Identification of α-fetoprotein-derived peptides recognized by cytotoxic T lymphocytes in HLA-A24+ patients with hepatocellular carcinoma

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α-Fetoprotein (AFP) has been proposed as a potential target for T-cell-based immunotherapy for hepatocellular carcinoma (HCC), but the number of its epitopes that have been identified is limited and the status of AFP-specific immunological responses in HCC patients has not been well-characterized. To address the issue, we examined the possibility of inducing AFP-specific cytotoxic T cells (CTLs) using novel HLA-A*2402-restricted T-cell epitopes (HLA, human leukocyte antigen) derived from AFP and then analyzed the relationship between its frequency of occurrence and clinical features associated with patients having HCC. Five AFP-derived peptides containing HLA-A*2402 binding motifs and showing high binding affinity to HLA-A*2402 induced CTLs to produce IFN-γ and kill an AFP-producing hepatoma cell line. The frequency of AFP-specific CTLs was 30–190 per 1 × 10⁶ peripheral blood mononuclear cells, which was the same as that of other immunogenic cancer associated antigen-derived epitopes. Analyses of the relationships between AFP-specific CTL responses and clinical features of patients with HCC revealed that AFP epitopes were more frequently recognized by CTLs in patients with advanced HCC correlating to tumor factors or the stage of TNM classification. The analyses of CTL responses before and after HCC treatments showed that the treatments changed the frequency of AFP-specific CTLs. In conclusion, we identified five HLA-A*2402-restricted T-cell epitopes derived from AFP. The newly identified AFP epitopes could be a valuable component of HCC immunotherapy and for analyzing host immune responses to HCC.

Key words: immune response; epitope; CD8; HLA-A24; hepatitis

Introduction
Hepatocellular carcinoma (HCC) is a malignancy¹,² and has gained major clinical interest because of its increasing incidence.⁵ Several current advances in therapeutic modalities such as surgical hepatic resection, percutaneous tumor ablation by ethanol injection or radiofrequency (RF), transcatheater arterial embolization (TAE), chemo-therapy and liver transplantation have improved the prognosis of HCC patients.⁴⁻⁹ However, the survival of those who have advanced HCC is still not satisfactory, since most of these patients have numerous tumors or vascular invasions, which conventional therapeutic modalities cannot eradicate completely and therefore keep recurring. Therefore, the development of new antitumor therapies for advanced HCC patients remains an urgent and important field of research.

To eradicate HCC and to protect the patients from its recurrence, tumor antigen-specific immunotherapy is an attractive strategy like the immunotherapy of melanoma and other cancers.¹⁰,¹¹ Tumor-specific immune responses are mediated by CD⁸⁺ and CD⁸⁻ T-cell responses. CD⁸⁺ T cells mediate antigen-specific and major histocompatibility complex (MHC)-restricted cytotoxic effects by recognition of peptides presented by MHC class I molecules through their TCR complex. Although many tumor-specific antigens have been identified in various cancers, the number of HCC-specific antigens known is still limited.

α-Fetoprotein (AFP) is a nonmutated oncofetal protein with tumor-selective expression that is frequently expressed in HCC, and its measurement in the serum is important for the diagnosis and monitoring of responses to treatment.¹² On the other hand, AFP expression in the normal liver is low or not detectable. Therefore, AFP is a target of interest for immunotherapy.

Recently, several results regarding AFP-specific cytotoxic T-cell responses were reported for human and mice studies.¹³⁻¹⁶ These reports revealed that AFP-specific cytotoxic T cells (CTLs) induced by stimulation with peptides or DNA-based immunization kill AFP-producing hepatoma cell lines, suggesting that AFP-reactive T-cell clones are not deleted from the human T-cell repertoire and that AFP may be a useful tumor-specific antigen as a target for T-cell-based immunotherapy against HCC. However, the number of AFP epitopes that have been identified is limited and the status of AFP-specific immunological responses has not been well-characterized in patients with HCC.

In the current study, using novel HLA-A*2402-restricted T-cell epitopes (HLA, human leukocyte antigen) derived from AFP, we found that AFP-specific T-cell responses exist in patients with HCC but are weak during the early stage of the tumor, and that anticancer treatment can enhance host immune responses. By studying peripheral blood mononuclear cells (PBMCs) from 38 patients, we have shown that the induction of AFP-specific T cells is possible independent of hepatitis viral infection and that the number of AFP-specific T cells is as frequent as that of other tumor associated antigens in patients with advanced HCC. Moreover, HCC treatment dramatically changes the strength of AFP-specific immune responses, mostly by increasing the frequency of AFP-specific CD⁸⁺ T-cell responses. These results provide a rationale for T-cell-based immunotherapy for HCC and suggest that the identified AFP epitopes could be a valuable component of HCC therapy and for analyzing host immune responses to HCC.

Material and methods

Patient population
In our study, we examined 38 HLA-A24 positive patients with HCC who were admitted to Kanazawa University Hospital between January 2002 and August 2003, consisting of 30 men and 8 women ranging from 46 to 80 years, with a mean age of 68.6 ± 7.0. HCCs were detected by imaging modalities such as dynamic CT scan, MR imaging, and abdominal arteriography. The diagnosis of HCC was histologically confirmed by taking US-guided needle biopsy specimens from 17 patients, surgical resection from 3 patients and autopsy from 4 patients. For the remaining 14 patients, diagnosis was made by typical hypervascular tumor staining on angiography in addition to using typical findings, which showed hyperattenuation areas in the early phase and hypotension in the late phase on dynamic CT.¹⁷ All subjects were negative for Abs to human immunodeficiency virus (HIV) and gave informed consent to this study in accordance with the Helsinki declaration. Eleven healthy blood donors with HLA-A24, who did not have a history of cancer and were negative for HBsAg and anti-hepatitis C virus antibody (HCVAb) served as controls.

Treatment of HCC
After diagnosis, 12 patients were treated by percutaneous tumor ablation using percutaneous ethanol injection therapy or RF ablation, 3 by TAE, 4 by chemotherapy, 3 by surgical operation, 13 by...
Severity of liver disease (stage of fibrosis) was evaluated according
clinical and pathologic study of primary liver cancer.18 The
Japan, Tokyo, Japan) and pathological grading of tumor cell dif-
ficient-dependent microcytotoxicity, using HLA typing trays
by standard enzyme immunoassay (EIA) techniques for the detec-
Laboratory and virologic testing
were drawn from patients before and 1–3 months after treatment.
follow-ups were conducted
characteristics of
Combination of TAE and chemotherapy. The characteristics of
Internationale Contre Le Cancer (UICC) classification system (6th version).–4NBNC, nonB, nonC.
Histological degree of HCC.–2Tumor size was divided into either
well, well differentiated; mod, moderately differentiated; por, poorly differentiated; ND, not determined.
Serum AFP level was measured by EIA (AxSYM AFP, Abbott
Blood samples were tested for HBsAg, HCVAb and HIVAb by
by analytical HPLC.
Synthetic peptides
To identify potential HLA-A24-binding peptides within AFP
restricted epitopes derived from HIV envelope protein.20 EBV
restricted epitope derived from AFP21 was used as a control pep-
type for HLA-A24 stabilization assay. Peptides were synthesized at
Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals
restricted epitope from EBV envelope protein22 were used as con-
Cell lines
Three human hepatoma cell lines, HepG2, Huh7 and HLE, were
cultured in DMEM (Gibco, Grand Island, NY) with 10% fetal calf
serum (FCS) (Gibco).
T2-A24 cells, which were transfected with HLA-A*2402 mole-
cule into T2 cells,22 were cultured in RPMI 1640 medium contain-
After another 4 washes, streptavidin-AP was added for 2 hr. Medium containing 5% FCS for 2 hr at 25°C (MFI) or % MFI increase, which was calculated as follows: %MFI = (cytotoxic activity in the presence of antigen) / (cytotoxic activity in the absence of peptide).

Preparation of PBMCs
Blood samples were diluted twice in phosphate-buffered saline (PBS) and loaded on ficoll gradients (AXIS-SHIELD PoC AS, Oslo, Norway) in 50 ml tubes. After centrifugation at 900g for 22 min at room temperature, PBMCs were harvested from the interphase, resuspended in PBS and centrifuged again at 600g. Each cell pellet was resuspended in PBS, centrifuged at 300g for 8 min and finally resuspended in complete culture medium consisting of RPMI, 10% heat inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Fresh PBMCs were used for CTL assay, and the remaining PBMCs were resuspended in RPMI 1640 containing 80% FCS and 10% dimethyl sulfoxide (Sigma) and cryopreserved until use.

MHC binding assay
Peptide binding assays were performed as previously described,24 with the following modification. T2-A24 cells (transfected with the HLA-A*2402 molecule, and human hepatoma cell lines were used as target cells for CTL lines. C1R-A24 cells transfected with the HLA-A*2402 molecule, and human hepatoma cell lines were used as target cells for CTL lines. C1R-A24 cells were incubated overnight with 100 μg/ml hygromycin B (Sigma, St Louis, MO), and K562 was cultivated in RPMI 1640 medium containing 10% FCS. All medium contained 100 U/ml penicillin and 100 μg/ml streptomycin (GibcoBRL).

Stimulation of PBMC with synthetic peptides
AFP-derived peptide-specific T cells were expanded from PBMCs in 96-well round bottom plates (NUNC, Naperville, IL) as previously described.25 Briefly, 400,000 cells/well were stimulated with synthetic peptides at 10 μg/ml, 10 ng/ml rIL-7 and 100 pg/ml rIL-12 (Sigma) in RPMI 1640 supplemented with 10% heat inactivated human AB serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were restimulated with 10 μg/ml peptide, 20 U/ml rIL-2 (Sigma) and 10μg/ml mytomycin C treated autologous PBMCs on days 7 and 14. On days 3, 10 and 17, 100 μl of RPMI with 10% human AB serum and 10 U/ml rIL-2 (final concentration) was added to each well.

Cytotoxicity assay
C1R-A24 cells, which are human lymphoblastoid HMYC1R cells transfected with the HLA-A*2402 molecule, and human hepatoma cell lines were used as target cells for CTL lines. C1R-A24 cells were incubated overnight with 10 μg/ml synthetic peptides and labeled with 25 μCi of 51Cr (Amersham, Arlington Heights, IL) for 1 hr. Hepatoma cell lines were labeled with 25 μCi of 51Cr for 1.5 hr without incubation with peptides. After 3 washes with PBS, the target cells were plated at 3,000 cells/well with complete medium in round-bottom 96-well plates. Unlabeled K562 cells at 120,000 cells/well were added to reduce nonspecific lysis. Stimulated PBMCs from patients were added at effector to target ratios of 100:1, 50:1, 25:1, 13:1, 6:1 and 3:1, respectively. For Ab-blocking assay, effector cells of 51Cr-labeled target cells were preincubated with each monoclonal antibody (MAB) for 20 min at room temperature. The percent cytotoxicity was determined from the formula: 100 × [(experimental release – spontaneous release) / (maximum release – spontaneous release)], and maximum release was determined by lysis of 51Cr-labeled targets with 5% Triton X-100 (Sigma Chemical). Spontaneous release was <15% of maximum release for all experiments. The specific cytotoxic activity was calculated as follows: (cytotoxic activity in the presence of peptide) – (cytotoxic activity in the absence of peptide).

### TABLE II – PEPTIDES

| Peptide | Source | Start position | Amino acid sequence | HLA restriction | Score1 |
|---------|--------|----------------|---------------------|----------------|--------|
| AFP403  | AFP    | 403            | KYIQESQAL           | HLA-A24        | 720    |
| AFP424  | AFP    | 424            | EYYLOQAFNL          | HLA-A24        | 200    |
| AFP434  | AFP    | 434            | AYTKKAPQQL          | HLA-A24        | 200    |
| AFP557  | AFP    | 557            | EYSSRHRPQQL         | HLA-A24        | 200    |
| AFP580  | AFP    | 580            | SYANRPPCF           | HLA-A24        | 100    |
| AFP591  | AFP    | 591            | CFAEEGQKL           | HLA-A24        | 32     |
| AFP614  | AFP    | 614            | RSCGLFOQQL          | HLA-A24        | 15     |
| AFP617  | AFP    | 617            | IFLFLLLNF           | HLA-A24        | 15     |
| AFP622  | AFP    | 622            | KPEGLSPN1           | HLA-A24        | 14     |
| HIVenv584| HIV  | 584            | RYLRRQQL           | HLA-A24        | 720    |
| EBV1m287| EBV latent membrane | 287 | TYGPVPFSL          | HLA-A24        | 403    |
| CMVpp65 | CMV pp65 | 328 | QYDPVAAALF         | HLA-A24        | 120    |
| AFP132  | AFP    | 132            | PLFQVPFPV           | HLA-A2 | 3      |

1Estimated half-time of dissociation from the HLA-A24 allele (min).
Statistical analysis

Fisher’s exact test (2-sided p-value) and the unpaired Student’s t-test were used to analyze the effect of variables on immune responses in HCC patients.

Results

Patient profiles

The clinical profiles of the patients are shown in Table I. Thirty were positive for AFP ranging from 10 to 50,800 ng/ml. The tumors of 24 patients were histologically diagnosed as HCC, and their differentiation was well, moderate and poor for 11, 12 and 1 cases, respectively. Other tumors were diagnosed as being HCC by typical CT findings and AFP elevation. The tumor size was categorized as “small” (≤2 cm) for 10 cases or “large” (>2 cm) for 28 cases, and tumor multiplicity was categorized as “multiple” (≥2 nodules) for 23 cases or “solitary” (single nodule) for 15 cases. Vascular invasion of HCC was observed in 11 cases. The TNM stage was classified according to the Union Internationale Contre Le Cancer classification system (6th version), where 13, 13, 5, 1, 2 or 4 patients had Stage I, II, IIIa, IIIb, IIIc or IV tumors, respectively. Thirty-seven patients received HCC treatment as described in Material and methods.

Selection of potential HLA-A24-binding peptides within AFP

To identify potential HLA-A24-binding peptides, the amino acid sequences of AFP were analyzed using a computer program designed to predict HLA-binding peptides (available at BIMAS website) based on the estimation of the half-time dissociation of the HLA-peptide complex. Ten peptides were selected according to the order of the high half-time dissociation scores (Table II). Next, MHC stabilization assays were performed to test these peptides for HLA-A*2402 binding capacity using T2-A24 cells. Most peptides increased HLA-A24 expression on the cells, indicating that they bound and stabilized the HLA complex on the cell surface except for peptides AFP591, AFP5 and AFP322 (Fig. 1a). Peptide CMVpp65328, which is identified as a strong binder of the HLA-A24 molecule, increased HLA-A24 expression, but peptide AFP137, which is HLA-A2 restricted, did not increase the expression, suggesting that the assay was specific for HLA-A24.

To confirm these results, a HLA-A24 stabilization assay was performed at different concentrations using several representative peptides. As shown in Figure 1b, a positive control peptide and representative AFP-derived peptides increased HLA-A24 expression depending on the concentrations, but this did not occur for the HLA-A2-restricted peptide.

Immunogenicity of AFP peptides assessed by IFN-γ ELISPOT analysis

To determine whether these HLA-A24 binding peptides could be recognized by the T cells of patients with HCC, IFN-γ ELISPOT responses were evaluated with ex vivo PBMCs. Seven of 10 AFP-derived peptides were recognized by PBMCs of at least
177 peptide-specific IFN-γ-producing cells were detected in 5 (32.2%), 3 (7.9%), 8 (21.1%), 7 (18.4%), 2 (5.1%), 3 (7.9%) and 2 (5.1%) of the 38 patients for peptides AFP403, AFP424, AFP434, AFP591, AFP594 and AFP414, respectively. Peptides AFP403, AFP7 and AFP322 were not recognized by any patient. Among the peptides, AFP403, AFP7 and AFP322 displayed a relatively low binding affinity for the HLA-A*2402 molecule compared with the other peptides (Fig. 1a). In contrast, peptides AFP150, AFP504 and AFP414, which were derived from EBV latent membrane or CMV pp65 protein, respectively, and are strongly immunogenic. These data show that AFP-derived peptides with a high binding affinity for the HLA-A*2402 molecule were also immunogenic.

The strength of the AFP-specific T-cell responses assessed by the frequency of IFN-γ-producing cells in the PBMC population is shown in Figure 2a. The maximum response was quantitated as 177 peptide-specific IFN-γ-producing cells per 3 × 10^6 PBMCs. Most patients, however, displayed between 10 and 60 specific cells per 3 × 10^6 PBMCs. The frequency of positive T-cell responses was lower than that of peptides EBV Bl287 and CMV pp65, which were derived from EBV latent membrane protein and CMV pp65 protein, respectively, and are strongly immunogenic. All the patients who showed positive T-cell responses against EBV Bl287 or CMV pp65 were sero-positive for EBV or CMV, respectively. No patient exhibited positive T-cell responses against peptides HIVenv584 derived from the HIV envelope protein, suggesting that these T-cell responses were antigen-specific.

In contrast to the results for the HCC patients, the ELISPOT assays for the normal donors did not show any IFN-γ-producing cells against AFP-derived peptides (Fig. 2b), but the ratio of normal donors who showed positive T-cell responses for EBV or CMV protein-derived peptides and the frequency of T cells were not significantly different from those of the HCC patients (Fig. 2b). On the basis of these results, we selected peptides AFP403, AFP424, AFP434, AFP591, AFP504 and AFP414 as possible peptides that contain a CD8 T-cell epitope.

Identification of AFP-derived peptides that elicit a primary CTL response

The 7 selected AFP-derived peptides were tested for their potential to induce HLA-A24-restricted CTLs using the PBMCs from the HCC patients with HLA-A24. Each peptide was tested on at least three patients. After 3 rounds of stimulation, responder cells that had been stimulated with peptides AFP403, AFP424, AFP434, AFP357 and AFP414 lysed the peptide-pulsed C1R-A24 and K562 that did not express HLA molecules (Figs. 3a and 3b). Thus, the CTLs induced by the selected peptides contained an epitope that is endogenously processed within the AFP producing cells.

CTLs incubated without any Ab did not show cytotoxicity against K562 that did not express HLA molecules (Figs. 4a and 4b). Thus, we confirmed that the AFP-derived peptide-specific T-cell response was mediated by CD8+ T cells and restricted by HLA-A24. In addition, together with the results of the ELISPOT assay, the data revealed that the peptide contains an epitope that is endogenously processed within the AFP producing cells.

AFP-specific T-cell responses and clinical features of HCC patients

To evaluate the status of AFP-specific T-cell responses in patients with HCC, we analyzed the relationships between the frequency of peptides AFP403, AFP424, AFP434, AFP591 or AFP414-specific T cells and the clinical features of patients by IFN-γ ELISPOT assay. AFP-specific IFN-γ producing cells in the peripheral blood were observed in 14 of the 30 (47%) patients with AFP-positive serum and were also observed in 4 of the 8 (50%) patients with AFP-negative serum. In 2 of the 4 patients who showed serum AFP negative but positive AFP-specific IFN-γ producing cells in the peripheral blood, serum AFP increased during the follow-up period. One out of the 4 patients could not be followed up because the patient had died. Thus, only 1 patient was confirmed to continuously have serum AFP below the detection limit during the follow-up period. In addition, analysis of the relationship between serum AFP levels and the positive rate of patients who had AFP-specific IFN-γ producing cells did not show a statistical correlation (Table III). These results suggest that the amount of AFP in serum is not associated with the induction of AFP-specific T cells.

Tumor factors indicated by the TNM classification (T2–T4 vs. T1) or TNM stage (Stage II–IV vs. Stage I) for the group with positive T-cell responses were significantly more advanced (p = 0.006) than those for the group without positive T-cell responses (Table III). Positive T-cell responses for the 5 peptides were observed in only 2 patients with TNM Stage I. Also, tumor multiplicity showed the same tendency between the 2 groups, although it was not significant. Differentiation of HCC, vascular invasion, histology of the nontumor liver, liver function and the type of viral infection were not associated with AFP-specific host immune responses (Table III).

Effect of anticancer treatment on AFP-specific T-cell responses

To analyze the effect of anticancer treatment on AFP-specific T-cell responses, we prospectively evaluated the T-cell responses for peptides AFP403, AFP424, AFP434, AFP591, AFP504 or AFP414 in 17 randomly selected patients undergoing HCC treatment. The frequency of AFP-specific T cells increased from 2 to 25 fold in 7 of the 17 patients after treatments (Fig. 6). In contrast, HIV-specific T-cell responses did not increase in all patients and CMV-specific T-cell responses increased in only 2 patients (Patients 14 and 31) (Fig. 6). These results suggest that the effect of anticancer treatment on the T-cell response is specific for AFP. The clinical profiles of the patients with or without increasing AFP-specific T-cell responsiveness after HCC treatment are shown in Table IV. The analyses of both patient groups showed that there were no differences in clinical factors except for the TNM stage. The ratio of

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FIGURE 2.
patients with TNM Stage I or II was greater for patients with increasing T-cell responsiveness than for those without (Table IV). Furthermore, 5 of the 7 patients who showed increasing AFP-specific T-cell responsiveness after HCC treatment did not show a response before treatment.

Discussion

AFP is a sugar-containing protein ~70 kDa in molecular weight\(^2\) and is produced at high levels by the yolk sac and fetal liver. In adults, AFP is produced by 80% of HCC and certain germ cell tumors, and production increases in benign liver diseases such as chronic hepatitis and cirrhosis.\(^\text{29,30}\) Furthermore, the expression of AFP in cancerous tissue is related to the biological malignancy of HCC.\(^\text{31}\) Recent studies reported that AFP-specific T-cell clones are not deleted during ontogeny and that AFP is recognized by murine\(^\text{16}\) and human T cells\(^\text{13–15}\) and serves as a tumor rejection antigen in a murine tumor model.\(^\text{16}\) Therefore, AFP has the potential of being a target of immunotherapy for HCC. However, the number of AFP epitopes that have been identified is limited and the status of AFP-specific immunological responses has not been well-characterized for patients with HCC. To address this issue, we tried to identify HLA-A*2402-restricted T-cell epitopes derived from AFP and to analyze the relationship between AFP-specific immunological responses and clinical features in HCC patients.

First, we attempted to identify AFP epitopes restricted by HLA-A24 that are present in 60% of Japanese, 20% of Caucasians and 12% of Africans,\(^\text{32,33}\) using a combined computer-based and immunological approach. Analysis of amino acid sequences of AFP by computer revealed a number of potential HLA-A24-binding peptides, and most of them functionally stabilized HLA-A*2402 molecules expressed in the peptide transporter-deficient cell line T2-A24. Five AFP-derived peptides (Peptides AFP403, AFP424, AFP434, AFP357 and AFP414) showing HLA-A*2402 binding affinity induced IFN-\(\gamma\) production of PBMCs and T-cell lines that showed cytotoxicity against the peptide-pulsed C1R-A24 cells. In addition, these T-cell lines showed cytotoxicity against hepatoma cell lines that expressed HLA-A*2402 and AFP, but did not show it against other hepatoma cell lines without HLA-A*2402 or AFP expression, suggesting that the cytotoxicity was HLA-A24-restricted and AFP-specific. Taken together with the result that cytotoxicity was inhibited by incubation with anti-CD8 Mb and anti-HLA-A24 MAb, we confirmed that the 5 peptides contained HLA-A24-restricted AFP epitopes that were endogenously processed within the AFP producing cells.

To study the status of host immunological responses to AFP in HCC patients, we examined the frequency of AFP-specific T cells in the peripheral blood by ELISPOT assay with the 5 epitopes, and analyzed the relationships between the frequency and the clinical features of the patients. ELISPOT assay showed that the frequency of reactive T cells to a single AFP epitope was 30–190 per 1 \(\times\) 10\(^6\) PBMCs. On the other hand, ELISPOT assay using HIV envelope-derived peptide did not show any positive T-cell responses. In addition, all the patients who showed positive T-cell responses against EBVIm\(_{287}\) or CMVpp65\(_{528}\) were sero-positive.
for EBV or CMV, respectively. These results suggest that the
ELISPOT responses are correlated with their serological results
and these peptides may be recall antigens. In previous reports
regarding the frequency of T cells specific for a single tumor asso-
ciated antigen epitope, the number of specific T cells for tyrosi-
nase, MelanA/MART-1, gp100 or CEA in patients with melanoma
or colorectal cancer was found to be 11–130 per $1 \times 10^6$
PBMCs. In addition, single AFP epitope-specific IFN-γ pro-
ducing cells were detected in 5.1–21.1% of the patients for peptide
AFP$_{403}$, AFP$_{424}$, AFP$_{434}$, AFP$_{357}$ or AFP$_{314}$. These rates are
similar to previously reported epitopes for tyrosinase, MelanA/
MART-1, gp100, Her-2/neu and CEA. Comparing the present

**Figure 4** – Cytotoxicity of AFP-specific T-cell lines on cancer
cell lines that do or do not express HLA-A*2402 or AFP. The cyto-
toxicity was determined using a standard 6 h cytotoxic assay (E/T
ratio of 50:1).

**Figure 5** – Inhibition of cytotoxicity of AFP-specific T-cell
lines by specific antibodies. T-cell lines were generated from PBMC
of HCC patients by stimulation with AFP$_{357}$. Inhibition of cytotox-
icity was determined using a standard 6 h cytotoxic assay against
C1R-A*2402 cells pulsed with AFP$_{357}$ (a) or HepG2 cells (b)
incubated with anti-CD4, -CD8 or -HLA-A24 MAbs (E/T ratio, (a)
50:1; (b) 20:1). Cytotoxicity of AFP-specific T-cell lines against
K562 cells was also examined for the same E/T ratio.
results with those reports, we believe that AFP-specific T-cell responses in patients with advanced HCC are as strong as other tumor associated antigen-specific T-cell responses, and that the newly identified AFP epitopes are immunogenic.

For the analysis of clinical factors and frequency of AFP-specific IFN-\(\gamma\) producing cells, we obtained evidence that the frequency of the patients with advanced tumor stages for the group with AFP-specific immune responses was significantly higher (\(p = 0.006\)) than that for the group without the responses (Table III). In other words, tumor stages were associated with AFP-specific immune responses. These results might be explained by the invasion of tumor cells into micro vessels, extra capsules or lymph nodes that can induce T cells. In accordance with our results, a higher frequency of T cells against epithelial cell adhesion molecule, her-2/neu or CEA was also reported among patients with advanced colorectal cancer.\(^4\),\(^5\)

Other factors, including serum AFP levels, histology of the non-tumor liver, liver function and hepatitis viral infections were not significantly different between patients with and without positive T-cell responses. Specially, the frequency of peripheral AFP-specific T cells was not correlated with serum AFP levels. This result is consistent with the previously demonstrated results that frequencies and function of AFP-specific T cells were not reduced in HCC patient independent of serum AFP levels.\(^4\),\(^0\) In the present study, AFP-positive T-cell responses were observed even in 4 of 8 (50%) patients with AFP-negative serum, and 2 of the 4 patients with AFP-negative serum but who were positive for AFP-specific T cells in the peripheral blood showed an increase in serum AFP levels.

**Peptide**

**FIGURE 6** – The induction of AFP-specific T-cell responses in HCC patients after treatment of HCC. Direct *ex vivo* analysis (IFN-\(\gamma\) ELISPOT assay) of peripheral blood T-cell responses to AFP-, HIV- or CMV-derived peptides were performed before (open bar) and after (solid bar) HCC treatment. Only patients with a significant change in the T-cell response to peptides AFP\(_{303}\), AFP\(_{424}\), AFP\(_{577}\), or AFP\(_{314}\) were included in the figure. A significant change in the IFN-\(\gamma\) response was defined as a more than twofold increase and the presence of more than 10 specific spots after HCC treatment. The data are expressed as the number of IFN-\(\gamma\) producing cells before and after treatment. The characteristics of the patients are shown in Table I, and the peptide sequences are described in Table II. * denotes 188 specific spots; **, 177 specific spots; ***, 59 specific spots; ****, 68 specific spots and ******, 81 specific spots.

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**TABLE III – UNIVARIATE ANALYSIS OF THE EFFECT OF VARIABLES ON THE T-CELL RESPONSE AGAINST AFP**

|                         | Patients with a positive T-cell response | Patients without a positive T-cell response | \(p\)-value |
|-------------------------|------------------------------------------|--------------------------------------------|-------------|
| No. of patients         | 18                                       | 20                                         |             |
| Age (years)\(^3\)       | 68.1 ± 6.8                               | 65.5 ± 8.9                                 | NS          |
| Sex (M/F)               | 15/3                                     | 15/5                                       | NS          |
| AFP level (\(\leq 20/\> 20\)) | 6/12                                     | 9/11                                       | NS          |
| Diff. degree of HCC (well/moderate or poor/ND) | 5/9/4                                    | 6/5/9                                      | NS          |
| Tumor multiplicity (multiple/solitary) | 14/4                                     | 9/11                                       | NS          |
| Vascular invasion (+/−) | 7/11                                     | 4/16                                       | NS          |
| TNM factor              | (T1/T2–4)                                | (N0/N1)                                    |             |
|                         | 2/16                                     | 11/9                                       | 0.006       |
|                         | (M0/M1)                                  | 18/0                                       | NS          |
|                         | 14/4                                     | 17/0                                       | NS          |
| TNM stage (I/II–IV)     | 2/16                                     | 11/9                                       | 0.006       |
| Histology of nontumor liver (LC/chronic hepatitis) | 16/2                                     | 17/3                                       | NS          |
| Liver function (Child A/B/C) | 12/6/0                                | 12/6/2                                     | NS          |
| Etiology (HCV/HBV/others) | 14/3/1                                 | 18/1/1                                     | NS          |

NS, no statistical significance; ND, not determined.

\(^3\) Data expressed as mean ± SD.
during the follow-up period. In addition, it has been noted that tissue-AFP in HCC is positive in some patients with lower or negative-AFP. 

Taken together, these results suggest that AFP-specific IFN-γ producing cells in the peripheral blood are more useful than serum AFP to detect HCC producing AFP at an early stage. Furthermore, the frequency of T cells reactive toward a single AFP epitope was equal or higher than that for a single HCV epitope. These results suggest that immunotherapy of HCC could be possible independent of hepatitis viral infection, which causes host immune disorders because of the impairment of dendritic cells.

Further, to understand host immune responses for HCC, the newly identified AFP epitopes were then used to analyze the immunological effects of HCC treatments, including tumor ablation, TAE and chemotherapy. The question regarding whether inhibition of HCC aided by antitumor treatments affects host cellular immune responses remains unknown. In the present study, we found that the frequency of AFP-specific T cells increased in 7 patients after HCC treatments and only increased for AFP but not for viral antigens. These results indicate that the effect of treatments on the host immune response is specific for HCC associated antigens.

For the analysis of factors associated with altered AFP-specific T-cell responses, we found that the ratio of patients with TNM Stage I or II is greater for patients with increasing T-cell responsiveness than for those without. Furthermore, 5 of the 7 patients did not show an AFP-specific T-cell response before treatment, but showed one afterward. These results suggest that HCC treatments have the possibility to restore tumor-specific T-cell responses, which are weak in patients with early stage HCC. Consistent with our findings, increased numbers of lymphocytes, natural killer cells and macrophages has been reported to be present at the tumor site after percutaneous microwave coagulation therapy (PMCT). The mechanisms that enhance host immune responses because of HCC treatment are unknown, but the following are suggested. First, AFP antigen recognized by T cells may increase because of destruction of the tumor. Second, the inhibition of host immune responses by HCC is relaxed because of tumor ablation. Finally, the factors that enhance host immune responses, including cytokines, are induced by inflammation caused by HCC treatment.

Although further studies are necessary to understand the precise mechanisms, these results suggest that HCC treatments might be able to enhance host immune responses and that the newly identified AFP epitopes could be useful for analyzing host immune responses for HCC.

In conclusion, we identified and characterized novel HLA-A*2402-restricted T-cell epitopes derived from AFP. The newly identified epitope-specific T cells can be detected and induced by PBMC stimulation with these peptides in HCC patients. The frequency of AFP-specific T cells is the same as that of other immunogenic cancer associated antigen-derived epitopes in patients with advanced HCC, but is lower during the early stages of the tumor. On the other hand, antitumor treatments have the possibility to enhance the host immune responses and restore weak responses. These results may provide a rationale for T-cell-based immunotherapy against HCC, and suggest that the identified AFP epitopes could be a valuable component for HCC immunotherapy and for analyzing host immune responses.

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