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Expression of Chondroitin-glucuronate C5-epimerase and Cellular Immune Responses in Patients with Hepatocellular Carcinoma

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Abbreviations: SART, squamous cell carcinoma antigen recognized by T cells; HLA, human leukocyte antigen; IFN, interferon; PBMC, peripheral blood mononuclear cells; TIL, tumor infiltrating lymphocytes; HCV, hepatitis C virus; ELISPOT, enzyme-linked immunospot

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Short title: SART-specific T Cell Responses in HCC

ABSTRACT

Background & Aims: Chondroitin-glucuronate C5-epimerase is an enzyme that converts D-glucuronic acid to L-iduronic acid residues in dermatan sulfate biosynthesis. It is also identified to be a tumor-associated antigen recognized by cytotoxic T cells (CTLs) and its enhanced expression in many cancers has been reported. In the present study, we investigated the usefulness of this molecule as an immunotherapeutic target in hepatocellular carcinoma (HCC).

Methods: The expression of chondroitin-glucuronate C5-epimerase in hepatoma cell lines and HCC tissues was confirmed by immunofluorescence and immunohistochemical analysis. CTL responses were investigated by several immunological techniques using peripheral blood mononuclear cells (PBMCs) or tumor-infiltrating lymphocytes. To determine the safety of immunotherapy using chondroitin-glucuronate C5-epimerase-derived peptide, 12 patients with HCC were administered s.c. vaccinations of the peptides and analyzed.

Results: Chondroitin-glucuronate C5-epimerase was expressed in HCC cell lines and human tissues including alpha-fetoprotein (AFP)-negative individuals. Chondroitin-glucuronate C5-epimerase-specific CTLs could be generated by

stimulating PBMCs of HCC patients with peptides and they showed cytotoxicity against HCC cells expressing the protein. The frequency of CTL precursors investigated by enzyme-linked immunospot (ELISPOT) assay was 0-34 cells/3X10^5 PBMCs and the infiltration of interferon-gamma-producing CTLs into the tumor site was confirmed. In the vaccination study, no severe adverse events were observed and the peptide-specific CTLs were induced in 4 of 12 patients tested.

Conclusions: Chondroitin-glucuronate C5-epimerase is a potential candidate for tumor antigen with immunogenicity and the peptides derived from this antigen could be useful in HCC immunotherapy.

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Keywords: epitope, immunotherapy, CTL, tumor-associated antigen, cancer, peptide vaccine

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and has gained much clinical interest because of its increasing incidence (1). It is treatable by hepatectomy or percutaneous ablation when the lesion is localized to some extent, and radical therapeutic effects can be obtained when resection or cauterization with a safety margin can be performed (2). However, the recurrence rate is very high (3), because active hepatitis and cirrhosis in surrounding non-tumor liver tissues have the potential to generate HCC de novo.

To protect against recurrence, tumor antigen-specific immunotherapy is an attractive option. Many tumor-associated antigens and their epitopes recognized by cytotoxic T cells (CTLs) have been identified during the last two decades. However, only a few HCC-specific tumor antigens and their antigenic epitopes have been used for human trials (4, 5).

Chondroitin-glucuronate C5-epimerase is an enzyme that converts D-glucuronic acid to L-iduronic acid residues in dermatan sulfate biosynthesis and identical to squamous cell carcinoma antigen recognized by T cells 2 (SART2) (6). It is expressed in many

malignant tumor cell lines and various histological types of cancer tissues and function as tumor rejection antigens (7). In addition, peptides containing chondroitin-glucuronate C5-epimerase epitopes are capable of generating CTLs, and therefore, have been used for immunotherapy to treat several kinds of cancers (8, 9). These reports suggest chondroitin-glucuronate C5-epimerase to be useful as a target antigen in HCC immunotherapy. Furthermore, in previous study, we compared T cell immune responses against the various tumor-associated antigen (TAA)-derived peptides (10). The results of the study showed that CTLs of HCC patients were frequently responsive against a single chondroitin-glucuronate C5-epimerase-derived peptide. Regarding tumor immunotherapy, it has recently been reported that strong immune responses can be induced at an earlier post-vaccination time using, as peptide vaccines, epitopes that frequently occur in peripheral blood CTL precursors (11). These results also suggest that chondroitin-glucuronate C5-epimerase is useful as a target for HCC immunotherapy.

In the present study, we examined chondroitin-glucuronate C5-epimerase expression in various hepatoma cell lines and HCC tissues of patients, and analyzed immune responses to the antigen using peripheral blood mononuclear cells (PBMCs) and tumor-infiltrating lymphocytes (TILs). Furthermore, to investigate the usefulness of HCC immunotherapy targeting chondroitin-glucuronate C5-epimerase, we analyzed the

safety and cellular immune responses in the patients vaccinated with chondroitin-glucuronate C5-epimerase-derived peptide.

MATERIALS AND METHODS

Patients

Forty-four HLA-A24-positive HCC patients were examined for the expression of chondroitin-glucuronate C5-epimerase and cellular immune responses. Twelve HCC patients were enrolled in vaccination study. Informed consent was obtained from each patient included in the present study and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the regional ethics committee.

The diagnosis of HCC was histologically confirmed by taking US-guided needle biopsy specimens in 17 cases, surgical resection in 9 cases, and autopsy in 5 cases. For the remaining 13 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 (12). The pathological grading of tumor cell differentiation was assessed according to the general

rules for the clinical and pathologic study of primary liver cancer (13). The severity of liver disease was evaluated according to the criteria of Desmet et al. using biopsy specimens of liver tissue (14). Eleven normal blood donors and 23 chronic hepatitis C patients (11 liver cirrhosis) with HLA-A24, who were diagnosed by liver biopsy, served as controls.

Cell lines

Four human hepatoma cell lines (HLF, Hep3B, HLE and Huh7) and Paca-2, which is a pancreatic cancer cell line, were cultured in DMEM (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Gibco). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24) was cultured in RPMI 1640 medium containing 10% FCS and 500 μg/ml of hygromycin B (Sigma, St Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS (15).

Immunofluorescence and immunohistochemical analysis

The expression of chondroitin-glucuronate C5-epimerase was examined in 4 different Hepatoma cell lines. A pancreatic cancer cell line (Paca 2) was used as a positive control. They were fixed in acetone with methanol for 5 min and incubated with rabbit

anti-human chondroitin-glucuronate C5-epimerase (ProteinTech Group, Inc, Chicago, IL; diluted 1:50) or mouse anti-human AFP (Nichirei Bioscience, Tokyo, Japan) antibody overnight at 4°C. For immunofluorescence analyses, Alexa Fluor 488-conjugated anti-rabbit and anti-mouse IgG (Invitrogen, Tokyo, Japan) were used for chondroitin-glucuronate C5-epimerase and AFP detection, respectively.

The expression in HCC tissue was examined in 26 patients. Non cancerous tissues were also obtained by a paired liver biopsy or surgical resection from the non neoplastic liver tissue. The tissues were fixed in buffered zinc formalin (Anatech Ltd, Battle Creek, MI), embedded in paraffin, sectioned (at 3 μm), and stained with hematoxylin and eosin. The sections were deparaffinized, treated in a pressure cooker for 1–4 min, and incubated with rabbit anti-human chondroitin-glucuronate C5-epimerase or AFP (DakoCytomation, Inc, Carpinteria, CA) antibody overnight at 4°C. The tissue sections were visualized using the DAKO EnVision^{TM}+ System (DakoCytomation, Inc, Carpinteria, CA). The expression levels were semi-quantitatively classified into four categories (negative to low, moderate and high; negative: no staining, low: <20% of the area stained, moderate: 20%-80% of the area stained, high: >80% of the area stained).

ELISPOT assay

PBMCs and TILs were isolated as described previously (16). ELISPOT assays were performed as reported previously with the following modifications (16). Three different peptides (Peptide 1; DYSARWNEI, Peptide 2; AYDFLYNYL, Peptide 3; SYTRLFLIL) derived from chondroitin-glucuronate C5-epimerase were used for the detection of CTLs. Negative controls consisted of a HIV envelope-derived peptide ($HIVenv_{584}$) (17). Positive controls consisted of 10 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$) (18). The peptides were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). 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 derived form chondroitin-glucuronate C5-epimerase in HCC patients were considered positive if the number of specific spots was more than the mean + 3SD of that in normal donors and if the number of spots in the presence of an antigen was at least twofold greater than the number in its absence. Responses to peptides $HIVenv_{584}$ and $CMVpp65_{328}$ 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 twofold that in its absence. ELISPOT assays were also performed in

12 patients whose PBMCs were available for analysis at 2-4 weeks after RFA.

CTL induction and Cytotoxicity assay

Peptide 3 (SYTRLFLIL), which correspond to HLA-A24 restricted CTL epitope (7, 19), was used to produce chondroitin-glucuronate C5-epimerase-specific T cells. CTLs were expanded from PBMCs as detailed previously (16). C1R-A24 cells and human hepatoma cell lines were used as targets. Cytotoxicity assay was performed by chromium-release assay. Percent cytotoxicity was calculated as previously described (16). For the assay using hepatoma cell lines, cytotoxicity was considered positive when it was higher than that of CTLs against K562 which show non-specific lysis.

Vaccination study

Twelve HLA-A24-positive HCC patients who were treated by radiofrequency ablation (RFA) and obtained complete necrosis of tumor with safety margin were enrolled in this vaccine study (Trial registration: UMIN000004540). They were vaccinated with peptide 3 (SYTRLFLIL) into the subcutaneous tissue of the armpit 4 weeks after RFA. The peptide utilized in the present study was prepared under conditions of Good Manufacturing Practice (NeoMPS, San Diego, CA). One milliliter

of the peptide, which was supplied in vials containing 0.04-4mg/ml sterile solution, was mixed with an equal volume of incomplete Freund's adjuvant (Montanide ISA-51; Seppic, Paris, France) and emulsified in 5-ml syringes. 1.5 ml of the preparing peptide was injected and the patients received three biweekly vaccinations. Toxicity was assessed every 2 weeks using the National Cancer Institute's Common Toxicity Criteria. To evaluate the immunological effect, ELISPOT assay was performed before and 4 weeks after the final vaccination. Responses to vaccination were considered positive if more than 10 specific spots were detected and if the number of spots after vaccination was at least twofold that before vaccination. After final vaccination, HCC recurrence was evaluated by dynamic CT or MRI every 3 months.

Statistical analysis

Data are expressed as the mean ± SD. The Mann-Whitney's U test was used for statistical analyses of chondroitin-glucuronate C5-epimerase expression in HCC and non-cancerous liver tissues. The x2 test with Yates' correction and the unpaired *t*-test were used for univariate analysis of the effect of variables on the T cell response against chondroitin-glucuronate C5-epimerase. A level of $P<0.05$ was considered significant.

RESULTS

Expression of chondroitin-glucuronate C5-epimerase in hepatoma cell lines and HCC tissues

Chondroitin-glucuronate C5-epimerase was expressed in all hepatoma cells (Fig. 1A) and its cellular distribution was cytoplasmic, similar to that in Paca-2, a pancreatic cancer cell line reported to express the protein (7). The expression was observed even in the hepatoma cell lines not expressing AFP, namely HLF and HLE.

The expression of chondroitin-glucuronate C5-epimerase in HCC tissues was examined in 26 HCC patients. A representative result for one HCC patient is shown in Fig. 1B. In this case, the expression of chondroitin-glucuronate C5-epimerase was observed in HCC tissue but not in non-cancerous areas. In addition, AFP was not detected in HCC tissue. To compare the expression levels of this protein between cancerous and non-cancerous tissues, the expression was semi-quantitatively classified into four categories as described in materials and methods, and analyzed. The expression levels were higher in HCC tissue than in the non-cancerous tissue ($p < 0.0001$) (Fig. 1C). The expression in liver tissue was also observed in the patients with

chronic hepatitis and liver cirrhosis (Control), however, the expression levels were lower than those in HCC tissue (p=0.0137). The expression of chondroitin-glucuronate C5-epimerase and AFP in HCC tissue was observed in 26 (100%) and 12 (46%) of 26 patients, respectively (Fig. 1D). The expression of chondroitin-glucuronate C5-epimerase was observed even in the HCC tissues without AFP expression.

Detection of chondroitin-glucuronate C5-epimerase-specific T cells by IFN-γ ELISPOT analysis

The clinical profiles of the 11 healthy normal donors, 12 patients with chronic hepatitis C, 11 patients with liver cirrhosis and 44 patients with HCC analyzed in the present study are shown in Table 1.

To determine whether a significant number of T cells specifically reacted with the chondroitin-glucuronate C5-epimerase-derived peptides (peptide 1, 2 and 3) in HCC patients, ELISPOT assays were performed using PBMCs from 11 healthy donors (Fig. 2A). The number of specific spots was 1.0±1.3, 1.5±1.3 and 1.0±1.4/3X10^5 PBMCs, respectively. Similarly, cells that specifically reacted with the peptides were counted among chronic hepatitis C and liver cirrhosis patient-derived PBMCs. Regarding a value larger than the mean + 3SD of the number of T cells that specifically reacted with

the peptide in healthy donor-derived PBMCs as a significant response, 1 of 23 (4.3%) patients showed a significant response to each of the chondroitin-glucuronate C5-epimerase-derived peptides (Fig. 2A).

In the same analysis of HCC patients, 10.8, 16.2 and 27.0% of the patients showed significant responses to peptide 1, 2 and 3, respectively (Fig. 2B). A significant response specific to CMVpp65$_{328}$ was detected in 36.4%, 34.8% and 45.9% of healthy donors, disease control groups and HCC patients, respectively, with no significant difference among the three groups. On the other hand, no significant response to HIVenv$_{584}$ was observed in all groups.

To clarify the clinical characteristics of chondroitin-glucuronate C5-epimerase-specific T cell responses in HCC patients, the clinical background was compared between patients who showed positive responses to chondroitin-glucuronate C5-epimerase-derived peptides and those who did not. The clinical features of both groups were not statistically different in terms of age, sex, serum AFP levels, differentiation of HCC, tumor multiplicity, vascular invasion, TNM factors and stages, histology of the non-tumor liver, liver function and the type of viral infection (Table 2). Chondroitin-glucuronate C5-epimerase-specific T cells had been generated even in the early stages of HCC.

Next, to examine the existence of chondroitin-glucuronate C5-epimerase-specific T cells among TILs, we performed a similar analysis in another 7 patients from whom samples of both PBMCs and TILs could be obtained. In the assay using PBMCs and TILs, 4 of 7 (57.1%) and 5 of 7 (71.4%) patients, respectively, showed significant responses to chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) (Fig. 3A). A positive T cell response in TILs was observed even in one patient without a positive T cell response in PBMCs (patient 39).

Cytotoxic activity of chondroitin-glucuronate C5-epimerase-specific CTLs against hepatoma cell lines

Whether the chondroitin-glucuronate C5-epimerase-derived peptides used were capable of generating peptide-specific CTLs from PBMCs was investigated in 18 HCC patients. The CTLs specific to chondroitin-glucuronate C5-epimerase could be induced in 8 of 18 (44.4%) patients (Fig.3B and C). They exhibited cytotoxicity against hepatoma cell lines with the HLA-A24 molecule and expression of chondroitin-glucuronate C5-epimerase, which correspond to HLF and HLE, but not against Hep3B and Huh7 cells without HLA-A24 (Fig. 3D).

Clinical safety of chondroitin-glucuronate C5-epimerase-derived peptide and its immunological effects

The clinical profiles of the 12 HCC patients with vaccination are shown in Table 3. The treatment was well-tolerated and there were no treatment-related serious adverse events. The most common adverse event was grade 1 injection-site reaction manifesting as pain, pruritus, skin induration and rubor. The worsening of hepatitis or liver function was not observed in any of the vaccinated patients.

In the analysis of ELISPOT assay using PBMCs of patients with vaccination, 4 patients demonstrated an immune response with increasing IFN-γ producing T cells responded with the corresponding peptide in PBMCs after vaccination (Fig. 4A and Table 3). All of the patients that responded were immunized with 3.0 mg of peptide. None of the patients immunized with 0.03 or 0.3 mg of peptide showed an enhancement of peptide-specific immune response. The enhancement of immunological response to HIVenv$_{584}$ and CMVpp65$_{328}$ was not observed in any patients except patient A2.

To examine whether similar occurs for the immune response in HCC patients with only RFA, we analyzed chondroitin-glucuronate C5-epimerase-derived peptide-specific T cell responses in 12 HCC patients without vaccination, whose PBMCs were available for analysis at 2-4 weeks after RFA. In this analysis, we observed an increase of the

frequency of chondroitin-glucuronate C5-epimerase-derived peptide-specific T cells in 2 of 12 patients (Fig. 4B). The frequency of the patients who showed an increase in the number of chondroitin-glucuronate C5-epimerase-derived peptide-specific T cells was higher in the patients with vaccination of 3 mg of peptide (66.7%) than in those without vaccination (16.7%).

Finally, we examined the HCC recurrence rate after RFA between the patients with and without the peptide-specific CTL response to examine the clinical effect of an increase of chondroitin-glucuronate C5-epimerase-derived peptide-specific CTLs after vaccination. In the analysis, the recurrence rate in the patients with an increase of the peptide-specific CTLs after vaccination (2 of 4 patients, 50%) was lower than that in the patients without immune response (6 of 8 patients, 75%) at 300 days after RFA, although there was no statistical significance due to the small number of patients.

DISCUSSION

Many tumor-associated antigens and their epitopes capable of inducing HLA-class I-restricted CTLs have been identified in various cancers. Some of the epitopes have been under investigation for the treatment of cancer, with major clinical responses in some trials (11, 20-22).

With regard to immunotherapy for HCC, AFP is considered a useful tumor-associated antigen and AFP-derived peptides have actually been used in clinical trials (5, 23-25). However, in general, the production of AFP depends on the size of the tumor, with AFP expressed in only 0-40% of HCCs less than 30mm in size (26). Therefore, for immunotherapy for HCC in cases where AFP is not expressed in tumor tissue, it is necessary to identify other tumor-associated antigens.

In the present study, the expression of chondroitin-glucuronate C5-epimerase was observed in all of the HCC tissues examined and independent of differential degree, size, TNM stage and the expression of AFP in the tumor. These results suggest the advantage of these antigens as a target for immunotherapy of HCC.

On the other hand, the expression of this protein was also observed in non-cancerous

tissue of HCC patients, although less frequently and at lower levels than in HCC tissue. Our results are consistent with the recent finding that chondroitin-glucuronate C5-epimerase is expressed in some normal tissues including liver tissue (6). Such results imply that immunotherapy targeting chondroitin-glucuronate C5-epimerase may have adverse effects on liver tissue expressing the protein. Therefore, we next examined the existence and specificity of chondroitin-glucuronate C5-epimerase-specific CTLs in HCC patients.

The presence of chondroitin-glucuronate C5-epimerase-recognizing CTLs has been reported as SART2-specific CTLs in lung, gastric and pancreatic cancer patients (7, 27, 28). However, to our knowledge, there has been no report of the presence of chondroitin-glucuronate C5-epimerase-specific CTLs in HCC patients except our recent study using only one SART2-derived peptide (10). In this study, we used 3 different HLA-A24 restricted peptides which were previously identified and derived from naturally processed squamous cell carcinoma antigen. The HLA-A24 allele is found in 60% of Japanese (29), and therefore, to use HLA-A24 restricted peptides has the advantage of analyzing CTL responses to tumor associated antigens in Japanese patients.

We showed that chondroitin-glucuronate C5-epimerase-specific CTLs could be

generated by stimulating PBMCs with peptides, and the CTLs were cytotoxic to hepatoma cell lines. Chondroitin-glucuronate C5-epimerase-specific immune responses were observed frequently only in HCC patients and the frequency of CTLs was higher in HCC patients than control groups, indicating that the immune responses are specific to HCC. Furthermore, the CTLs were also detected among TILs, suggesting that they infiltrate the tumor. Based on these findings, we confirmed that chondroitin-glucuronate C5-epimerase-specific CTL precursors exist in HCC patients and the immune responses are specific for HCC.

In previous study, we reported that the frequency of TAA-derived peptide-specific CTLs in HCC patients was 0-92 cells/3X10^5 PBMCs and the frequency of the patients who showed immune responses to each peptide was 0-19% (10). In the present study, the frequency of chondroitin-glucuronate C5-epimerase-derived peptide-specific CTLs in HCC patients was 0-30 cells/3X10^5 PBMCs and the frequency of the patients who showed immune responses to the peptides was 11-27%. These results show that the frequencies of chondroitin-glucuronate C5-epimerase-specific CTLs in PBMCs and the patients with CTLs responsive to the TAA are very similar to those of previously identified immunogenic TAA-derived epitopes and suggest that the antigen and its CTL epitope are immunogenic. In addition, the CTLs were generated even in the early stages

of HCC. These results suggest the advantages of using chondroitin-glucuronate C5-epimerase-derived peptides as a vaccine for immunotherapy of HCC.

For the next step to investigate the usefulness of chondroitin-glucuronate C5-epimerase as an immunotherapeutic target in HCC, we examined the safety and efficacy of chondroitin-glucuronate C5-epimerase-derived peptide as a cancer vaccine. In previous studies using chondroitin-glucuronate C5-epimerase-derived peptides for several cancers, they were reported to be safe. However, most patients with HCC have chronic liver disease. Therefore, safety of the peptide vaccine should be confirmed in the patients with chronic hepatitis or cirrhosis. The present vaccination study included 9 patients with chronic liver diseases (4 chronic hepatitis and 5 cirrhotic patients) confirmed by histological examination and there was no severe adverse event in all patients vaccinated. The induction of chondroitin-glucuronate C5-epimerase-specific CTLs was observed in 4 of 6 (66.7%) patients vaccinated with 3mg of peptide, which is similar to the frequency of responded patients reported in other peptide vaccination studies (11, 20).

Apart from induction of CTLs, the efficacy of chondroitin-glucuronate C5-epimerase-derived peptides as a vaccine for advanced HCC is still unclear. In previous vaccine studies for advanced HCC, AFP, hTERT, and glypican-3 have been

targeted as tumor-associated antigens for the treatment (25, 30-32). In these studies, peptide-specific CTLs were reported to be induced in 10-80% of vaccinated patients. However, in spite of the induction of peptide-specific CTLs, it has been reported that the anti-tumor effect was very limited. Recent studies have shown that the frequency of myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) is increased in HCC patients and the cells inhibit the function of T cells (33, 34). Therefore, controlling their function might be important to develop more effective vaccination for advanced HCC.

In contrast, other recent studies using chondroitin-glucuronate C5-epimerase-derived peptides for other advanced cancers have shown the induction of cellular immune responses and clinical responses for certain patients (9, 11). In the analysis of the prognosis of patients with RFA and chondroitin-glucuronate C5-epimerase-derived peptide vaccination in the present study, the recurrence rate in the patients with an increase of the peptide-specific CTLs after vaccination was lower than that in the patients without immune response. Although further studies are necessary to evaluate the efficacy of chondroitin-glucuronate C5-epimerase-derived peptides for HCC, the results of our study suggest that chondroitin-glucuronate C5-epimerase is a potential candidate for a target of HCC immunotherapy.

In conclusion, chondroitin-glucuronate C5-epimerase is a potential candidate for a tumor antigen with immunogenicity, and peptides derived from the protein would be useful for immunotherapy in cases of HCC.

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Table 1 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	Etiology (B/C/ Others)	Child Pugh (A/B/C)	Diff. degree[a] (wel/mod/por/ND)	Tumor size[b] (large/small)	Tumor multiplicity (multiple/solitary)	Vascular Invasion (+/-)	TNM stage (I/II/IIIA/IIIB/IIIC/IV)
Normal donors	11	8/3	35± 2	ND	ND	ND	ND	ND	ND	ND	ND	ND
Chronic hepatitis	12	7/5	54± 11	104±119	12± 4	0/12/0	12/0/0	ND	ND	ND	ND	ND
Liver cirrhosis	11	5/6	60± 11	83±73	79± 140	1/7/3	6/5/0	ND	ND	ND	ND	ND
HCC	44	35/9	66± 8	67±32	1629±7874	8/34/2	28/14/2	11/17/3/13	29/15	25/19	12/32	13/17/5/1/2/6

[a] Histological degree of HCC; wel: well-differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.
[b] Tumor size was divided into either ‘small’ (≦2 cm) or ‘large’ (＞2 cm).

Table 2 Univariate analysis of the effect of variables on the T cell response against chondroitin-glucuronate C5-epimerase

	Patients with positive T cell response	Patients without positive T cell response	*p*-value [a]
No. of patients	15	22	
Age (years) [b]	64.6±9.8	68.7±5.9	NS
Sex (M/F)	14/1	15/7	NS
AFP (ng/ml)	3569.7±13070.0	580.7±2394.2	NS
Diff. degree of HCC (well/moderate or poor/ND) [c]	3/7/5	8/6/8	NS
Tumor multiplicity (multiple/solitary)	10/5	13/9	NS
Vascular invasion (+/-)	5/10	6/16	NS
TNM factor			
(T1/T2-4)	4/11	8/14	NS
(N0/N1)	14/1	22/0	NS
(M0/M1)	13/2	20/2	NS
TNM stage (I/II-IV)	4/11	8/14	NS
Histology of non-tumor liver (LC/Chronic hepatitis)	12/3	20/2	NS
Liver function (Child A/B/C)	11/4/0	13/7/2	NS
Etiology (HCV/HBV/Others)	11/3/1	20/1/1	NS
T cell response against to CMV $pp65_{328}$ (+/-)	9/6	9/13	NS

[a] NS: not significant.
[b] Data are expressed as the mean ± SD.
[c] ND: not determined.

Table 3 Patient characteristics

Patient	Peptide Dose (mg)	Age	Sex	Etiology	Stage of HCC	ALT (IU/L)	AFP (ng/ml)	Child-Pugh (A/B/C)	Histology of liver	Treatment	Immune response	Toxicity * (grade)
A1	0.03	73	F	HCV	I	26	12	A	F4A2	RFA	-	Pa(1)
A2	0.03	78	F	HCV	I	45	10	B	F4A2	RFA	-	P(1)
A3	0.03	59	M	NBNC	II	30	10	A	ND	RFA	-	None
B1	0.3	79	M	HCV	I	40	61	A	F3A1	RFA	-	R(1), S(1)
B2	0.3	72	M	NBNC	II	24	66	A	ND	RFA	-	R(1), S(1), P(1), H(1)
B3	0.3	78	M	HCV	II	45	10	A	F3A2	RFA	-	P(1)
C1	3.0	67	M	HCV	I	111	49	A	F3A1	RFA	+	P(1), S(1)
C2	3.0	73	M	NBNC	I	30	5	A	ND	RFA	-	None
C3	3.0	78	F	HCV	I	23	24	A	F4A2	RFA	+	P(1)
C4	3.0	75	M	HBV	I	21	15	A	F3A1	RFA	+	R(1), P(1)
C5	3.0	49	M	HBV	I	18	14	A	F4A1	RFA	+	None
C6	3.0	69	F	HBV	II	42	84	A	F4A2	RFA	-	Pa(1)

Toxicity *; Pa: Pain, P: Pruritus, S: Skin induration, R: Rubor, H: Headache

FIGURE LEGENDS

Figure 1: Expression of chondroitin-glucuronate C5-epimerase. (A) immunofluorescence analysis for the expression of chondroitin-glucuronate C5-epimerase in hepatoma cell lines. Original magnification, X400. (B) Immunohistochemical analysis for the expression of chondroitin-glucuronate C5-epimerase and AFP in sequential non-cancerous and HCC tissue sections. Original magnification, X200 (left) and X400 (right). (C) Analysis of chondroitin-glucuronate C5-epimerase expression levels among the three groups (HCC; tumor tissue in HCC patients, Non-tumor; non-tumor tissue in HCC patients, Control; liver tissue in disease control groups). Closed and open circles show the level of chondroitin-glucuronate C5-epimerase expression in the patients with liver cirrhosis and chronic hepatitis, respectively. (D) The expression of chondroitin-glucuronate C5-epimerase was also compared with AFP expression in HCC tissues.

Figure 2: Immune responses of chondroitin-glucuronate C5-epimerase-specific T cells. (A) IFN-γ ELISPOT assay of PBMCs to chondroitin-glucuronate C5-epimerase-derived peptides (peptides 1, 2 and 3: solid bars) or control peptides (peptides $HIVenv_{584}$ and $CMVpp65_{328}$: open and grey bars, respectively) in normal donors and disease control groups. “N” denotes normal donors. “C” denotes the patients with chronic hepatitis. “L” denotes the patients with liver cirrhosis. % shows the ratio of the patients who showed positive responses. *denotes more than 30 specific spots. (B) IFN-γ ELISPOT assay in HCC patients. *denotes more than 30 specific spots.

Figure 3: Characteristics of chondroitin-glucuronate C5-epimerase-specific CTLs. (A) IFN-γ ELISPOT assay of PBMCs and TILs to one of the chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) in 7 HCC patients. Open and solid bars show the frequency of chondroitin-glucuronate C5-epimerase-specific T cells in PBMCs and TILs, respectively. *denotes 114 specific spots. **denotes 42 specific spots. (B) Representative results of the CTL assay. The closed and open circles show the cytotoxicity against C1R-A*2402 cells pulsed with and without a peptide, respectively. (C) CTL assays (E/T ratio of 50:1) were performed in 18 HCC patients. Solid bars show the result for one patient. The results are shown as specific cytotoxic activity, which was calculated as follows: (cytotoxic activity in the presence of peptide) - (cytotoxic activity in the absence of peptide) and considered positive when higher than 10%. (D) Cytotoxicity of chondroitin-glucuronate C5-epimerase-specific T-cell lines derived with peptides was also measured against hepatoma cell lines. The cytotoxicity was considered positive when it was higher than that against K562 which shows non-specific lysis (E/T ratio of 50:1).

Figure 4: IFN-γ ELISPOT assays of PBMCs to chondroitin-glucuronate C5-epimerase-derived peptide (peptide 3) or control peptides (peptides HIVenv$_{584}$ and CMVpp65$_{328}$) in HCC patients with RFA. (A) The assays were performed in the patients with peptide 3 vaccination. White and black bars show the T cell responses before and after vaccination, respectively. (B) The assays were also performed in the patients without vaccination. White and black bars show the T cell responses before and after RFA, respectively. *denotes more than 50 specific spots.

Table 1 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	Etiology (B/C/ Others)	Child Pugh (A/B/C)	Diff. degree[a] (wel/mod/por/ND)	Tumor size[b] (large/small)	Tumor multiplicity (multiple/solitary)	Vascular Invasion (+/-)	TNM stage (I/II/IIIA/IIIB/IIIC/IV)
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(M0/M1)	13/2	20/2	NS
TNM stage (I/II-IV)	4/11	8/14	NS
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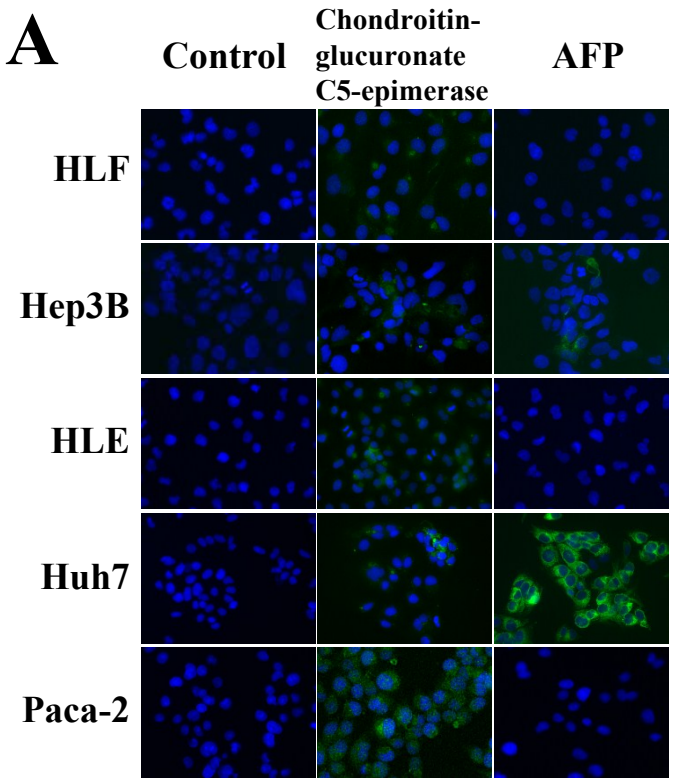
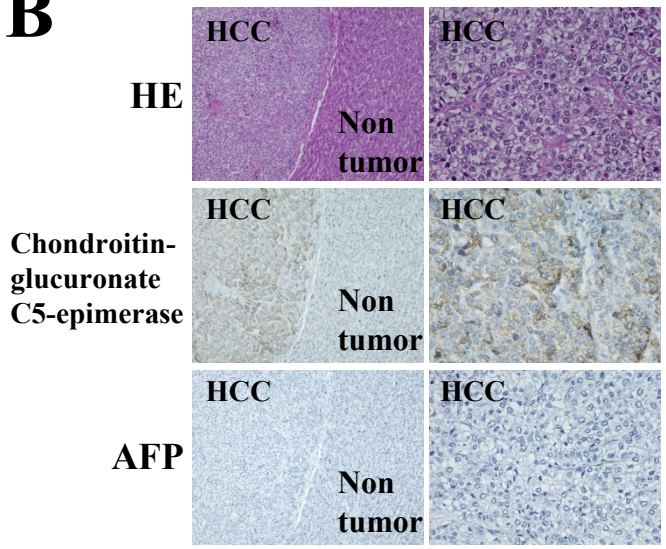
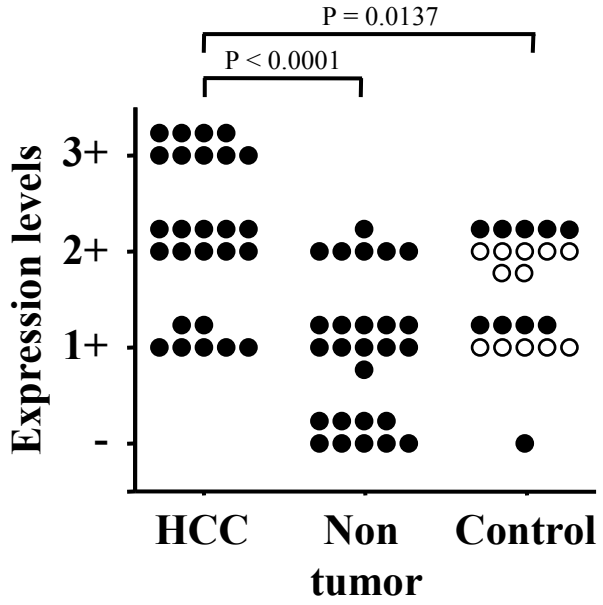
C5	3.0	49	M	HBV	I	18	14	A	F4A1	RFA	+	None
C6	3.0	69	F	HBV	II	42	84	A	F4A2	RFA	-	Pa(1)

Toxicity *; Pa: Pain, P: Pruritus, S: Skin induration, R: Rubor, H: Headache

Figure 1







D

		Chondroitin-glucuronate C5-epimerase expression	
		+	−
AFP expression	+	12	0
	−	14	0

Figure 2

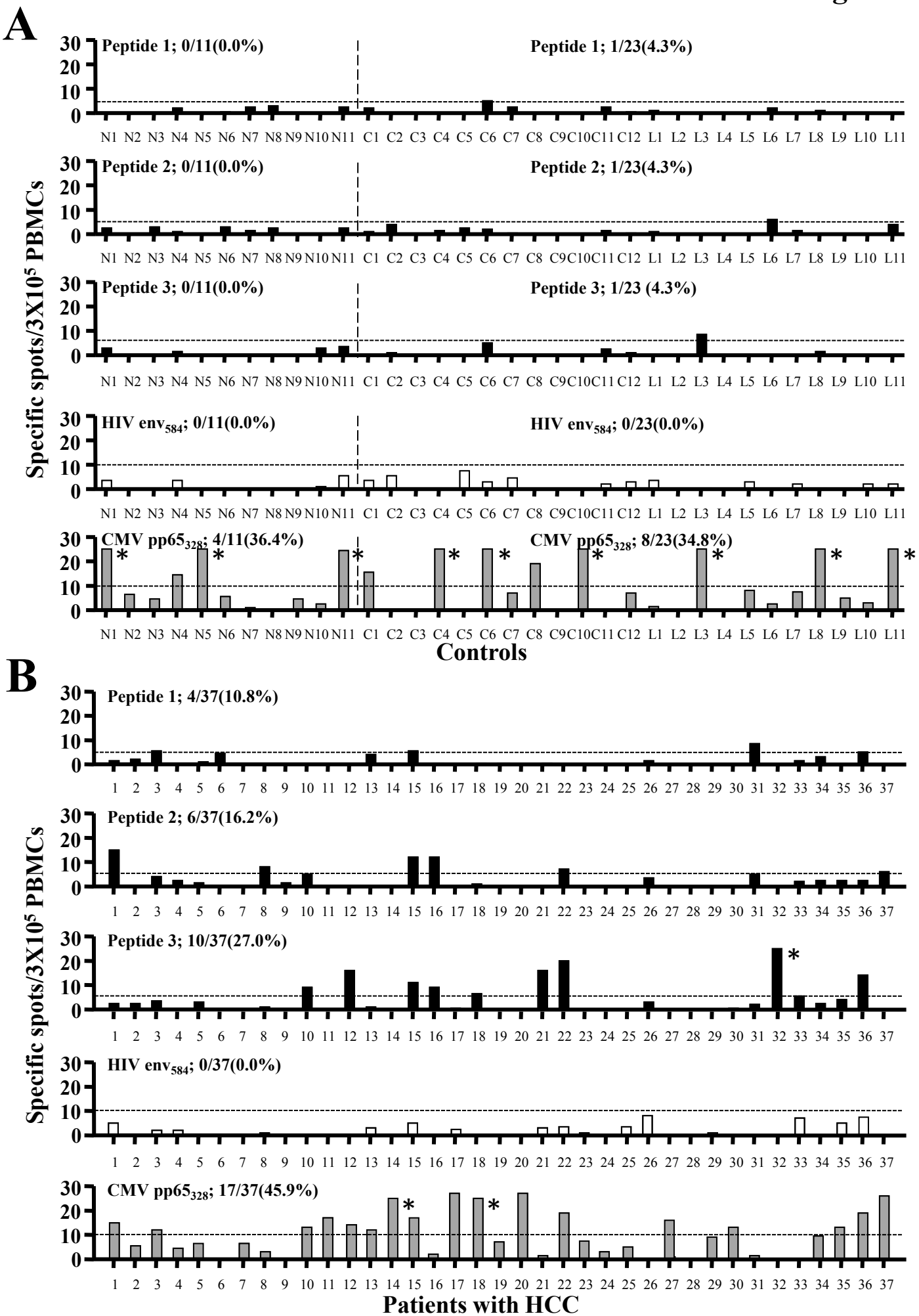

A
Peptide 1; 0/11(0.0%)
Peptide 1; 1/23(4.3%)
Peptide 2; 0/11(0.0%)
Peptide 2; 1/23(4.3%)
Peptide 3; 0/11(0.0%)
Peptide 3; 1/23 (4.3%)
HIV env584; 0/11(0.0%)
HIV env584; 0/23(0.0%)
CMV pp65328; 4/11(36.4%)
CMV pp65328; 8/23(34.8%)
Specific spots/3X10^5 PBMCs
Controls
B
Peptide 1; 4/37(10.8%)
Peptide 2; 6/37(16.2%)
Peptide 3; 10/37(27.0%)
HIV env584; 0/37(0.0%)
CMV pp65328; 17/37(45.9%)
Specific spots/3X10^5 PBMCs
Patients with HCC

Figure 3

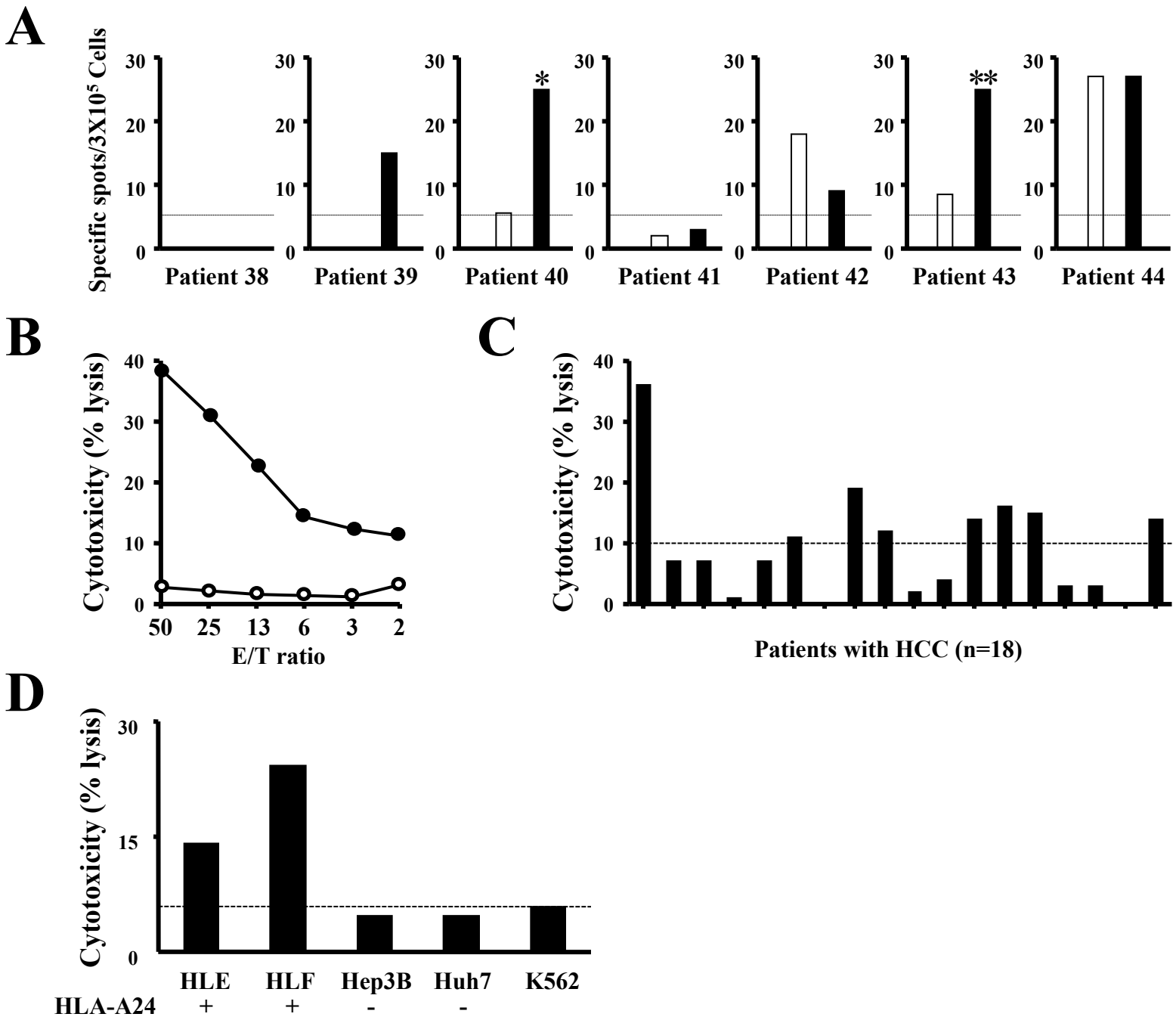
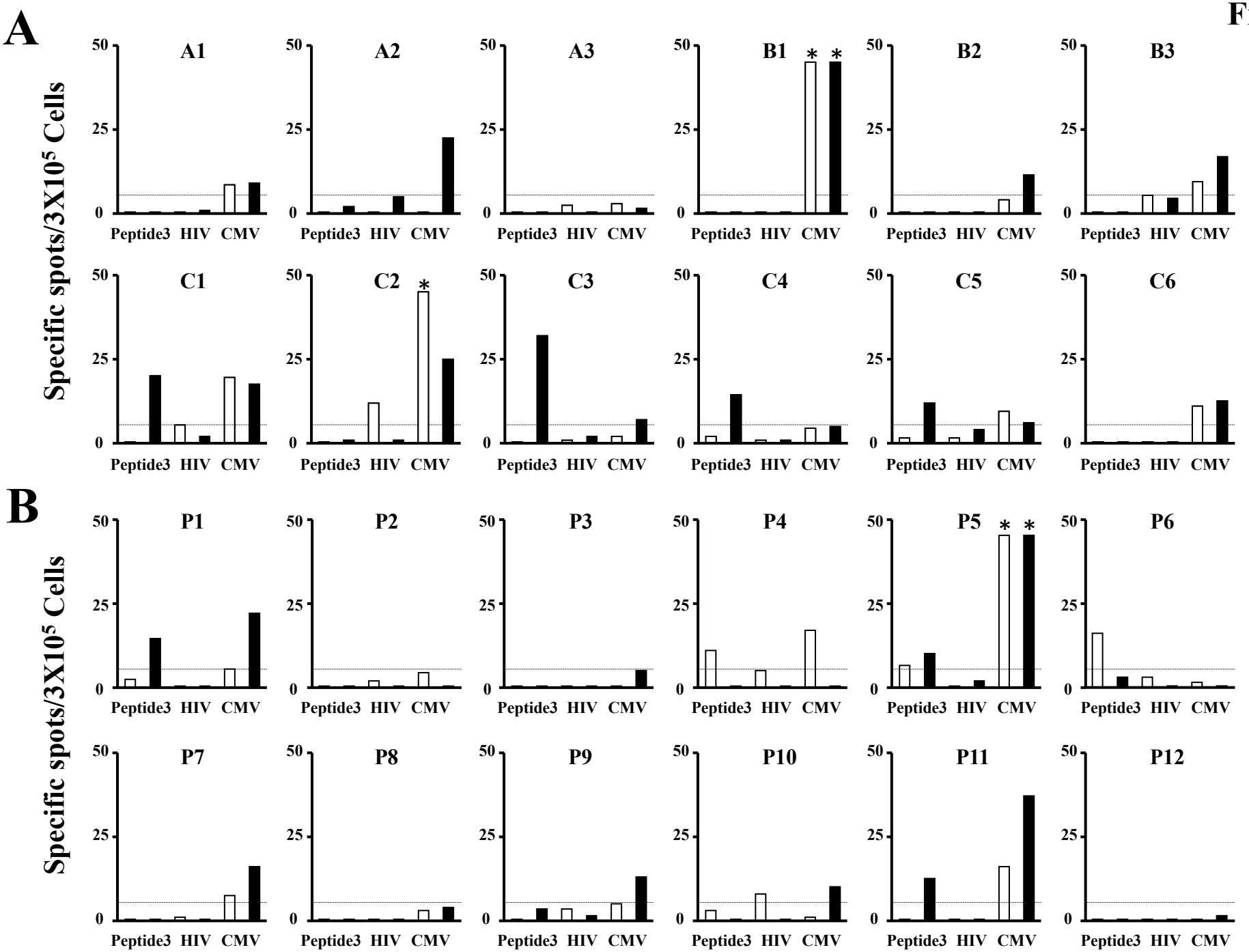

A
Specific spots/3X10^5 Cells
30
20
10
0
*
**
Patient 38
Patient 39
Patient 40
Patient 41
Patient 42
Patient 43
Patient 44
B
Cytotoxicity (% lysis)
40
30
20
10
0
50 25 13 6 3 2
E/T ratio
C
Cytotoxicity (% lysis)
Patients with HCC (n=18)
D
Cytotoxicity (% lysis)
30
15
0
HLE HLF Hep3B Huh7 K562
HLA-A24 + + - -

Figure 4


A
Specific spots/3X10^5 Cells
A1
A2
A3
B1
B2
B3
C1
C2
C3
C4
C5
C6
B
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
50
25
0
Peptide3 HIV CMV
*