand the precise mechanism that RFA enhances TAA-specific T cell responses, we analyzed the factors that affected the number of TAA-specific T cells after RFA. Among the factors analyzed, the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs after RFA was inversely correlated with the number of TAA-specific T cells, suggesting these MDSCs may have a negative effect on TAA-specific immune responses. Regarding the function of MDSCs in cancer patients, it has been reported that they inhibit T lymphocyte responses.[25] In HCC patients, it is reported that the frequency of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs in

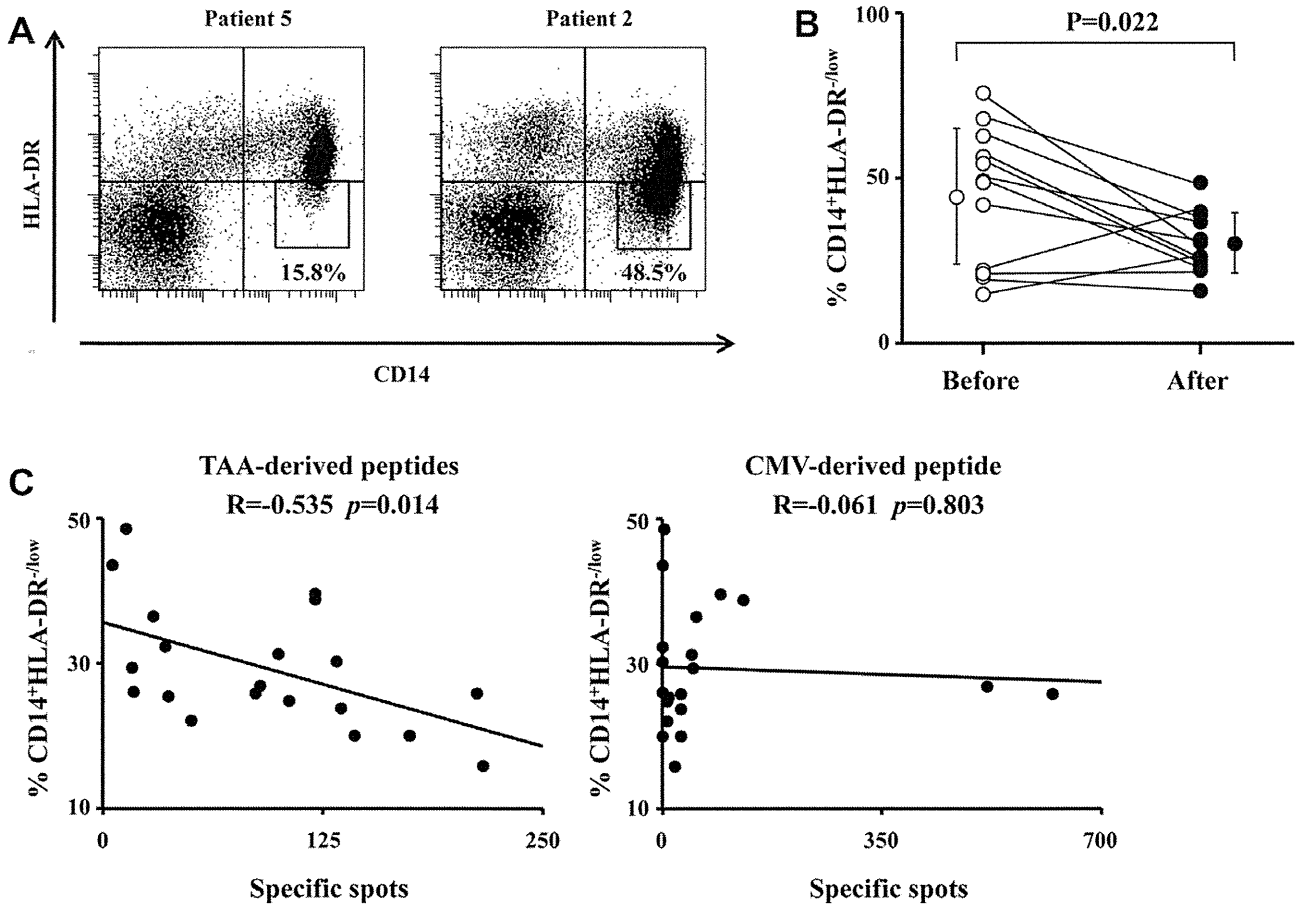


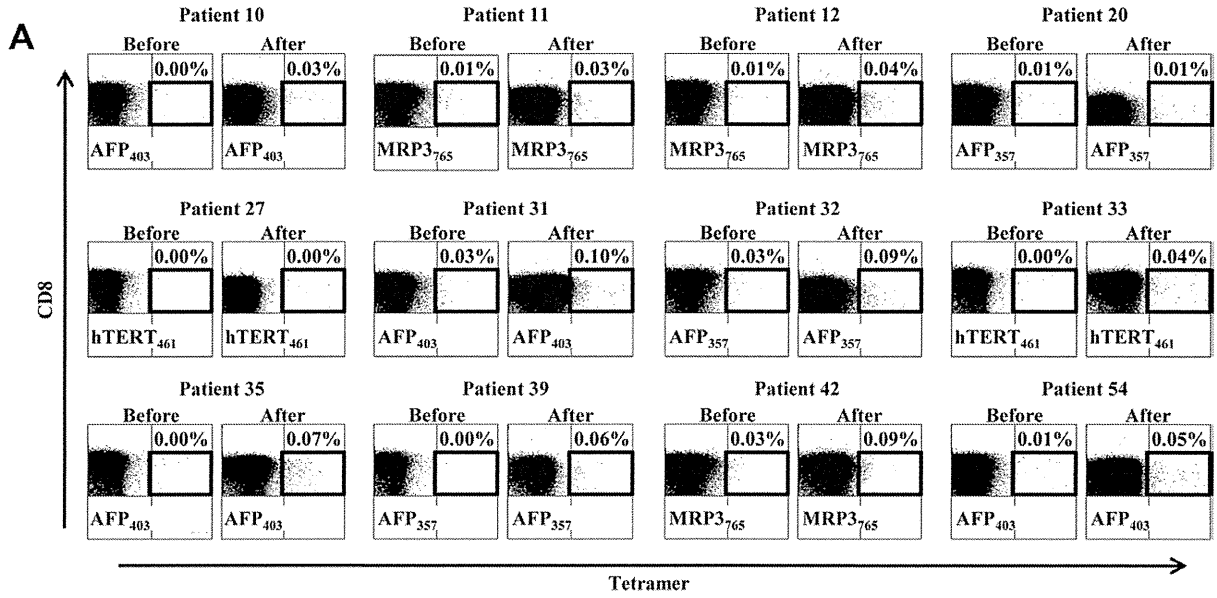
Fig. 3. Frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs and its relationship with the frequency of TAA-specific T cells after RFA. (A) The frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs was measured in 20 randomly selected patients by FACS analysis after RFA. The representative results of two patients are shown. (B) The frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs was also measured in 12 of 20 randomly selected patients before RFA and compared with that after RFA. (C) Relationship between the frequency of $CD14^{+}HLA\text{-}DR^{-/low}$ MDSCs and the frequency of TAA- and CMV-derived peptide-specific T cells after RFA.

PBMCs is significantly increased in comparison with healthy controls and they exert immunosuppressive function via induction of regulatory T cells.[26] Taken together with our results, these reports suggest that an additional immunological approach to inhibit the function of MDSCs after RFA may enhance TAA-specific immune responses.

On the other hand, patients with a high number of TAA-specific T cells were not completely protected from HCC recurrence. To examine the mechanisms behind the failure to control HCC recurrence completely by RFA-induced TAA-specific immune responses, we performed phenotypic and kinetic analysis of T cells enhanced by RFA. The results showed that the frequency of T cells with each memory phenotype depended on the patient, and the ratio of these cells changed after RFA. The memory phenotype of T cells that showed a more than two-fold increase was the $CD45RA^{-}/CCR7^{+}$ (central memory) phenotype, which required secondary stimulation by antigen to exert stronger antitumor effects.[17] Interestingly, they were newly induced, suggesting that RFA may modify not only the frequency but also the phenotype of TAA-specific T cells.

The frequencies of TAA-derived peptide-specific T cells decreased in most of the patients at 24 weeks after RFA, suggesting that RFA could not induce long-lived T cells. In a previous study, it was reported that tumor-specific immune responses induced by RFA could not protect from HCC recurrence completely because of tumor immune escape.[27] In addition to this mechanism, our results suggest that one of the reasons that RFA-induced tumor-specific immune response is insufficient for controlling HCC recurrence is the weak induction of long-lived T cells.

Taken together with these results, the present study suggests that the antitumor effect of TAA-specific T cells induced by RFA should be enhanced by an additional immunological approach. In recent studies of cancer immunology, cancer vaccines consisting of TAA-derived protein or peptide, recombinant virus, and



B

	Patient 10		Patient 11		Patient 12		Patient 20		Patient 27		Patient 31	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CD45RA-CCR7+	ND	2.1	7.4	10.3	0.0	4.2	0.0	2.2	ND	ND	13.5	2.2
CD45RA-CCR7-	ND	27.1	14.8	17.6	47.8	41.7	7.4	13.0	ND	ND	18.9	51.7
CD45RA+CCR7-	ND	22.9	44.4	33.8	21.7	10.1	74.1	69.6	ND	ND	35.1	42.2

	Patient 32		Patient 33		Patient 35		Patient 39		Patient 42		Patient 54	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
CD45RA-CCR7+	0.0	7.6	ND	6.2	ND	3.9	ND	6.2	0.0	6.9	0.0	3.1
CD45RA-CCR7-	29.6	38.6	ND	27.2	ND	4.5	ND	8.3	19.6	22.4	20.0	23.1
CD45RA+CCR7-	44.4	49.7	ND	11.1	ND	67.0	ND	41.7	80.4	63.8	60.0	51.5

Fig. 4. Phenotypic analysis of T cells induced by RFA. (A) Enhancement of TAA-specific T cell responses was also analyzed by tetramer assay. The results of all patients examined are shown. Peptide $MRP3_{765}$-, AFP_{357}-, AFP_{403}-, and $hTERT_{461}$- specific tetramers were used. The frequency of tetramer-positive cells is shown as the percentage in $CD8^+$ cells. (B) The memory phenotype of tetramer-positive cells was analyzed using the criterion of CD45RA/CCR7 expression. The box numbers show the percentage of cells in tetramer-positive cells. ND means that the experiments are not available because of the small number of tetramer-positive cells. Results with increased frequency after RFA are shown in gray boxes.

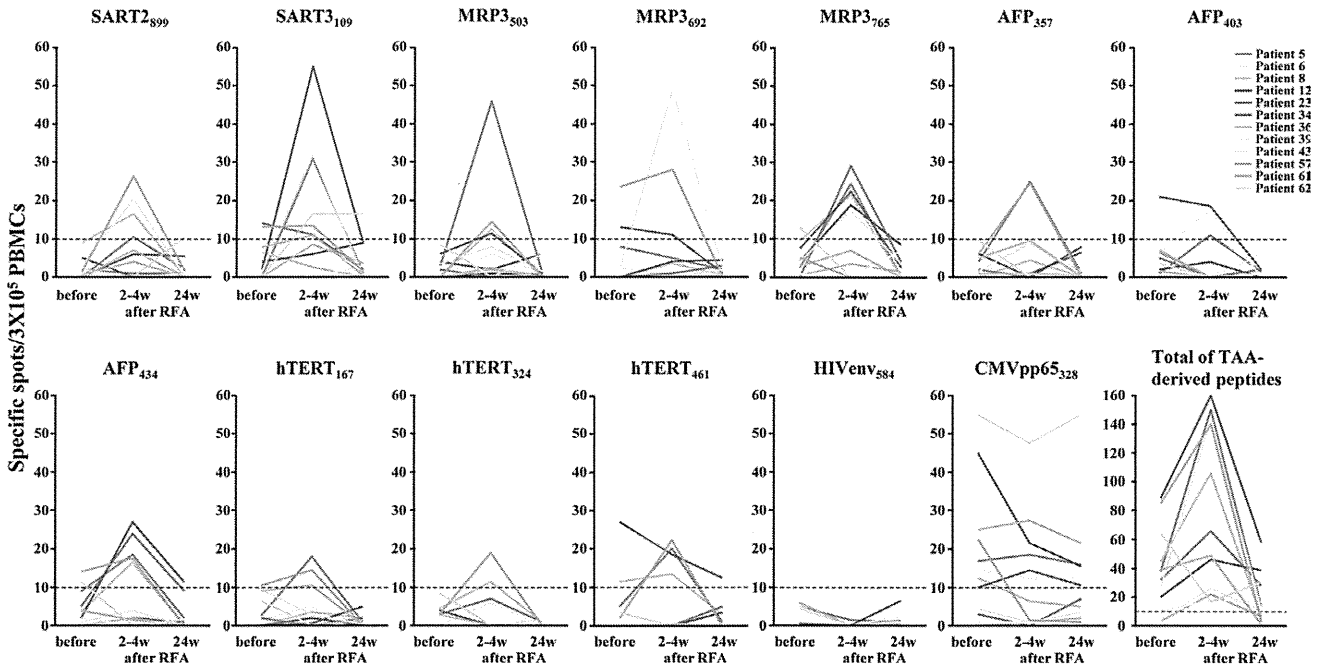


Fig. 5. Kinetics of TAA-specific T cell responses determined via IFN-γ ELISPOT assay. PBMCs were obtained at three different time points: (1) before RFA, (2) 2-4 weeks after RFA, and (3) 24 weeks after RFA. Each graph indicates the kinetics of T cells specific for each peptide in each patient.

engineered tumor cells have been considered as candidates to enhance host immune responses.[28] Alternatively, immunomodulating antibodies such as anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) and anti-programmed cell death 1 (PD-1) have been considered to reactivate T cell function.[28,29] These approaches may also be effective to enhance the antitumor effect induced by RFA.

In conclusion, the results of this study show that RFA can enhance various TAA-specific T cell responses and the number of T cells induced is associated with HCC recurrence-free survival. To maintain the TAA-specific T cell responses induced by RFA and to improve the immunological effect for HCC, additional treatment by vaccine or immunomodulatory drugs might be useful.

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Clinical Cancer Research

Association of *Interleukin-28B* Genotype and Hepatocellular Carcinoma Recurrence in Patients with Chronic Hepatitis C

Yuji Hodo[1], Masao Honda[1,3], Akihiro Tanaka[1], Yoshimoto Nomura[1], Kuniaki Arai[1], Taro Yamashita[1], Yoshio Sakai[1], Tatsuya Yamashita[1], Eishiro Mizukoshi[1], Akito Sakai[1], Motoko Sasaki[2], Yasuni Nakanuma[2], Mitsuhiko Moriyama[4], and Shuichi Kaneko[1]

Abstract

Purpose: Several single-nucleotide polymorphisms (SNP) in the interleukin-28B (*IL-28B*) locus have recently been shown to be associated with antiviral treatment efficacy for chronic hepatitis C (CHC). However, such an association with hepatocellular carcinoma (HCC) is unkno3 we investigated the association between the *IL-28B* genotype and the biology and clinical outcome of patients with HCC receiving curative treatment.

Experimental Design: Genotyping of 183 patients with HCC with CHC who were treated with hepatic resection or radiofrequency ablation (RFA) was carried out, and the results were analyzed to determine the association between the *IL-28B* genotype (rs8099917) and clinical outcome. Gene expression profiles of 20 patients with HCC and another series of 91 patients with CHC were analyzed using microarray analysis and gene set enrichment analysis. Histologic and immunohistochemical analyses were also conducted.

Results: The TT, TG, and GG proportions of the rs8099917 genotype were 67.8% (124 of 183), 30.6% (56 of 183), and 1.6% (3 of 183), respectively. Multivariate Cox proportional hazard analysis showed that the *IL-28B* TT genotype was significantly associated with HCC recurrence ($P = 0.007$; HR, 2.674; 95% confidence interval, 1.16–2.63). Microarray analysis showed high expression levels of IFN-stimulated genes in background liver samples and immune-related genes in tumor tissues of the *IL-28B* TG/GG genotype. Histologic findings showed that more lymphocytes infiltrated into tumor tissues in the TG/GG genotype.

Conclusions: The *IL-28B* genotype is associated with HCC recurrence, gene expression, and histologic findings in patients with CHC. *Clin Cancer Res; 19(7); 1827–37. ©2013 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the seventh most common cancer worldwide and the third most common cause of cancer mortality (1). HCC usually develops in patients suffering from chronic hepatitis B or chronic hepatitis C (CHC). Although hepatic resection has been considered the most efficient therapy for HCC, it is only suitable for 20% to 35% of patients because of poor hepatic reserve (2). Radiofrequency ablation (RFA) has therefore been introduced as a minimally invasive therapy for such cirrhotic patients and is widely applicable with little effect on hepatic reserve. Moreover, randomized (3, 4) and nonrandomized (5, 6) controlled studies revealed no statistical difference in patient survival between resection and RFA.

Despite these curative treatments of HCC, its recurrence remains common. Several studies have identified potential risk factors for HCC recurrence, including the presence of cirrhosis, high α-fetoprotein (AFP) levels, large tumor foci, and tumor multiplicity (7, 8).

The interleukin-28B (*IL-28B*) gene, also known as IFN-λ3, is a newly described member of the family of IFN-related cytokines (9) and shares the same biologic properties as type I IFNs (10). Recently, several single-nucleotide polymorphisms (SNP) in the *IL-28B* locus have been associated with the effectiveness of pegylated-IFN and ribavirin combination therapy for CHC (11, 12). We previously confirmed this relationship and revealed that the *IL-28B* genotype is associated with the expression of hepatic IFN-stimulated genes (ISG) in patients with CHC (13). Others have also described an association between the *IL-28B* genotype and the outcome of CHC therapy, biochemical factors, and histologic findings (14, 15); however, the relationship between the *IL-28B* genotype and the biology and clinical course of HCC remains unknown. In this study,

Authors' Affiliations: Departments of [1]Gastroenterology and [2]Human Pathology, Kanazawa University Graduate School of Medical Science; [3]Department of Advanced Medical Technology, Kanazawa University Graduate School of Health Medicine, Kanazawa, Ishikawa; and [4]Third Department of Internal Medicine, Nihon University School of Medicine, Tokyo, Japan

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Corresponding Author: Shuichi Kaneko, Department of Gastroenterology, Kanazawa University Graduate School of Medical Science, 13-1 Takara-Machi, Kanazawa, Ishikawa 920-8641, Japan. Phone: 81-76-265-2235; Fax: 81-76-234-4250; E-mail: skaneko@m-kanazawa.jp

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Translational Relevance

Several single-nucleotide polymorphisms (SNP) in the interleukin-28B (*IL-28B*) locus have recently been shown to be associated with antiviral treatment efficacy in chronic hepatitis C (CHC). In this study, we investigated the association between the *IL-28B* genotype (rs8099917) and the biology and clinical outcome of patients with hepatocellular carcinoma (HCC) receiving curative treatment. Patients with the *IL-28B* TT genotype had a significantly higher incidence of HCC recurrence than patients with the TG/GG genotype. Gene expression profile and histologic analysis showed that the immune response and chronic hepatitis inflammation were more severe in patients with the TT genotype. Conversely, the expression of IFN-stimulated genes was upregulated and the immune response to tumors was more intense in those with the TG/GG genotype. These finding suggest that such molecular mechanisms may affect HCC recurrence.

therefore, we investigated the association between the *IL-28B* genotype and clinical outcome after initial curative treatment of HCC and clarified the molecular features in relation to the *IL-28B* genotype.

Materials and Methods

Patients

A total of 852 patients were admitted to the Department of Gastroenterology, Kanazawa University Hospital, Kanazawa, Japan between January 2000 and March 2012 for the treatment of developed HCC. The major background liver disease was hepatitis C virus (HCV; $n = 502$), followed by hepatitis B virus ($n = 148$). Treatment of HCC included surgical resection in 175 patients and RFA in 390 patients. The choice of treatment procedure was determined according to the extent of the tumor and the hepatic functional reserve as assessed by Child's classification that forms the Japanese HCC Guidelines (16, 17). In some cases indicated for surgical resection, we conducted RFA on patients who refused surgical resection, and we consequently excluded these patients on the basis of Japanese HCC guidelines.

Study inclusion criteria were: (i) Child–Pugh class A or B; (ii) the presence of up to 3 tumors, each 3 cm or less; (iii) HCV infection (positive for HCV RNA, patients with sustained viral response were excluded); (iv) radical treatment by either surgical resection or RFA; and (v) availability of blood samples for genetic analyses (Supplementary Fig. S1). Consequently, 183 patients were studied and their baseline characteristics are reported in Table 1. Informed consent was obtained from all patients before therapy. The experimental protocol was approved by the Human Genome, Gene Analysis Research Ethics Committee of Kanazawa University (Approval No. 260), and the study was conducted in accordance with the Declaration of Helsinki.

Diagnosis of HCC

HCC diagnosis was based predominantly on image analysis. Patients underwent dynamic computed tomography (CT) and/or dynamic MRI and abdominal angiography with CT imaging in the arterial and portal flow phase. HCC was diagnosed if a liver nodule showed hyperattenuation in the arterial phase and washout in the portal or delayed phase or showed typical hypervascular staining on digital subtraction angiography (18).

Table 1. Clinical features of 183 patients with HCC at entry by *IL-28B* genotype

Variables	*IL-28B* TT genotype ($n = 124$)	*IL-28B* TG/GG genotype ($n = 59$)	*P*
Sex (male:female)	76:48	32:27	0.422
Age, y (≤70:>70)	64:60	32:27	0.754
Platelet count ($\times 10^4$/mm^3; ≤10:>10)	68:56	28:31	0.429
ALT, IU/L (≤40:>40)	44:80	25:34	0.416
γ-GTP, IU/L (≤50:>50)	46:78	21:38	0.871
Albumin, g/dL (≤3.5:>3.5)	41:83	12:47	0.084
Protrombin activity, % (≤70:>70)	28:96	9:50	0.325
Total bilirubin, mg/dL (≤2:>2)	7:117	1:58	0.440
Child–Pugh class (A:B)	77:29	43:10	0.352
Therapy (resection: RFA)	19:105	10:49	0.830
Period of therapy (2000-05:2006-12)	41:83	21:38	0.741
History of IFN therapy (yes:no)	56:68	26:33	0.999
Tumor number (solitary: 2–3)	80:44	42:17	0.406
Tumor size, mm (≤20:>20)	83:41	36:23	0.508
AFP, ng/mL (≤20:>20)	60:64	37:22	0.082
DCP, AU/L (≤40:>40)	75:49	39:20	0.516

Method of treatment

Hepatic resection was carried out under intraoperative ultrasonographic monitoring and guidance. Anatomic resection was conducted in 9 patients and nonanatomic resection was conducted in 20 patients. Curative resection was defined as removal of all recognizable tumors with a clear margin (19). RFA was conducted using either the radiofrequency interstitial tumor ablation system (RITA; RITA Medical Systems Inc.) or the cool-tip system (Tyco Healthcare Group LP). All procedures were conducted according to the manufacturer's protocol. In the case of RFA, dynamic CT was conducted 1 to 3 days after therapy and the ablated area was evaluated. Complete ablation was defined as no enhancement in the ablated area on the dynamic CT. When complete ablation was not achieved, additional ablation was considered.

Follow-up

All patients were followed up by ultrasound and contrast enhancement 3-phase CT or MRI every 3 months. Local tumor progression was defined as the reappearance of tumor progression adjacent to the treated site and distant recurrence as the emergence of one or several tumor(s) not adjacent to the treated site. Patients with confirmed recurrence received further treatment such as resection, RFA, transarterial chemoembolization, and chemotherapy depending on the condition. Time to recurrence (TTR) was defined as the period from the date of therapy until the detection of tumor recurrence, death, or the last follow-up assessment. For TTR analysis, the data were censored for patients without signs of recurrence.

Genetic variation of the *IL-28B* polymorphism

Genomic DNA was extracted from peripheral blood samples using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. An *IL-28B* SNP (rs8099917) was determined using TaqMan Pre-Designed SNP Genotyping Assays as described previously (12). A custom assay was created by Applied Biosystems for rs12979860. We determined *IL-28B* genetic variations in all patients included in this study.

Affymetrix genechip analysis

Resected cancer and noncancerous liver tissue specimens were immediately frozen in liquid nitrogen and kept at −80°C until required for RNA preparation. Liver tissue RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Isolated RNA was stored at −70°C until required. The quality of isolated RNA was estimated after electrophoresis using an Agilent 2001 Bioanalyzer. Microarray analysis using an Affymetrix Human 133 Plus 2.0 microarray chip was conducted as described previously (13). The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at National Center for Biotechnology Information (NCBI, Bethesda, MD; accession number GSE41804).

Gene set enrichment analysis

Affymetrix GeneChip array data were normalized, preprocessed, and analyzed using R (20) and Bioconductor (21) software. Raw CEL file data were normalized using the MAS 5.0 algorithm as implemented in the *affy* package. Normalized data were $\log_2$ transformed and assessed using gene set enrichment analysis (GSEA), which is a bioinformatics method to assess whether genes with known biological/molecular function are concomitantly upregulated or downregulated in a certain gene expression dataset (22). GSEA was conducted using a parametric analysis of gene set enrichment (PAGE; ref. 23). The Gene Ontology gene set collection C5 of the Molecular Signatures Database (22) was downloaded from the Broad Institute and loaded into the R environment.

We also investigated the gene set differentially expressed HCC-infiltrating mononuclear inflammatory cells studied previously (24). *Z* scores and *P* values of all gene sets were calculated using the *PGSEA* package and an estimate was made as to whether certain gene sets, and therefore functional gene categories, were differentially regulated in HCC tissue from patients with the *IL-28B* TT genotype and the *IL-28B* TG/GG genotype.

Hierarchical clustering

Hierarchical clustering was conducted with Cluster software using Pearson's correlation distance metric and average linkage followed by visualization in Treeview software.

Histologic liver analysis

Noncancerous liver tissue that had been surgically resected from patients with HCC and liver specimens obtained by needle biopsy from the background liver of patients with HCC were fixed in 10% buffered formalin and embedded in paraffin. Each paraffin-embedded specimen was sliced into 3 to 4 μm sections and stained with hematoxylin and eosin. Each specimen was semiquantitatively analyzed by assigning a score according to each of the following features: (i) severity of inflammatory cell infiltration (0 for none, 1 for minimal, 2 for mild, 3 for moderate, and 4 for severe) in the periportal, intralobular, and portal areas; (ii) the severity of the F stage of fibrosis (0 for F0, 1 for F1, 2 for F2, 3 for F3, and 4 for F4; ref. 25); the degree of lymphoid aggregates in the portal area (0 for none, 1 for mild, 2 for scattered, 3 for clustered, 4 for lymph follicle without germinal center, and 5 for lymph follicle with germinal center); the severity of portal sclerotic change, perivenular fibrosis, and pericellular fibrosis (on a scale of 0–4 with 0 for none to 4 for severe); the severity of damage to the bile duct (on a scale of 0–4 with 0 for none to 4 for disappearance); the existence of bridging necrosis (0 for none and 1 for existence); the severity of irregular regeneration of hepatocytes as described previously (on a scale of 0–4 with 0 for none to 4 for severe; ref. 26); the grade of steatosis (on a scale of 0–4 with 0 for none to 4 for severe). The irregular regeneration score was based on the findings of a map-like distribution, anisocytosis, and pleomorphism

of the hepatocytes, bulging of the regenerated hepatocytes and proliferation of atypical hepatocytes and oncocytes.

Immunohistochemistry

Paraffin-embedded specimens were sliced into 3 to 4 μm sections, deparaffinized, and subjected to heat-induced epitope retrieval at 98°C for 40 minutes. After blocking endogenous peroxidase activity using 3% hydrogen peroxide, the slide was incubated with appropriately diluted primary antibodies. Antihuman CD4, antihuman CD8 and antihuman CD14 mouse monoclonal antibodies were used to evaluate the immunoreactivity of HCC using a DAKO EnVision+TM kit, as described in the manufacturer's instructions.

We semiquantitatively analyzed tumor tissues by assigning a score to the severity of CD4-positive and CD8-positive lymphocyte infiltration in the tumor tissue (0 for none, 1 for mild, 2 for moderate, and 3 for severe).

Statistical analysis

Fisher exact probability test was used to compare categorical variables and the Mann–Whitney *U* test was used to compare continuous variables; a *P* value of less than 0.05 was considered statistically significant. The TTR survival curve was analyzed using the Kaplan–Meier curve and compared by the log-rank test. Univariate Cox regression analysis was conducted to identify TTR predictors out of clinical and biologic parameters [sex, age, *IL-28B* genotype, therapy, platelet count, alanine aminotransferase (ALT), γ-GTP, albumin, prothrombin activity, bilirubin, Child–Pugh class, history of IFN therapy, AFP, and des-γ-carboxy prothrombin (DCP)] and tumor factors (size and number).

Multivariate analysis was conducted using the Cox regression model with backward elimination (27). The significance level for removing a factor from the model was set to 0.05. A bootstrap technique was applied to confirm the choice of variables (27). One thousand bootstrap samples were generated using resampling with replacement and Cox regression analysis with backward elimination was applied to each sample. The percentage of samples (from the total of 1,000) for which each variable was included in the model was calculated. In multivariate analysis, we evaluated two models that contained either Child–Pugh class or its components to avoid multicollinearity. Data analysis was conducted with R software. We used functions from the Regression Modeling Strategies library for validation with the bootstrap technique (28).

Results

Patient characteristics and *IL-28B* genotype frequency

We genotyped 183 patients with HCC for the *IL-28B* rs8099917 TT, TG, and GG genotypes and observed respective proportions of 67.8% (124 of 183), 30.6% (56 of 183), and 1.6% (3 of 183), which is a similar distribution to that observed in several Japanese studies of patients with CHC (13, 14, 29, 30). Although the prevalence of the TG/GG genotype was higher than that of the general population (12%–16%; refs. 12, 31, 32), there was no significant difference between our result and that of HCV-infected patients in a previous study. There was also no significant difference in clinical variables between the TT and TG/GG genotypes (Table 1).

We next genotyped 160 of 183 cases for rs12979860 and our findings were largely in concordance with those of rs8099917, with the exception of 1 case (0.6%). The haplotype of the case showed that rs8099917 was TT and rs12979860 was CT suggesting that these 2 loci are in a haplotype block with a high level of linkage disequilibrium, as previously reported (13, 30). Genotype distribution analysis showed that rs8099917 was in Hardy–Weinberg equilibrium, so we selected it for further investigation.

During the median follow-up period of 2.5 years (range, 0.3–7.2 years), 118 of 183 patients developed HCC recurrence. Local tumor progression was seen in 13 patients treated by RFA and in only 1 patient treated by resection. The local tumor progression rate and distant recurrence rate were 2.6% and 21.2% in the first year and 8.3% and 54.2% within 2.5 years, respectively. These results are comparable with previous reports by others (33, 34). The type of recurrence was also comparable between *IL-28B* genotype groups.

Associations between the *IL-28B* genotype and HCC clinical outcome

HCC TTR was also analyzed using multivariate Cox regression analysis using 15 clinical parameters and the *IL-28B* genotype. With a significance level of 0.05 for removing a variable in a Cox regression with backward elimination, the *IL-28B* genotype was selected as the final model (Table 2). To confirm this decision, a bootstrapping technique was applied. The percentages of inclusion among the 1,000 samples created by the bootstrapping technique for variables are shown in Table 2. The percentage of inclusion for the *IL-28B* genotype was 80.4%. Frequencies of another variable were lower than 40%. The bootstrap procedure result confirmed the variables chosen for the final model.

In univariate Cox regression analyses, the *IL-28B* genotype was associated with HCC recurrence (Table 2). The TTR survival curve was analyzed using the Kaplan–Meier curve and log-rank test (Fig. 1), and patients with the *IL-28B* TT genotype showed a significantly shorter median TTR (1.61 years) than those with the *IL-28B* TG/GG genotype (2.58 years; $P = 0.007$).

Histologic analysis of noncancerous liver tissues of *IL-28B* TT and TG/GG genotypes

To clarify the molecular mechanism influencing HCC recurrence, we histologically analyzed 141 noncancerous liver tissues according to previously published criteria (Table 3; ref. 26). The mean score of the degree of inflammatory cell infiltration in the periportal area was significantly higher in TT genotype patients (2.804) than TG/GG genotype patients (2.513; $P = 0.025$); the degree of inflammatory cell infiltration in the intralobular area was also

Table 2. Cox regression analysis and relative frequency of variables inclusion with $P < 0.05$ (in 1,000 bootstrap samples)

Variables	Univariate		Multivariate		Frequency (%)
	HR (95% CI)	*P*	HR (95%CI)	*P*	
IL-28B allele: major vs. minor	2.674 (1.161–2.627)	0.007	2.674 (1.161–2.627)	0.007	80.4
Tumor size, mm: >20 vs. ≤20	1.303 (0.881–1.880)	0.193			39.8
AFP, ng/mL: >20 vs. ≤20	1.674 (0.948–1.968)	0.094			33.2
γ-GTP, IU/L: >50 vs. ≤50	1.188 (0.865–1.804)	0.235			32.8
Therapy: RFA vs. resection	1.218 (0.826–2.266)	0.223			31.6
DCP, AU/L: >40 vs. ≤40	1.524 (0.920–1.945)	0.127			27.4
ALT, IU/L: >40 vs. ≤40	0.277 (0.721–1.544)	0.782			23.6
Child–Pugh class: A vs. B	0.025 (0.653–1.515)	0.980			19.2
Period of therapy: 2000-05 vs. 2006-12	0.886 (0.818–1.701)	0.375			15.8
History of IFN therapy: yes vs. no	0.570 (0.771–1.605)	0.569			15.8
Sex: male vs. female	0.108 (0.697–1.496)	0.914			14.6
Tumor number: solitary vs. 2-3	0.263 (0.845–1.857)	0.263			13.4
Platelet count ($\times 10^4/mm^3$): >10 vs. ≤10	0.118 (0.680–1.407)	0.906			12.6
Age: per 1 y	0.621 (0.986–1.028)	0.534			8.4

higher in the TT genotype (2.522) than the TG/GG genotype (2.308), although this did not reach statistical significance ($P = 0.08$). Furthermore, the mean score of the degree of hepatocyte anisocytosis was significantly higher in the TT genotype (1.891) than the TG/GG genotype (1.385; $P = 0.024$). Anisocytosis is characterized by viability of cell size with focal dysplastic change and indicates irregular regeneration of hepatocytes. The irregular regeneration score was higher in the TT genotype (2.207) than the TG/GG genotype (1.795), albeit not significantly ($P = 0.105$).

IL-28B TT and TG/GG genotype gene expression profiles in the noncancerous liver

We next compared the gene expression profile of HCC tissues and noncancerous liver tissues of both the *IL-28B* TT and *IL-28B* TG/GG genotype. Ten patients with HCC were selected from each *IL-28B* genotype and their gene expression was determined using Affymetrix genechip analysis (Supplementary Table S1). We recently reported that expression of hepatic ISGs is downregulated in individuals with the *IL-28B* TT genotype, whereas the expression of other immune response-related genes was shown to be upregulated (13). Therefore, to validate our expression data, we compared the expression of ISGs and other immune response-related genes in the present study with that of the previous study. We analyzed the expression data of 20 patients from the current study in addition to another series of 91 patients with CHC from our previous study.

One-way hierarchical clustering using 28 representative ISGs showed that patients with the *IL-28B* TG/GG genotype

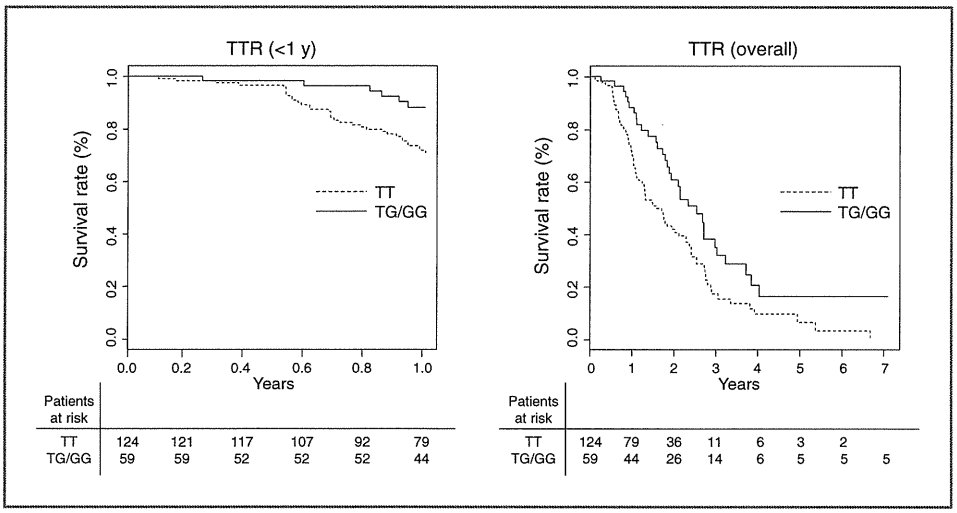

Figure 1. Kaplan–Meier curves of early and overall TTR in relation to *IL-28B* genotype. The patients with the *IL-28B* TT genotype showed a significantly shorter median TTR (1.61 years) than those with the *IL-28B* TG/GG genotype (2.58 years; $P = 0.007$).

Table 3. Comparison of liver histology between *IL-28B* major and minor genotypes

Variable	*IL-28B* TT genotype ($n = 92$)	*IL-28* TG/GG genotype ($n = 39$)	*P* value
Score of inflammatory cell infiltration			
Periportal	2.804	2.513	0.032
Intralobular	2.522	2.308	0.082
Portal	2.946	2.846	0.322
Fibrosis	3.587	3.436	0.428
Portal lymphoid reaction	4.098	3.949	0.363
Damage of bile duct	0.380	0.256	0.216
Portal sclerotic change	0.076	0.077	0.990
Perivenular fibrosis	1.133	1.000	0.447
Pericellular fibrosis	1.163	0.821	0.045
Bridging fibrosis	0.957	0.641	0.106
Irregular regeneration	2.207	1.795	0.105
Anisocytosis	1.891	1.385	0.024
Bulging	0.326	0.436	0.485
Map-like distribution	1.370	1.333	0.881
Oncocytes	1.326	1.051	0.227
Nodularity	1.185	1.231	0.849
Atypical hepatocytes	0.467	0.692	0.304
Steatosis	1.707	1.692	0.951

NOTE: Data shown as mean.

had higher expression of hepatic ISGs, whereas patients with the TT genotype showed lower expression of hepatic ISGs in CHC tissues and noncancerous background liver tissue, confirming our previous data (Fig. 2A and Supplementary Table S2). Expression of hepatic ISGs in HCC tissues was lower than in background liver tissues, with no relationship to the *IL-28B* genotype. Hierarchical clustering of 51 representative immune response-related genes from the Gene Ontology gene set of the Molecular Signatures Database indicated that their expression was upregulated in TT genotype compared with TG/GG genotype tissues, with the exception of HCC tissues (Fig. 2B and Supplementary Table S2). Upregulation of immune response-related genes suggests that hepatic inflammation is more severe in TT genotype patients, which is consistent with our histologic findings and recent studies that reported an association between high serum ALT levels and the *IL-28B* TT genotype (14, 29).

Gene expression profile of HCC tissues from *IL-28B* TT and TG/GG genotypes

We applied PAGE to identify gene sets differentially regulated between the different *IL-28B* genotypes from the whole gene expression profiles derived from HCC tissues. Analysis of groups of genes involved in a specific function enables significant differences to represent a biologically meaningful result (23). Many gene sets associated with the immune system (e.g., the immune system process, T-cell activation, regulation of T-cell activation, and T-cell proliferation) showed a significant increase in their expression in patients with HCC with the *IL-28B* TG/GG genotype (Supplementary Table S3). This PAGE profile was consistent with the hierarchical clustering of 51 immune response-related genes (Fig. 2B) and suggests that the immune response to tumors might be more intensive in *IL-28B* TG/GG genotype HCC than *IL-28B* TT genotype HCC.

Lymphocyte infiltration into HCC tissues with the *IL-28B* TG/GG genotype

To verify our PAGE profile, we histologically compared HCC tissue of 20 cases of the *IL-28B* TT genotype and 12 cases of the TG/GG genotype using immunohistochemical staining with antibodies against helper T cells (CD4) and cytotoxic T cells (CD8). The mean score of the degree of $CD8^+$ lymphocyte infiltration in the tumor tissue was significantly higher in the TG/GG genotype (1.75) than the TT genotype (1.175; $P = 0.047$; Supplementary Table S4). A representative case is shown in Fig. 3. There was no morphologic alteration associated with the *IL-28B* genotype. Immunohistochemical analysis showed intratumoral infiltration of $CD4^+$ and $CD8^+$ lymphoid cells and slight infiltration of monocytes/macrophages in HCC of the *IL-28B* TG/GG genotype, compared with little infiltration of lymphocytes or monocytes/macrophages in HCC of the *IL-28B* TT genotype.

Furthermore, the gene set differentially expressed in HCC-infiltrating mononuclear inflammatory cells from our previous study (24) was upregulated in HCC of the *IL-28B* TG/GG genotype (Z score, -9.879; $P < 0.001$). One-way hierarchical clustering was carried out of 122 genes involved in the gene set differentially expressed in HCC-infiltrating

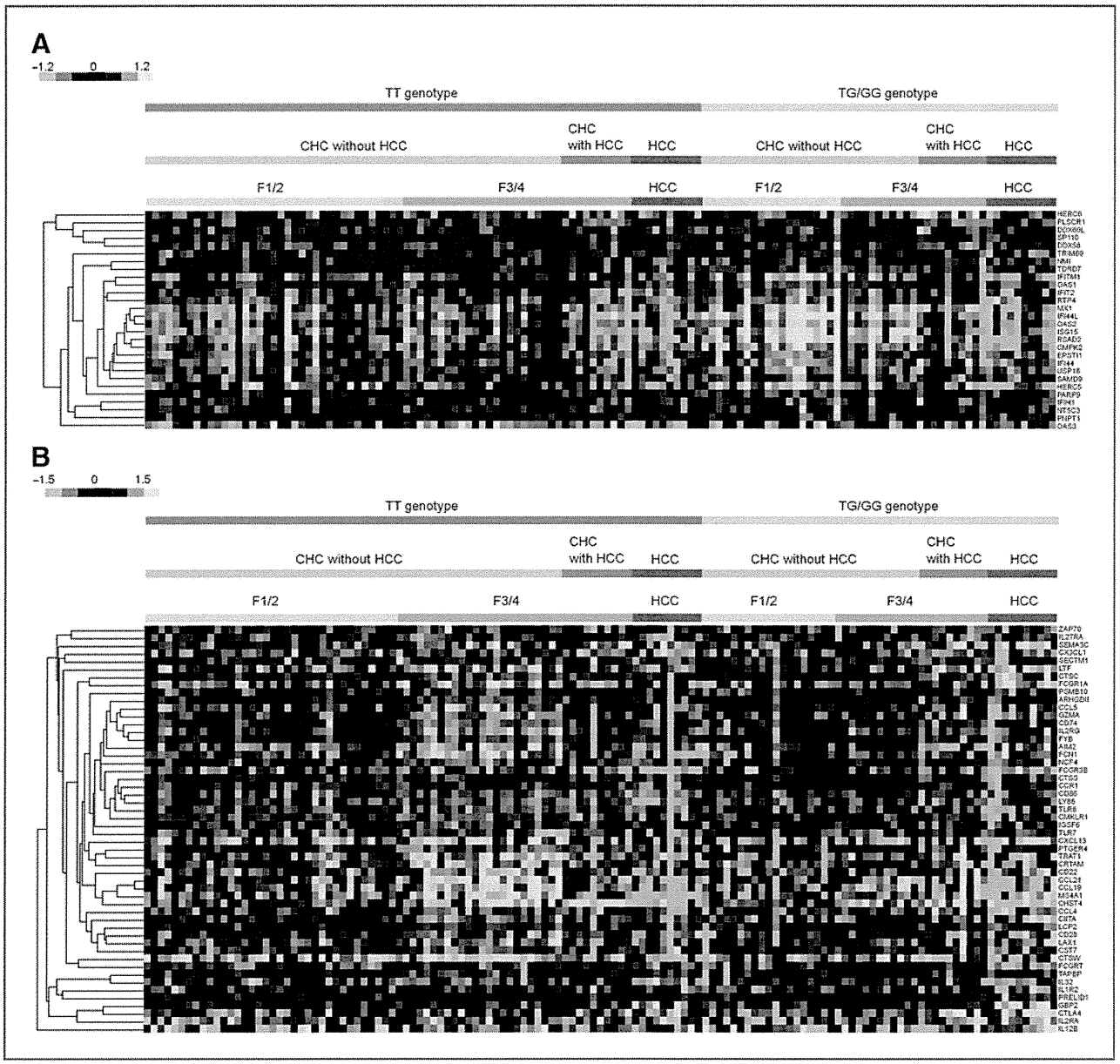

Figure 2. A, one-way hierarchical clustering of 28 representative ISGs of 111 patients with the *IL-28B* genotype. B, one-way hierarchical clustering analysis of 51 representative immune response-related genes of 111 patients with the *IL-28B* genotype.

mononuclear inflammatory cells. Most of the 122 genes were expressed at high levels in many HCC tissues of *IL-28B* TG/GG genotype patients (Supplementary Fig. S2).

Discussion

IL-28B is a recently identified, novel IFN-λ family member that shares the same biologic properties as type I IFNs (9). Recent reports have shown a significant association between *IL-28B* allelic variants and treatment outcome in CHC (11, 12). *IL-28B* genotyping is therefore considered to be a suitable pretreatment predictor of treatment response for individual patients, and also an indicator of biochemical and histologic findings in patients infected with HCV (14). In this study, we determined that the *IL-28B* genotype is associated with HCC recurrence in patients with CHC as patients with the *IL-28B* TT genotype showed a significantly higher incidence of recurrence than those with the *IL-28B* TG/GG genotype after curative therapy. To our knowledge, this is the first study to reveal an association between the *IL-28B* genotype and HCC recurrence and molecular features in patients with CHC.

To date, there are several contradicting results about the association of the *IL-28B* genotype and progression of liver disease including the development of HCC. Fabris and colleagues and Eurich and colleagues reported that

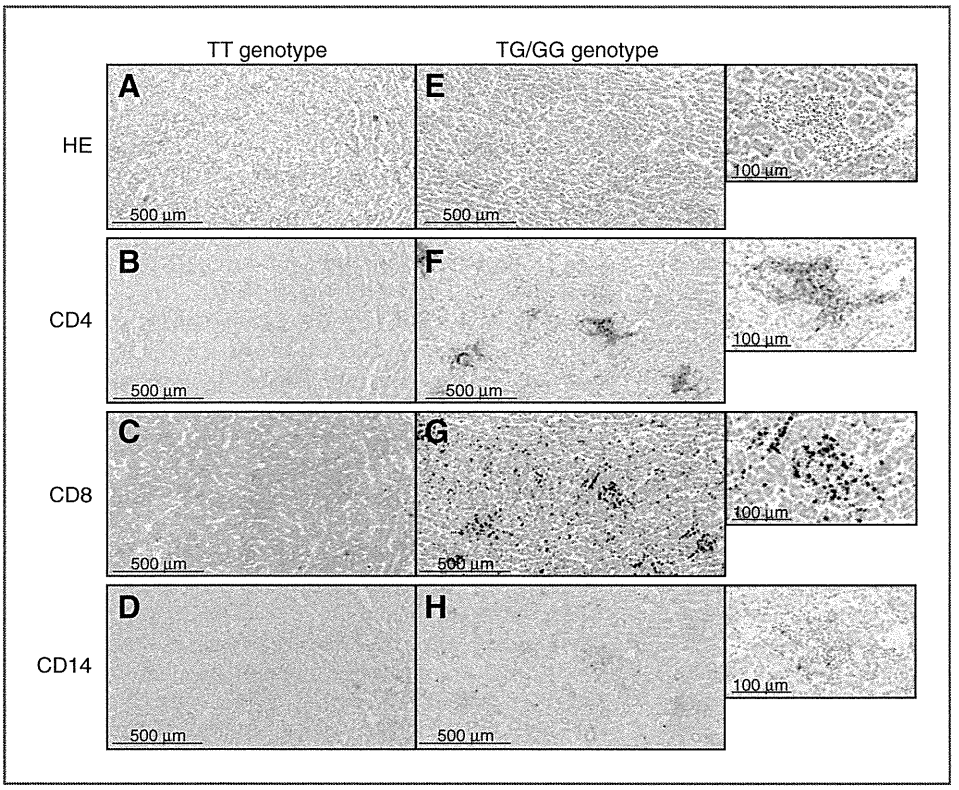

Figure 3. Expression of CD4, CD8, and CD14 in tumor-infiltrating mononuclear cells in HCC tissues. Immunohistochemical analysis of noncancerous liver tissues of *IL-28B* TT (A–D) and TG/GG genotypes (E–H). Samples were analyzed by hematoxylin and eosin staining (A and E), CD4 staining (B and F), CD8 staining (C and G), and CD14 staining (D and H).

patients with a T allele in rs12979860 (G allele in rs8099917) were at a high risk of progressing to liver cirrhosis and HCC (35, 36), however, these reports have not yet been confirmed by others. A large-scale European genome-wide association study (GWAS) recently identified a weak protective role for the rs12979860 T allele in the progression of fibrosis during HCV infection (37), whereas a Japanese GWAS identifying a susceptibility locus for HCV-induced HCC found no association of rs12979860 and rs8099917 SNPs with HCC (38). In support of these findings, Joshita and colleagues reported no association between the *IL-28B* genotype and the incidence of primary HCC (39). These results show a good concordance with those of the present study, which revealed that the *IL-28B* genotype was not associated with HCC incidence before treatment (Table 1). Furthermore, closer histologic assessment showed a high score of periportal inflammation and pericellular fibrosis in the rs8099917 TT genotype (CC in rs12979860). This suggests that our patient selection process was not biased, and that our results are in agreement with the Japanese study and are comparable with the European study.

To date, the reasons for contradicting results about the association of the *IL-28B* genotype and progression of liver disease have not been clear, however, clinical bias such as patient number, history of treatment, virus genotype, and titer and racial differences may affect the results. It should be noted that significant differences in genotype frequencies with respect to ethnic/racial groups have previously been reported for *IL-28B* SNPs (11). To overcome these limitations, a future cross-sectional prospective study should be conducted.

Several risk factors for HCC recurrence have previously been reported, including the presence of cirrhosis, high AFP levels, and multiplicity of tumors 7, 8). However, multivariate analysis and the bootstrap procedure of the present study revealed that the *IL-28B* genotype was independent indicators for recurrence, suggesting that it is stronger predictors of HCC recurrence than other factors.

The expression of hepatic ISGs was higher in *IL-28B* TG/GG genotype patients than *IL-28B* TT genotype patients with CHC in this study. This confirms our previous findings in a different cohort and those of another research group (13, 40). Several ISGs have been reported to have antiproliferative and proapoptotic functions (41, 42), and IFN-α (type I IFN) has also been found to inhibit metastasis and human HCC recurrence after curative resection mediated by angiogenesis (43). Indeed, *IL-28B* rs8099917 is associated with early HCC recurrence (<1 year), possibly because of the intrahepatic metastasis of HCC in this study (Fig. 1 and Supplementary Table S5). These reports and our findings suggest that high expression of hepatic ISGs might cause the low HCC recurrence in the *IL-28B* TG/GG genotype, although the mechanism of this association remains unknown.

Microarray, histologic, and immunohistochemical analysis in the present study showed that the immune response was more severe in chronic hepatitis and noncancerous tissue of *IL-28B* TT genotype compared with TG/GG genotype patients. Serum ALT levels were also higher in the

IL-28B TT genotype, albeit not significantly. These results support previous findings that showed higher serum ALT levels and more severe liver inflammation in TT genotype compared with TG/GG genotype patients with HCC (14, 29). Irregular regeneration of hepatocytes develops as a result of repeated cycles of necrosis and regeneration of hepatocytes and was previously reported to be an important predictive factor for the development of HCC (26). We histologically showed that the degree of hepatocyte anisocytosis was more severe in noncancerous livers of TT genotype than TG/GG genotype patients, perhaps because of *IL-28B* genotype-dependent hepatic inflammation. This might also affect the late recurrence of HCC (>1 year) as a result of the multicentric occurrence of HCC in background liver disease. In the late recurrence group, *IL-28B* TT genotype patients showed a shorter TTR than *IL-28B* TG/GG genotype patients although this did not reach statistical significance ($P = 0.086$; Supplementary Fig. S3; Supplementary Table S6).

Previous studies showed that the gene expression profile of noncancerous liver tissue was associated with late recurrence HCC and the multicentric occurrence of HCC (44). However, the gene set expression of these studies did not differ between the *IL-28B* TT and TG/GG genotypes in the present study. Although the reason for this discrepancy is unclear, the *IL-28B* genotype may affect early recurrence more than late recurrence, and the limited number of patients and the short follow-up period may affect statistical comparisons. Therefore, further investigations with a large series of patients are necessary to clarify whether *IL-28B* genotype-dependent inflammation influences HCC recurrence.

On the other hand, the gene expression profile and histologic analyses showed that more lymphocytes infiltrate into the tumor tissue of the *IL-28B* TG/GG genotype than the TT genotype. Chew and colleagues previously showed that 14 intratumoral immune gene signatures were able to identify molecular cues driving the tumor infiltration of lymphocytes and predict the survival of patients with HCC, particularly during the early stages of disease (45). We can confirm that the expression of some of these 14 genes was higher in TG/GG genotype than TT genotype patients (Supplementary Fig. S4), supporting the association of the *IL-28B* genotype, HCC recurrence, and histologic findings. The presence of lymphocyte infiltration in HCC was also reported as a negative predictor of HCC recurrence after liver transplantation (46), and this phenomenon may contribute to a lower incidence of HCC recurrence in the TG/GG genotype.

It may seem contradictory that the immune response in noncancerous liver was more severe in TT genotype than TG/GG genotype patients despite the fact that the expression of immune genes was higher in tumor tissue and more lymphocytes infiltrated the tumor in the TG/GG genotype compared with the TT genotype. Although we are unable to explain this contradiction, it is conceivable that the host immune reaction has a differential role between tumor and nontumor tissue. Moreover, HCV-specific T-cell immune responses, which are essential for disease control, are attenuated in patients with CHC, and T-cell exhaustion has recently been implicated in the deficient control of chronic viral infections. On the other hand, little is known on self- and tumor-specific T-cell responses in patients with HCC. While several reports have shown the existence of exhausted T cells in a tumor environment, impaired T-cell responses to tumors are unlikely to be simply explained by T-cell exhaustion (47).

Anergy or other functional statuses such as suppressive immunity by tumor cells should be considered in tumor immunity. Therefore, differences in immunity to viral antigens and self- and tumor-antigens could explain our findings, although further work should be carried out to confirm these conclusions. We have preliminarily confirmed that the ratio of regulatory T cells is higher in the peripheral blood of *IL-28B* TT genotype HCC patients than *IL-28B* TG/GG genotype patients, although there is no significant difference between non-HCC *IL-28B* TT genotype and *IL-28B* TG/GG genotype patients (data not shown). Although the cause of this phenomenon is unclear, our gene expression profile of noncancerous liver and tumor tissues suggests paradoxical roles for the immune response in CHC and HCC depending on *IL-28B* genotype; it will be necessary to clarify these mechanisms in future investigations.

Recently, a sustained virologic response (SVR) to CHC antiviral treatment was shown to be associated with a lower risk of HCC recurrence (48). Although we did not include patients with SVR in the current study, we nevertheless observed that they showed a longer recurrence-free survival than patients infected with HCV, independent of *IL-28B* genotype (data not shown). This result together with the association between the *IL-28B* genotype and response to antiviral treatment promotes recommendations for aggressive CHC antiviral treatment, especially in cases with the *IL-28B* TT genotype.

RFA is a recently developed technique and its efficacy has been reported equal to that of surgical resection, especially in early-stage HCC (3–6). In the European Association for the Study of the Liver–European Organisation for Research and Treatment of Cancer (EASL-EORTC) guidelines, RFA is considered the standard care for patients with Barcelona Clinic Liver Cancer stage 0-A tumors not suitable for surgery and whether or not RFA can be considered a competitive alternative to resection is uncertain (49). In our study, the local tumor progression rate was not statistically different between RFA and resection cases. However, further studies with an appropriate sample population are necessary to clarify the comparison of RFA and resection. The present study has some limitations. It was a retrospective cohort and a single-center study, so it was difficult to completely eliminate bias. Further prospective studies of a larger series of patients should be conducted to validate our results. As a consequence of the small sample size and even smaller number of patients undergoing surgical resection, we could not show an association between *IL-28B* genotype and HCC

recurrence in the surgical resection group (data not shown). However, we did find no significant difference in TTR between RFA and surgical resection, confirming previous findings.

In conclusion, we found that the *IL-28B* rs8099917 TT genotype is associated with shorter TTR in patients with HCC with CHC. Microarray analysis showed a high expression of ISGs in background liver and high expression of immune system-related genes in tumor tissues of the *IL-28B* TG/GG genotype. Histologic findings also showed that more lymphocytes infiltrated into tumor tissues in the TG/GG genotype. The *IL-28B* genotype therefore is a candidate useful genetic marker to predict HCC recurrence as well as the response to pegylated-IFN and ribavirin combination therapy for CHC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Honda, Y. Sakai, M. Moriyama, S. Kaneko
Development of methodology: M. Moriyama
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Hodo, A. Tanaka, K. Arai, T. Yamashita, T. Yamashita, A. Sakai, Y. Nakanuma, M. Moriyama, S. Kaneko
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Hodo, M. Honda, M. Sasaki, M. Moriyama
Writing, review, and/or revision of the manuscript: Y. Hodo, M. Honda, M. Moriyama
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Hodo, A. Tanaka, Y. Nomura, K. Arai, E. Mizukoshi, M. Sasaki, M. Moriyama, S. Kaneko
Study supervision: Y. Sakai, E. Mizukoshi, M. Moriyama

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