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Comparative Analysis of Various Tumor-Associated Antigen-Specific T-Cell Responses in Patients with Hepatocellular Carcinoma

Eishiro Mizukoshi, Yasunari Nakamoto, Kuniaki Arai, Tatsuya Yamashita, Akito Sakai, Yoshio Sakai, Takashi Kagaya, Taro Yamashita, Masao Honda, and Shuichi Kaneko

Many tumor-associated antigens (TAAs) recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials. However, there are very few in the field of immunotherapy for hepatocellular carcinoma (HCC) because there have not been comparative data regarding CTL responses to various TAAs. In the present study, using 27 peptides derived from 14 different TAAs, we performed comparative analysis of various TAA-specific T-cell responses in 31 HCC patients to select useful antigens for immunotherapy and examined the factors that affect the immune responses to determine a strategy for more effective therapy. Twenty-four of 31 (77.4%) HCC patients showed positive responses to at least one TAA-derived peptide in enzyme-linked immunospot assay. The TAAs consisting of cyclophilin B, squamous cell carcinoma antigen recognized by T cells (SART) 2, SART3, p53, multidrug resistance-associated protein (MRP) 3, alpha-fetoprotein (AFP) and human telomerase reverse transcriptase (hTERT) were frequently recognized by T cells and these TAA-derived peptides were capable of generating peptide-specific CTLs in HCC patients, which suggested that these TAAs are immunogenic. HCC treatments enhanced TAA-specific immune responses with an increased number of memory T cells and induced *de novo* T-cell responses to lymphocyte-specific protein tyrosine kinase, human epidermal growth factor receptor type 2, p53, and hTERT. Blocking cytotoxic T-lymphocyte antigen-4 (CTLA-4) resulted in unmasking of TAA-specific immune responses by changing cytokine and chemokine profiles of peripheral blood mononuclear cells stimulated by TAA-derived peptides. *Conclusion:* Cyclophilin B, SART2, SART3, p53, MRP3, AFP, and hTERT were immunogenic targets for HCC immunotherapy. TAA-specific immunotherapy combined with HCC treatments and anti-CTLA-4 antibody has the possibility to produce stronger tumor-specific immune responses. (HEPATOLOGY 2011;53:1206-1216)

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and becoming an important public health concern.[1,2] Although many kinds of treatments have been performed for HCC, their effects are limited because the recurrence rate of HCC is very high; therefore, the development of new therapeutic options to prevent recurrence is necessary.[3,4]

To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Many tumor-associated antigens (TAAs) and their epitopes recognized by cytotoxic T cells (CTLs) have been identified during the last two decades and some of them have been used in clinical trials for several cancers.[5-21] The epitopes have been under investigation for the treatment of cancer, with major clinical responses in some trials.[22,23] With regard to immunotherapy for HCC, few kinds of TAAs and their epitopes have been used and only clinical data of α-fetoprotein (AFP) have been reported.[24,25] In human trials targeting AFP, it is possible to raise an AFP-specific T-cell response using AFP-derived peptides, but this has shown little

Abbreviations: AFP, alpha-fetoprotein; CTL, cytotoxic T cell; ELISPOT, enzyme-linked immunospot; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HLA, human leukocyte antigen; hTERT, human telomerase reverse transcriptase; IFN, interferon; Lck, lymphocyte-specific protein tyrosine kinase; MRP, multidrug resistance-associated protein; PBMC, peripheral blood mononuclear cell; TAA, tumor-associated antigen.

From the Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan.

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Address reprint requests to: Shuichi Kaneko, M.D., Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-8641, Japan. E-mail: skaneko@m-kanazawa.jp; fax: 81-76-234-4250.

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antitumor effect. On the other hand, immunotherapy trials using autologous tumor lysate or dendritic cells have shown statistically significant improvements in the risk of HCC recurrence and recurrence-free survival.[26] These reports suggest that tumor antigen-specific immunotherapy is effective to reduce the recurrence rate after HCC treatment; therefore, it is necessary to find immunogenic antigens or their epitopes to develop more effective immunotherapy.

In addition, in the field of molecular targeting therapies, developments of monoclonal antibodies targeting immunomodulatory molecules to enhance antitumor immunity are progressing and some of these are under clinical trial.[27] In particular, clinical data of anti-cytotoxic T-lymphocyte antigen-4 (anti-CTLA-4) antibody have shown durable objective response and stable disease in melanoma patients.[28]

In the present study we performed comparative analysis of various TAA-specific T-cell responses in patients with HCC and examined the factors that affect the immune responses, including anti-CTLA-4 antibody. This approach offers useful information to select immunogenic TAAs and to develop a new strategy for HCC immunotherapy.

Patients and Methods

Patients and Laboratory Testing. In this study we examined 31 human leukocyte antigen (HLA)-A24-positive patients with HCC, 29 chronic hepatitis C patients without HCC, who were diagnosed by liver biopsy, and 11 healthy blood donors who did not have a history of cancer and were negative for hepatitis B surface antigen and anti-hepatitis C virus (HCV) antibody (Ab). The diagnosis of HCC was histologically confirmed in 21 patients. For the remaining 10 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 computed tomography (CT).[29]

HLA-based typing of peripheral blood mononuclear cells (PBMCs) from patients and normal blood donors was performed as described.[19] The pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer.[30] The severity of liver disease was evaluated according to the criteria of Desmet et al.[31] using biopsy specimens of liver tissue.

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, No. 829).

Table 1. Peptides

Peptide No.	Peptide Name	Source	Reference	Amino Acid Sequence	Number of Specific Spots in Normal Donors (Mean SD)
1	$ART1_{188}$	ART1	5	EYCLKFTKL	0.9 ± 1.1
2	$ART4_{161}$	ART4	6	AFLRHAAL	0.3 ± 0.5
3	$ART4_{899}$	ART4	6	DYPSLSATDI	0.6 ± 1.0
4	$Cyp\text{-}B_{109}$	Cyp-B	7	KFHRVIKDF	0.5 ± 0.9
5	$Cyp\text{-}B_{315}$	Cyp-B	7	DFMIQGGDF	1.2 ± 1.7
6	Lck_{208}	Lck	8	HYTNASDGL	0.3 ± 0.6
7	Lck_{486}	Lck	8	TFDYLRSVL	0.2 ± 0.8
8	Lck_{488}	Lck	8	DYLRSVLEDF	0.9 ± 1.5
9	$MAGE1_{135}$	MAGE-A1	9	NYKHCFPEI	1.0 ± 0.9
10	$MAGE3_{195}$	MAGE-A3	10	IMPKAGLLI	1.4 ± 1.7
11	$SART1_{1690}$	SART1	11	EYRGFTQDF	0.9 ± 1.3
12	$SART2_{899}$	SART2	12	SYTRLFLIL	1.0 ± 1.4
13	$SART3_{109}$	SART3	13	VYDYNCHVDL	2.1 ± 1.9
14	$Her\text{-}2/neu_8$	Her-2/neu	14	RWGLLLALL	1.4 ± 2.0
15	$p53_{125}$	p53	15	TYSPALNKMF	1.4 ± 1.5
16	$p53_{161}$	p53	16	AIYKQSQHM	0.4 ± 0.6
17	$p53_{204}$	p53	17	EYLDDRNTF	1.1 ± 1.5
18	$p53_{211}$	p53	17	TFRHSVVV	0.9 ± 1.9
19	$p53_{235}$	p53	17	NYMCNSSCM	2.1 ± 2.6
20	$MRP3_{503}$	MRP3	18	LYAWEPSFL	0.2 ± 0.5
21	$MRP3_{692}$	MRP3	18	AYVPQQAWI	1.5 ± 2.1
22	$MRP3_{765}$	MRP3	18	VYSDADIFL	0.9 ± 1.0
23	AFP_{357}	AFP	19	EYSRRHPQL	1.8 ± 2.0
24	AFP_{403}	AFP	19	KYIQESQAL	1.1 ± 1.5
25	AFP_{434}	AFP	19	AYTKKAPQL	0.8 ± 1.1
26	$hTERT_{167}$	hTERT	20	AYQVCGPPL	0.8 ± 1.1
27	$hTERT_{324}$	hTERT	20	VYAETKHFL	0.5 ± 0.7
28	HIV env_{584}	HIV env	32	RYLRDQQLL	1.3 ± 2.0
29	HCV $NS3_{1031}$	HCV NS3	33	AYSQQTRGL	ND
30	CMV $pp65_{328}$	CMV pp65	34	QYDPVAALF	13.3 ± 15.7

ND, not determined.

Peptides, Cell Lines, and Preparation of PBMCs. Twenty-seven peptides derived from 14 different TAAs (Table 1), human immunodeficiency virus (HIV) envelope-derived peptide ($HIVenv_{584}$),[32] HCV NS3-derived peptide ($HCVNS3_{1031}$),[33] and cytomegalovirus (CMV) pp65-derived peptide ($CMVpp65_{328}$),[34] which were identified as HLA-A24 restricted CTL epitopes in previous studies, were used. Peptides were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry and their purities were determined to be >80% by analytical high-performance liquid chromatography (HPLC). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 500 μg/mL hygromycin B (Sigma, St. Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS.[35] PBMCs were isolated before HCC treatments as described.[20] In 12 patients their PBMCs were also obtained 4 weeks after treatments.

Table 2. Characteristics of the Patients Studied

Clinical Diagnosis	No. of Patients	Sex M/F	Age (yr) Mean ± SD	ALT (IU/L) Mean ± SD	AFP (ng/ml) Mean ± SD	Child Pugh (A/B/C)	Diff. Degree* (wel/mod/ por/ND)	Tumor Size† (large/small)	Tumor Multiplicity (multiple/solitary)	Vascular Invasion (+/−)	TNM Stage (I/II/IIIA/IIIB/ IIIC/IV)
Normaldonors	11	8/3	35 ± 2	ND	ND	ND	ND	ND	ND	ND	ND
Chronichepatitis	29	16/13	59 ± 10	92 ± 94	31 ± 87	27/2/0	ND	ND	ND	ND	ND
HCC	31	23/8	71 ± 4	74 ± 33	1768 ± 9103	20/10/1	11/10/0/10	22/9	20/11	9/22	10/12/3/1/2/3

*Histological degree of HCC; wel: well differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
†Tumor size was divided into either "small" (≦2 cm) or "large" (>2 cm).

CTL Induction and Cytotoxicity Assay. CTL induction and cytotoxicity assays were performed as described.[20] Briefly, stimulated PBMCs were added at effector to target ratios of 100:1, 50:1, 25:1, 13:1, 6:1, and 3:1. In cases where the number of CTLs was insufficient, cytotoxicity assays were performed at effector to target ratios less than 100:1.

Interferon Gamma IFN-γ Enzyme-Linked Immunospot (ELISPOT) Assay. IFN-γ ELISPOT assays were performed as reported.[20] Responses to TAA-derived peptides were considered positive if more than 10 specific spots were detected, which is greater than the mean plus 3 standard deviations (SDs) of the baseline response detected in 11 normal blood donors (Table 1), and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Responses to HIV-, HCV-, and CMV-derived peptides were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. In ELISPOT assay with blocking CTLA-4, anti-human CTLA-4 (eBioscience, Tokyo, Japan) was added at a final concentration of 50 μg/mL, which has been described to have maximum effect in *in vitro* cultures.[36] As a control, functional grade mouse immunoglobulin G (IgG)2a isotype control was used. The assay with blocking CTLA-4 was performed in triplicate and the results were statistically analyzed using the unpaired Student's *t* test.

Cytokine and Chemokine Profiling. The effect of CTLA-4 antibody on TAA-specific T-cell responses was also analyzed by cytokine and chemokine profiling. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay (Bio-Rad, Hercules, CA). These included interleukin (IL)-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, RANTES, tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF). Eight standards (ranging from 2 to 32,000 pg/mL) were used to generate calibration curves for each cytokine. Data acquisition and analysis were carried out using Bio-plex Manager software v. 4.1.1.

Cytokine Secretion Assay. TAA-specific IFN-γ-producing T cells were also analyzed by cytokine secretion assay. The assay was performed with the MACS cytokine secretion assay (Miltenyi Biotec K.K., Tokyo, Japan), in accordance with the manufacturer's instructions. Briefly, 5,000,000 PBMCs were pulsed with TAA-derived peptides for 16 hours and then incubated with 20 μL of IFN-γ detection antibody, 10 μL of anti-CD8-APC Ab (Becton Dickinson, Tokyo, Japan), 10 μL of anti-CCR7-FITC Ab (eBioscience, Tokyo, Japan), and 10 μL of anti-CD45RA-PerCP-Cy5.5 Ab (eBioscience, Tokyo, Japan) for 10 minutes at 4°C. After washing with a cold buffer (phosphate-buffered saline/0.5% bovine serum albumin with 2 mM EDTA), the cells were resuspended with 500 μL of cold buffer and analyzed using FACSCalibur (Becton Dickinson, Tokyo, Japan). As a positive control, CMVpp65$_{328}$-specific IFN-γ-producing T cells were also analyzed by the same methods. The number of IFN-γ-producing T cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs.

Results

Patient Profile. The clinical profiles of the 11 healthy blood donors, 29 patients with chronic hepatitis C, and 31 patients with HCV-related HCC analyzed in the present study are shown in Table 2 and Fig. 1. Using TNM staging of the Union Internationale Contre Le Cancer (UICC) system (6th v.), 10, 12, 3, 1, 2, and 3 patients were classified as having stage I, II, IIIA, IIIB, IIIC, and IV tumors, respectively.

Detection of TAA-Specific T Cells in HCC Patients. First we examined the frequency of cells that specifically reacted with TAA-derived and control peptides in HCC patients. Fifty-one responses in total were observed against TAA-derived peptides. Twenty-

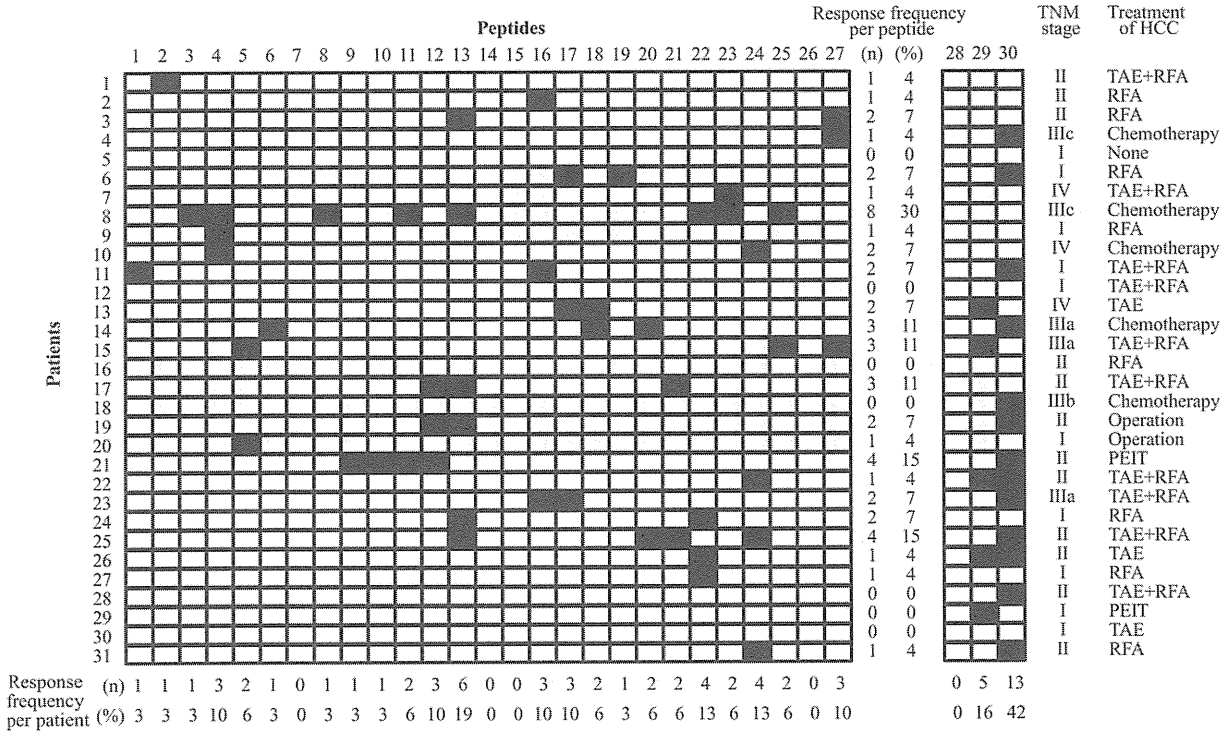

Fig. 1. TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses. Results of all HCC patients examined are shown. The T-cell responses were examined by IFN-γ ELISPOT assay. Responses to peptides were considered positive if more than 10 specific spots per 300,000 PBMCs were detected and if the number of spots in the presence of an antigen was at least 2-fold that in its absence. Black boxes indicate the presence of a significant IFN-γ T-cell response to peptides. Peptide sequences are described in Table 1 and characteristics of patients in Table 2.

four of 31 (77.4%) patients showed positive responses to at least one TAA-derived peptide and most of them showed responses to 1 to 4 kinds of TAA-derived peptide. Twenty-three of 27 (85.2%) TAA-derived peptides were recognized by T cells of at least one patient. Peptides 4, 12, 13, 16, 17, 22, 24, and 27 were recognized in more than two patients, suggesting that these peptides were immunogenic. Peptides 28 (HIV env_{584}), 29 (HCV $_{1031}$), and 30 (CMV $pp65_{328}$) were recognized by 0 (0%), 5 (16%), and 13 (42%) patients, respectively.

The magnitude of TAA-specific T-cell responses was assessed by the frequency of peptide-specific IFN-γ-producing T cells in the PBMC population (Fig. 2A). The range of TAA-derived peptide-specific T-cell frequency was 10-60.5 cells/300,000 PBMCs. Those specific to peptides 13 and 16 numbered more than 30 cells/300,000 PBMCs, suggesting that these peptides were immunogenic. The frequencies of T cells specific to HCV- and CMV-derived peptides were 12-22 cells and 12-92/300,000 PBMCs, respectively.

Whether these TAA-derived peptides were capable of generating peptide-specific CTLs from PBMCs was investigated in HCC patients. The seven peptides were selected according to the magnitude of TAA-specific T-cell responses determined by the frequency of T cells with a positive response. The CTLs generated with these peptides were cytotoxic to C1RA24 cells pulsed with the corresponding peptides (Fig. 2B).

Comparison of TAA-Specific T-Cell Responses Between the Patient Groups With and Without HCC. To characterize the immunogenicity and specificity of TAA-derived peptides, we compared T-cell responses to the peptides derived from TAA, HIV, HCV, and CMV among three groups consisting of normal blood donors, patients with chronic hepatitis C, and patients with HCV-related HCC. A significant TAA-specific T-cell response was not detected in normal blood donors (Fig. 3A). A response was detected in both chronic hepatitis C and HCC patient groups, but it was more frequently observed in HCC patients. HIV-specific T-cell response was not detected in any group. HCV-specific T-cell response rate was not different between the groups with chronic hepatitis C and HCC. CMV-specific T-cell response rates were similar among the three groups. Similar tendencies were observed in the analysis of individual peptides (Fig. 3B). We also examined the frequency of T cells responsive to peptides among the three groups. The mean frequency of TAA-specific T cells without *in vitro* expansion was higher in HCC patients than in

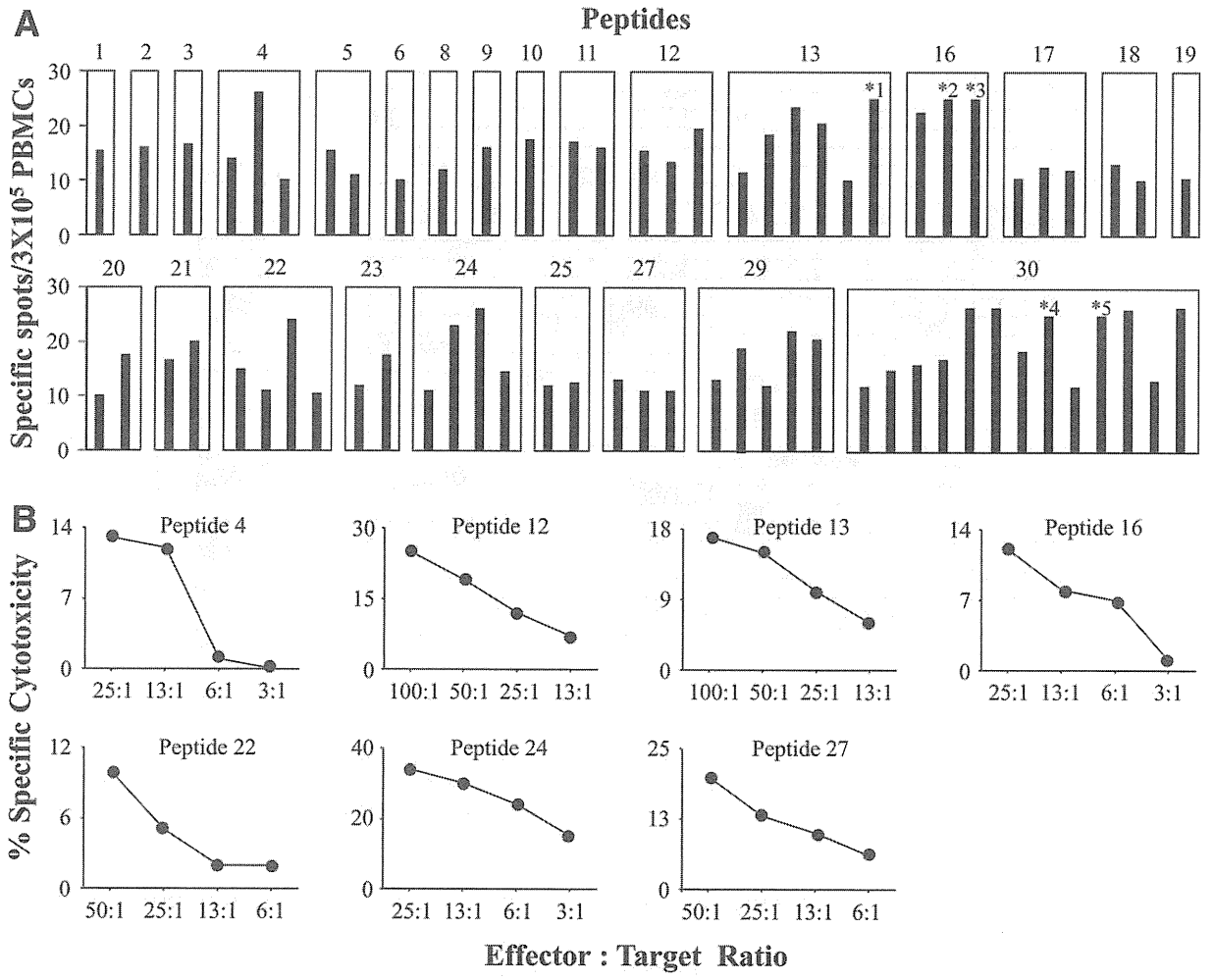

Fig. 2. Vigor of TAA-, HCV-, and CMV-derived peptide-specific T-cell responses. (A) The frequency of TAA-specific IFN-γ-producing T cells was analyzed by ELISPOT assay. Only positive responses are shown. Black bars indicate the response of one patient. *1, *2, *3, *4, and *5 denote 33, 60.5, 44, 92, and 67.5 specific spots, respectively. (B) Representative TAA-specific T-cell responses were also analyzed by CTL assay. T cell lines were generated from PBMC of the HLA-A24-positive HCC patients by stimulation with TAA-derived peptides (peptides 4, 12, 13, 16, 22, 24, and 27) (see Table 1). Expanded T cell lines were then tested for specific cytotoxicity against the corresponding peptides in a standard ^{51}Cr release assay at the indicated E:T ratios.

patients with chronic hepatitis C for 14 of 27 TAA-derived peptides (peptides 1, 2, 3, 4, 12, 16, 18, 19, 20, 21, 22, 24, 25 and 27) (Fig. 3C).

Enhancement of TAA-Specific T-Cell Responses After HCC Treatments. Several studies including our own have clarified that HCC treatments enhanced HCC-specific immune responses (19, 37, 38). In this study, we examined whether the enhancement was observed equally in all kinds of TAAs or specifically in some TAAs. For this purpose we measured the frequency of TAA-specific T cells before and after HCC treatment by ELISPOT assay in 12 cases who received transcatheter arterial embolization (TAE), radiofrequency ablation (RFA), or chemotherapy. The frequency of TAA-specific T cells increased in all patients and it was observed for 23 of 27 TAA-derived peptides (Fig. 4A). The enhancement was observed in the patients who received TAE, RFA, or chemotherapy and even in the patients without an increase in the frequency of CMV-specific T cells. Peptides 7, 14, 15, and 26, which were not recognized by T cells in all HCC patients before treatments (Fig. 1), were recognized by T cells in 1, 4, 1, and 5, respectively, of 12 patients after treatments. Representative results of enhancement of TAA-specific immune responses are shown in Fig. 4B. The frequency of TAA-specific T cells increased to 11-80 cells/300,000 PBMCs after treatments.

The enhancement of TAA-specific immune responses was also confirmed by cytokine secretion assay. Representative results are shown in Fig. 4C. In this patient (patient 25) the frequency of TAA-specific IFN-γ-producing $CD8^+$ T cells was increased from 0.4% to 1.4% of $CD8^+$ T cells after HCC treatment.

A

	TAA	HIV antigen	HCV antigen	CMV antigen
Normal donors	0/11(0%)	0/11(0%)	ND	4/11(36%)
Patients with HCV	10/29(34%)	0/29(0%)	8/29(28%)	15/29(52%)
Patients with HCC	24/31(77%)	0/31(0%)	5/31(16%)	13/31(42%)

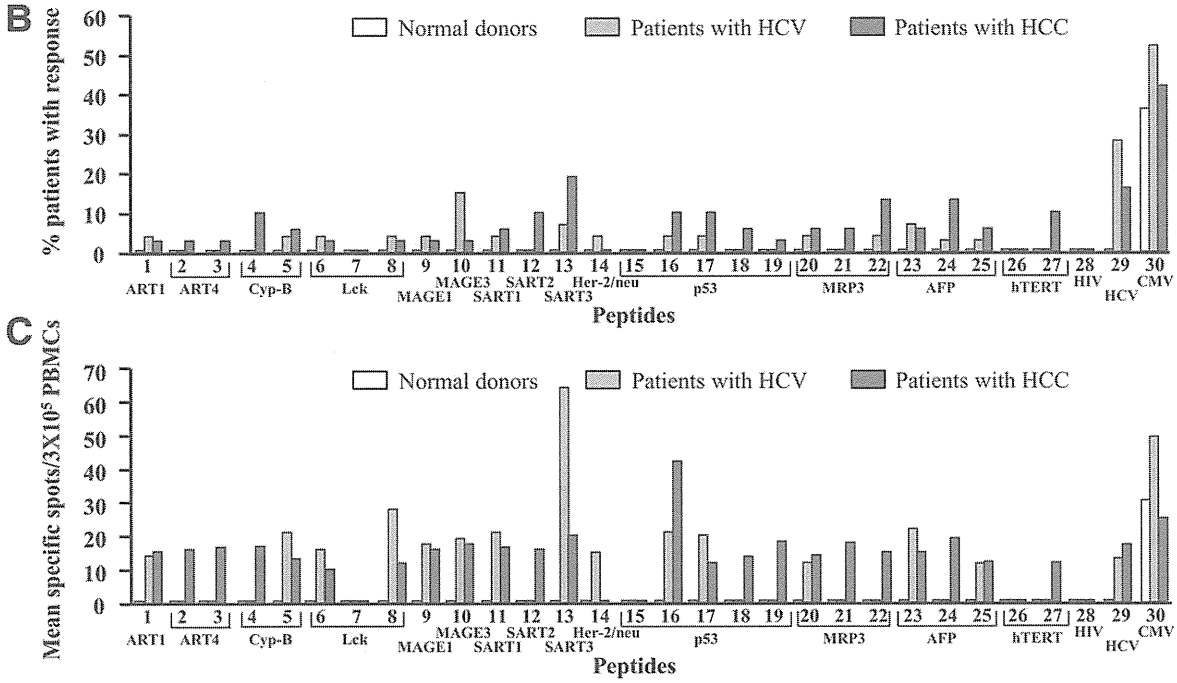

Fig. 3. Comparative analysis of TAA-, HIV-, HCV-, and CMV-derived peptide-specific T-cell responses among three groups of subjects: normal donors, patients with chronic hepatitis C not complicated by HCC, and HCC patients. (A) Summary of the number of patients with a significant IFN-γ T-cell response to tumor-associated, HIV, HCV, and CMV antigens in each group. (B) Graph shows the percentage of patients in each group who showed a significant IFN-γ T-cell response to individual peptides. Peptide sequences are described in Table 1. (C) Mean frequency of peptide-specific IFN-γ-producing T cells in each group. The frequency of IFN-γ-producing T cells was analyzed by ELISPOT assay.

In this assay we also examined the naïve/effector/memory phenotype of these cells by the criterion of CD45RA/CCR7 expression.[39] Phenotypic analysis of TAA-specific, IFN-γ-producing memory $CD8^+$ T cells before and after treatment showed that the frequency of $CD45RA^-/CCR7^+$ central memory T cells was the highest, indicating that the posttherapeutic increase in these T cells is due to the increase in cells with this phenotype (Fig. 4D). In this patient the number of T cells with the $CD45RA^-/CCR7^+$ phenotype increased from 73 cells/300,000 PBMCs before treatment to 316 cells/300,000 PBMCs after treatment. Similar results were noted in five patients.

Blocking CTLA-4 Restores TAA-Specific T-Cell Responses. In previous studies including our own,[19,20,24] the CTL epitopes that correlate with the prevention of tumor progression or prognosis of HCC patients have not been identified. One of the reasons for this is considered to be that the naturally occurring T-cell responses to the epitopes are weak; therefore, recent tumor immunotherapeutic studies are moving toward modulation of T-cell responses.

CTLA-4 is recognized as a critical negative regulator of immune response; therefore, its blockade has been considered to contribute to antitumor activity.[27] In a recent study it was reported that blocking of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of CTLA-4 antibodies.[40] To examine whether similar occurs for immune response in HCC patients, we analyzed 32 separate TAA-specific T-cell responses in 15 HCC patients using 13 TAA-derived peptides. Incubation of T cells with CTLA-4 antibodies resulted in an increase of the number of TAA-specific T cells in 18 of 32 (56%) responses and in 9 of 15 (60%) patients (Fig. 5A). Fourteen and four patients showed increases of 1-10 and more than 10 TAA-specific T cells, respectively. Representative results of six patients are shown

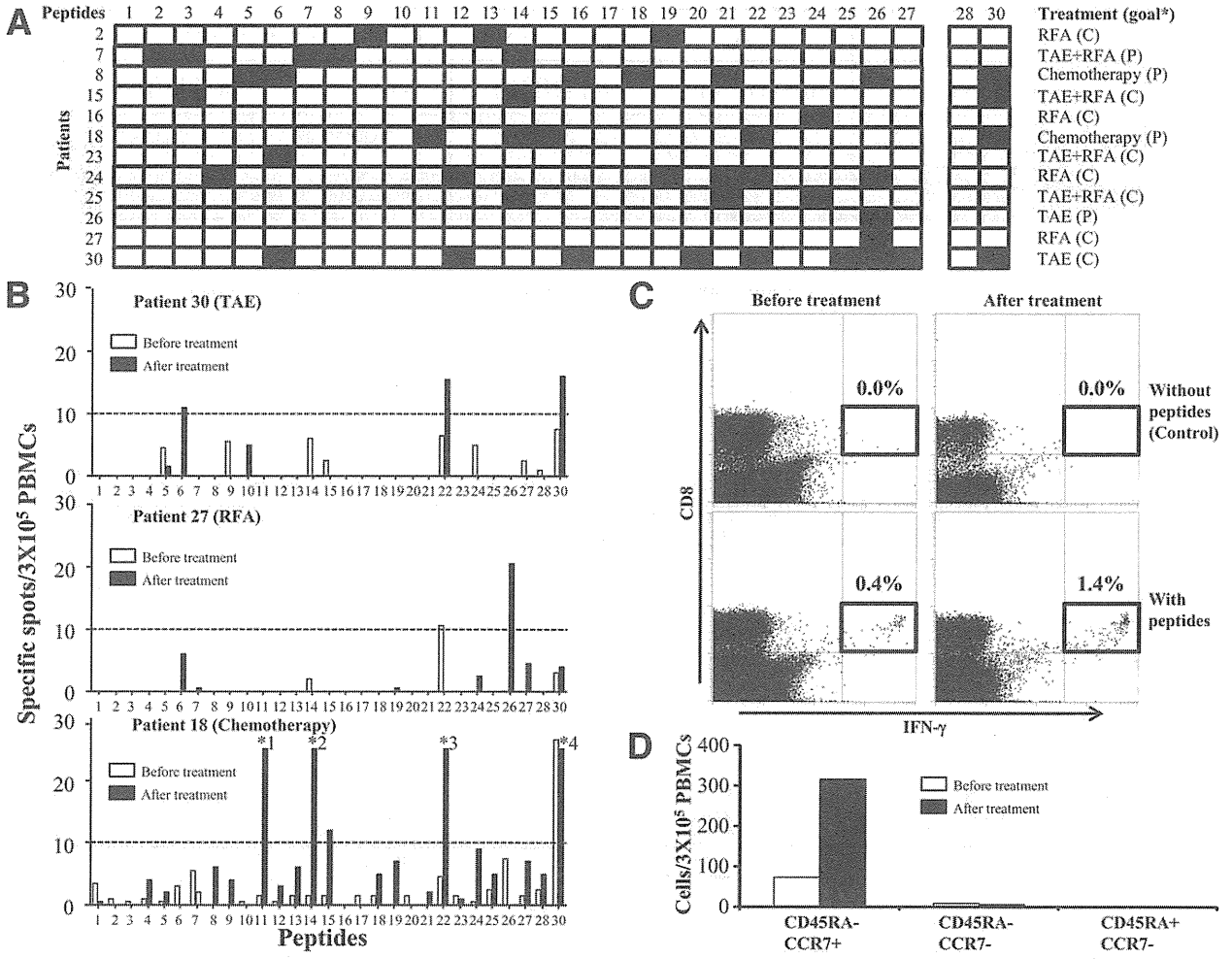

Fig. 4. Enhancement of TAA-specific T-cell responses in HCC patients after treatments. (A) Summary of patients and peptides with a significant increase of the number of IFN-γ-producing T cells (black boxes). A significant change in the IFN-γ response was defined as a more than 2-fold increase and the presence of more than 10 specific spots in ELISPOT assay after HCC treatments. The assays were performed in 12 HCC patients using 27 TAA-, HIV-, and CMV-derived peptides. Goal* shows the goal of HCC treatment. C and P denote "curative intention" and "palliative intention," respectively. (B) Representative results of ELISPOT assay are shown. White and black bars indicate the frequency of T cells before and after HCC treatments, respectively. *1, *2, *3, and *4 denote 53, 60, 80, and 121 specific spots, respectively. (C) Enhancement of TAA-specific T-cell responses was also analyzed by cytokine secretion assay. Representative results are shown (patient 25). PBMCs were pulsed with TAA-derived peptides (peptides 14, 21, and 24) for 16 hours and then analyzed for IFN-γ production. (D) IFN-γ-producing T cells were also examined for naïve/effector/memory phenotype by the criterion of CD45RA/CCR7 expression. The number of cells was calculated from the results of FACS analysis and is shown as a number per 300,000 PBMCs. White and black bars indicate the frequency of TAA-specific IFN-γ-producing T cells before and after HCC treatments, respectively. The experiments were performed in five patients and similar results were observed.

in Fig. 5B. The magnitude of TAA-specific T-cell increase was statistically significant in four patients.

To examine the effect of CTLA-4 antibodies for production of other cytokines by T cells, we measured 27 kinds of human cytokines and chemokines in the medium of ELISPOT assay. Figure 5C shows the results of cytokine production in the well with positive T-cell responses against TAA-derived peptides. The various cytokines consisting of IL-1β, IL-4, IL-6, IL-10, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, MIP-1α, MIP-1β, RANTES, and TNF-α were increased in the medium with CTLA-4 antibodies compared with that without CTLA-4 antibodies. In contrast, increased production of these cytokines in the well without positive T-cell responses against TAA-derived peptides was not observed in medium either with or without CTLA-4 antibodies (Fig. 5D).

Discussion

In recent years, specific TAAs and their CTL epitopes have been identified in many tumors.[21] Several TAAs and their CTL epitopes, such as AFP, MAGE, and human telomerase reverse transcriptase (hTERT) have also been reported in HCC.[19,20,24,41] Although AFP-targeting immunotherapy could induce TAA-

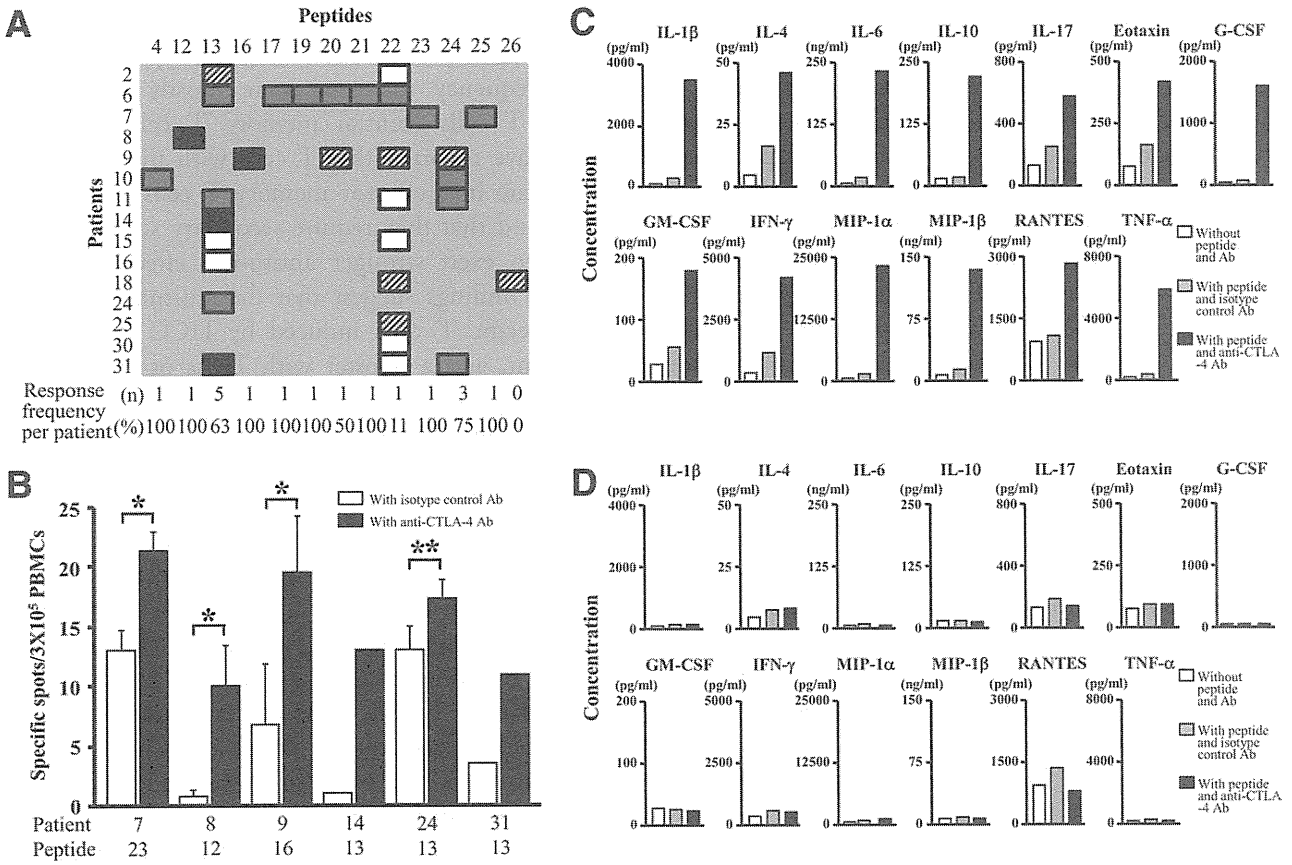

Fig. 5. Enhancement of TAA-specific T-cell responses in HCC patients by CTLA-4 antibodies. (A) Summary of patients and peptides with an increase of the number of IFN-γ-producing T cells. Black, gray, white, and hatched boxes indicate the immune responses with an increase of more than 10 specific spots, an increase of 1-10 specific spots, without change and a decrease of 1-10 specific spots, respectively. (B) Representative results of six patients are shown. Black and white bars indicate the results of assays incubated with CTLA-4 antibodies and mouse IgG2a isotype control, respectively. Data are expressed as the mean ± SD of specific spots, except for patients 14 and 31. (C) Effects of CTLA-4 antibodies on production of cytokine and chemokine. Cytokine and chemokine levels in the medium of ELISPOT assay were measured using the Bio-plex assay. The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 13 (medium in ELISPOT assay with enhancement of T-cell response) (see A,B). The increase of cytokines and chemokines after incubation with anti-CTLA-4 antibodies was confirmed in another three experiments using PBMCs of three other patients. (D) The graphs indicate the concentrations of cytokine and chemokine in the medium of ELISPOT assay using PBMCs of patient 31 and peptide 22 (medium in ELISPOT assay without enhancement of T-cell response) (see A).

specific CTLs, no patients achieved an objective tumor response; therefore, the search for TAAs as suitable targets for HCC immunotherapy and identification of their epitopes are important issues in therapy development. However, to date, T-cell responses to previously identified TAAs or their epitopes have been measured simultaneously and comparatively in only one study involving several patients with HBV-related HCC,[42] but no T-cell responses to the many other TAAs or their epitopes have been evaluated.

In this study we performed a simultaneous, comparative analysis of immune responses to 27 different CTL epitopes derived from 14 previously reported TAAs in the peripheral blood lymphocytes of 31 HCV-related HCC patients. We noted immune responses to epitopes (peptides 4, 12, 13, 16, 17, 22, 24, and 27) derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT in more than two patients (Fig. 1). These findings suggest the immunogenicity of these TAAs and their epitopes. In addition, the frequencies of peripheral blood CTLs specific to epitopes (peptides 4, 13, 16, 22, and 24) derived from CypB, SART3, p53, MRP3, and AFP, as detected by the ELISPOT assay, were high (≥20 specific spots/300,000 PBMCs), suggesting the high immunogenicity of these TAAs and their epitopes.

Among these immunogenic antigens the expression of p53, MRP3, AFP, and hTERT was reported in HCC.[18,19,43,44] We also previously confirmed that the expression of SART2 and SART3 was observed in 100% of human HCC tissue (data not shown). As for CypB, this protein is well known to be widely expressed in normal and malignant tissue[7]; therefore, it is considered to be expressed in HCC.

Regarding tumor immunotherapy, it has recently been reported that strong immune responses can be induced at an earlier postvaccination time using, as peptide vaccines, epitopes that frequently occur in peripheral blood CTL precursors.[23] The epitopes (peptides 4, 12, 13, 16, 22, 24, and 27) that were derived from CypB, SART2, SART3, p53, MRP3, AFP, and hTERT and considered to be highly immunogenic in this study were capable of inducing epitope-specific CTLs from the PBMCs of HCC patients, suggesting that these epitopes can be candidates for peptide vaccines.

Next, TAA-specific immune responses were compared among three groups of subjects: HCC patients, normal blood donors, and patients with chronic hepatitis C not complicated by HCC. The results showed that there were no differences in the positive rate of immune responses to CMV among the three groups and no difference in the positive rate of immune responses to HCV between chronic hepatitis C patients with and without HCC. However, TAA-specific immune responses were observed frequently only in HCC patients, indicating that these immune responses are specific to HCC.

In the present study we also analyzed factors influencing host immune responses to these TAA-derived epitopes. Previous studies have reported that treatments, such as RFA and TAE, enhance HCC-specific T-cell responses.[19,37,38] However, TAAs and their epitopes, to which these enhanced immune responses occur, have not been identified. Thus, we simultaneously measured immune responses to 27 different epitopes derived from 14 TAAs in 12 patients who were available for analysis before and after treatment. The results showed that the antigens and their epitopes to which treatment-enhanced T-cell responses occur were diverse and some of them were newly induced after HCC treatment, suggesting that HCC treatments could induce *de novo* T-cell responses and these TAAs and their epitopes can be candidates as targets for HCC immunotherapy.

Furthermore, it became clear that enhanced immune responses to TAAs were induced not only by previously reported RFA and TAE, but also by cytotoxic drug chemotherapy. The patients who received chemotherapy showed partial responses after the treatment; therefore, we considered that it induced release of TAA into the tumor environment by tumor necrosis and/or apoptosis such as the mechanism reported in RFA or TAE.[19,37,38] Thus, our findings suggest that combined cancer chemotherapy and immunotherapy is useful as a treatment for HCC.

Analysis of the memory phenotypes of the T cells thus induced showed that the phenotypes of T cells whose frequency increased were mostly $CD45RA^-/CCR7^+$ T cells (central memory T cells). Previous studies have reported that T cells with this phenotype differentiate into effector memory T cells and effector T cells, and that they require secondary stimulation by antigen to exert stronger antitumor effects.[39] Therefore, our findings suggest that the antitumor effect of tumor-specific T cells induced by HCC treatment is insufficient, and a booster with TAAs or epitope-containing peptides is a suitable method to further enhance antitumor effects.

Finally, we investigated the effect of anti-CTLA-4 antibodies, which have recently been in clinical trials as drugs enhancing antitumor immunity, on the host immune response to HCC. Regarding the mechanism of the antitumor activity of anti-CTLA-4 antibodies, it has been reported that they maximize the antitumor effect by blocking CTLA-4 on the surface of effector and regulatory T cells.[40] Because the number of peripheral blood regulatory T cells has been reported to increase in HCC patients,[45] TAA-specific CTLs that should be present but may not be detected by the ELISPOT assay. Therefore, in this study anti-CTLA-4 antibodies were added along with peptides to examine their effect on the ELISPOT assay.

The addition of anti-CTLA-4 antibodies resulted in an increase in the frequency of TAA-specific T cells in 60% of HCC patients. Although most patients showed an increase of only 1-10 TAA-specific T cells, the increased number of T cells was statistically significant. In addition, an increase of more than 10 TAA-specific T cells and a conversion from a negative to a positive response were observed in four patients. These results suggested that the anti-CTLA-4 antibodies unmasked IFN-γ production by CTLs. However, the function might be limited because the number of TAA-specific T cells was not changed and even decreased in some patients.

The cytokine and chemokine profiling showed that the addition of anti-CTLA-4 antibodies increased the production of not only IFN-γ but also cytokines, such as TNF-α, IL-1, and IL-6, and chemokines such as MIP-1; therefore, we speculate that the increased production of these antitumor immunity substances also plays a role in the unmasking of TAA-specific CTLs by anti-CTLA-4 antibodies. These results suggest that anti-CTLA-4 antibody is promising as a drug to enhance antitumor immunity, and that the ELISPOT assay with this antibody may serve as a more appropriate test tool to detect more HCC-specific TAAs or their epitopes.

On the other hand, recent studies have shown the important role of $CD4^+$ helper T cells in optimal function and proliferation of $CD8^+$ T cells.[46] Therefore, the lack of $CD4^+$ helper T cells or anergic $CD4^+$ T cells may explain the limited TAA-specific $CD8^+$ T-cell responses in HCC. Further studies using $CD4^+$ T-cell-depleted PBMCs or $CD8^+$ T cells expanded with TAA-derived peptide may enable identification of more immunogenic HCC-specific TAAs and their epitopes.

In conclusion, the results of this study suggest that CypB, SART2, SART3, p53, MRP3, AFP, and hTERT are promising TAAs in HCC immunotherapy, that the administration of these TAAs or peptides containing their epitopes as vaccines after HCC treatment is likely to be effective, and that the concomitant use of anti-CTLA-4 antibodies may further increase antitumor immunity. We believe that the results of this study provide useful information for the development of immunotherapy for HCC.

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