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

Eishiro Mizukoshi, Yasunari Nakamoto, Hirokazu Tsuji, Tatsuya Yamashita and Shuichi Kaneko*

Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa, Japan

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.
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Introduction

Hepatocellular carcinoma (HCC) is a malignancy[1,2] and has gained major clinical interest because of its increasing incidence.[3] Several current advances in therapeutic modalities such as surgical hepatic resection, percutaneous tumor ablation by ethanol injection or radiofrequency (RF), transcatheter arterial embolization (TAE), chemotherapy 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 CD4⁺ 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 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 hypoattenuation 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

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*Correspondence to: Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-8641, Japan.
Fax: +81-76-234-4250. E-mail: skaneko@m.kanazawa-u.ac.jp

TABLE I – CHARACTERISTICS OF THE PATIENTS STUDIED

Case	Age (years)	Sex (M/F)	AFP level (ng/ml)	Diff. degree[1]	Tumor size[2]	Tumor multiplicity	Vascular invasion	TNM stage[3]	Nontumor liver	Liver function	Etiology[4]	Treatment
1	67	M	46	Mod	Large	Multiple	−	II	Cirrhosis	Child A	HCV	TAE + RF
2	72	M	16	Wel	Large	Multiple	−	II	Cirrhosis	Child A	HCV	RF
3	69	M	22	ND	Small	Multiple	−	II	Cirrhosis	Child B	HCV	RF
4	58	F	50,800	ND	Large	Multiple	+	IIIc	Cirrhosis	Child B	HCV	Chemotherapy
5	65	F	94	ND	Small	Solitary	−	I	Cirrhosis	Child C	HCV	No treatment
6	72	M	12	ND	Small	Solitary	−	I	Cirrhosis	Child B	HCV	RF
7	66	M	471	ND	Large	Multiple	+	IV	Cirrhosis	Child A	HCV	TAE + RF
8	79	M	19	Mod	Large	Multiple	+	IIIc	Cirrhosis	Child A	HCV	TAE + PETT
9	63	M	154	Mod	Large	Solitary	−	I	Chronic hepatitis	Child A	HBV	Surgical resection
10	70	F	13	Wel	Large	Solitary	−	I	Cirrhosis	Child A	HCV	RF
11	67	M	41	Wel	Large	Multiple	−	IV	Cirrhosis	Child B	HCV	TAE + chemotherapy
12	67	F	330	Wel	Small	Solitary	−	I	Cirrhosis	Child A	HCV	TAE + RF
13	69	M	13	ND	Large	Solitary	−	I	Cirrhosis	Child A	HCV	TAE
14	73	M	332	Mod	Large	Solitary	+	IV	Cirrhosis	Child B	HCV	TAE + chemotherapy
15	56	M	<10	ND	Large	Multiple	+	IV	Chronic hepatitis	Child A	HBV	Chemotherapy
16	66	F	<10	ND	Large	Multiple	+	IIIa	Cirrhosis	Child A	HCV	Chemotherapy
17	55	M	<10	ND	Small	Solitary	−	I	Cirrhosis	Child B	HCV	RF
18	77	M	75	Wel	Small	Multiple	+	IIIa	Cirrhosis	Child A	HCV	TAE + RF
19	54	M	21	Mod	Large	Multiple	−	II	Cirrhosis	Child A	HCV	RF
20	71	M	<10	Mod	Large	Multiple	−	II	Cirrhosis	Child A	NBNC	RF
21	80	M	94	ND	Large	Multiple	−	II	Cirrhosis	Child A	HCV	TAE + PEIT
22	73	M	26	Mod	Large	Multiple	+	IIIb	Cirrhosis	Child A	HCV	Chemotherapy
23	59	M	1260	Wel	Large	Multiple	+	II	Cirrhosis	Child B	HCV	TAE + RF
24	59	M	<10	ND	Large	Solitary	−	IIIa	Cirrhosis	Child A	HBV	TAE + RF
25	46	M	287	Mod	Small	Solitary	−	I	Cirrhosis	Child A	HCV	Surgical resection
26	68	M	46	Wel	Small	Multiple	−	II	Cirrhosis	Child A	HCV	PEIT
27	52	M	11,291	Por	Large	Multiple	+	IIIa	Chronic hepatitis	Child C	HBV	TAE + RF
28	66	F	67	Mod	Large	Multiple	−	II	Cirrhosis	Child B	HCV	TAE
29	66	F	247	ND	Large	Multiple	+	IIIa	Cirrhosis	Child A	HCV	TAE
30	76	M	16	ND	Small	Solitary	−	I	Cirrhosis	Child A	HCV	RF
31	62	M	341	Mod	Large	Multiple	−	II	Cirrhosis	Child B	HCV	TAE + RF
32	71	M	<10	Wel	Large	Multiple	−	II	Chronic hepatitis	Child A	HCV	TAE + RF
33	76	M	<10	Wel	Large	Solitary	−	I	Chronic hepatitis	Child A	HCV	Surgical resection
34	79	M	22	Wel	Large	Multiple	−	II	Cirrhosis	Child B	HCV	TAE + RF
35	67	M	18	Wel	Large	Solitary	−	I	Cirrhosis	Child A	HCV	PEIT
36	70	F	30	Mod	Small	Solitary	−	I	Cirrhosis	Child B	HCV	RF
37	71	M	<10	Mod	Large	Multiple	−	II	Cirrhosis	Child A	HCV	RF
38	58	M	46	ND	Large	Solitary	−	I	Cirrhosis	Child B	NBNC	TAE + RF

wel, well differentiated; mod, moderately differentiated; por, poorly differentiated; ND, not determined.
[1]Histological degree of HCC.–[2]Tumor size was divided into either 'small' (≤ 2 cm) or 'large' (>2 cm).–[3]TNM stage according to the Union Internationale Contre Le Cancer (UICC) classification system (6th version).–[4]NBNC, nonB, nonC.

a combination of TAE and perucutaneous tumor ablation and 2 by a combination of TAE and chemotherapy. The characteristics of the patients are shown in Table I. The treatment efficacy was evaluated by complete necrosis of the tumor lesion using dynamic CT after the completion of the treatment. Follow-ups were conducted at outpatient clinics, using blood tests, USG and dynamic CT, every 3 months for 1 year. Blood samples, including lymphocytes, were drawn from patients before and 1–3 months after treatment.

Laboratory and virologic testing

Blood samples were tested for HBsAg, HCVAb and HIVAb by commercial immunoassays (Fuji Rebio, Tokyo, Japan). Epstein-Barr virus (EBV) and cytomegalo virus (CMV) serology was done by standard enzyme immunoassay (EIA) techniques for the detection of the specific IgG, using commercial assays. HLA typing of PBMC from patients and normal donors was performed by complement-dependent microcytotoxicity, using HLA typing trays purchased from One Lambda (Canoga Park, CA).

Serum AFP level was measured by EIA (AxSYM AFP, Abbott Japan, Tokyo, Japan) and pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathologic study of primary liver cancer.[18] The severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet et al.[19] using the biopsy specimens of liver tissue, where F4 was defined as cirrhosis.

Synthetic peptides

To identify potential HLA-A24-binding peptides within AFP (GenBank accession number J00077, J00076 and V01514), a computer-based program available at BioInformatics and Molecular Analysis Section (BIMAS) website was employed. The HLA-A24 restricted epitopes derived from HIV envelope protein,[20] EBV latent membrane protein 2A[21] and CMV pp65[22] were used as control peptides to test for T-cell responses, and the HLA-A2 restricted epitope derived from AFP[14] was used as a control peptide for HLA-A24 stabilization assay. 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 HPLC.

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 molecule into T2 cells,[22] were cultured in RPMI 1640 medium contain-

TABLE II – PEPTIDES

Peptide	Source	Start position	Amino acid sequence	HLA restriction	Score[1]
AFP_{403}	AFP	403	KYIQESQAL	HLA-A24	720
AFP_{424}	AFP	424	EYYLQNAFL	HLA-A24	200
AFP_{434}	AFP	434	AYTKKAPQL	HLA-A24	200
AFP_{357}	AFP	357	EYSRRHPQL	HLA-A24	200
AFP_{150}	AFP	150	AYEEDRETF	HLA-A24	180
AFP_{504}	AFP	504	SYANRRPCF	HLA-A24	100
AFP_{591}	AFP	591	CFAEEGQKL	HLA-A24	32
AFP_{414}	AFP	414	RSCGLFQKL	HLA-A24	15
AFP_7	AFP	7	IFLIFLLNF	HLA-A24	15
AFP_{322}	AFP	322	KPEGLSPNL	HLA-A24	14
$HIVenv_{584}$	HIV envelope	584	RYLRDQQLL	HLA-A24	720
$EBV1m_{287}$	EBV latent membarne	287	TYGPVFMSL	HLA-A24	403
$CMVpp65_{328}$	CMV pp65	328	QYDPVAALF	HLA-A24	120
AFP_{137}	AFP	137	PLFQVPEPV	HLA-A2	3

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

ing 10% FCS and 800 μg/ml G418 (GibcoBRL, Grand Island, NY). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24)[23] was cultured in RPMI 1640 medium containing 10% FCS and 500 μg/ml hygromycin B (Sigma, St Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS. All medium contained 100 U/ml penicillin and 100 μg/ml streptomycin (GibcoBRL).

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 PBMC 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 (transporter associated with antigen processing [TAP]-deficient human lymphoid-derived cells transfected with HLA-A*2402 molecule) were cultured for 16 hr at 26°C to enhance the expression of peptide-receptive cell surface molecules. After the addition of synthetic peptides, the cells were incubated at 37°C for 2 hr to unfold HLA-A*2402 molecules not stabilized by peptide binding. The cells were then washed and stained with anti-HLA-A24 monoclonal antibody (Sankojunyaku, Tokyo, Japan), anti-mouse immunoglobulin conjugated FITC (DAKO, Glostrup, Denmark) and 1 μg/ml of propidium iodide. Live cells were gated based on forward and side scattering and the exclusion of propidium iodide-positive cells. The data were expressed as the mean fluorescence intensity (MFI) or % MFI increase, which was calculated as follows: %MFI increase = (MFI with the given peptide – MFI without peptide) / (MFI without peptide) × 100.

Enzyme linked immunospot assay

Ninety-six-well plates (Millititer; Millipore, Bedford, MA) were coated with anti-human interferon-γ (IFN-γ) Ab Mabtech, Nacka, Sweden) at 4°C overnight and then washed 4 times with sterile PBS. The plates were next blocked with RPMI 1640 medium containing 5% FCS for 2 hr at 25°C. Three hundred thousand unfractionated PBMCs were added in duplicate cultures of RPMI 1640 containing 5% FCS together with the peptides at 10 μg/ml. After 24 hr, the plates were washed 8 times and incubated overnight with 100 μl of biotin conjugated anti-human IFN-γ Ab. After another 4 washes, streptavidin-AP was added for 2 hr.

Finally, the plates were washed again 4 times with PBS and developed with freshly prepared NBT/BCIP solution (Biorad, Hercules, CA). The reaction was stopped by washing with distilled water, and after drying at room temperature, colored spots with fuzzy borders, which indicated the presence of IFN-γ secreting cells, were counted. The number of specific spots was determined by subtracting the number of spots in the absence of antigen from the number of spots in the presence of antigen. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen. Positive controls for IFN-γ enzyme linked immunospots (ELISPOTs) consisted of 10 ng/ml phorbol 12-myristate 13-acetate (Sigma), 500 ng/ml ionomycin (Sigma) or the HLA-A24-restricted EBV late membrane or CMV pp65-derived peptides (Table II).

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 10^5 mytomicin 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 ^{51}Cr (Amersham, Arlington Heights, IL) for 1 hr. Hepatoma cell lines were labeled with 25 μCi of ^{51}Cr 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 or ^{51}Cr-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 ^{51}Cr-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).
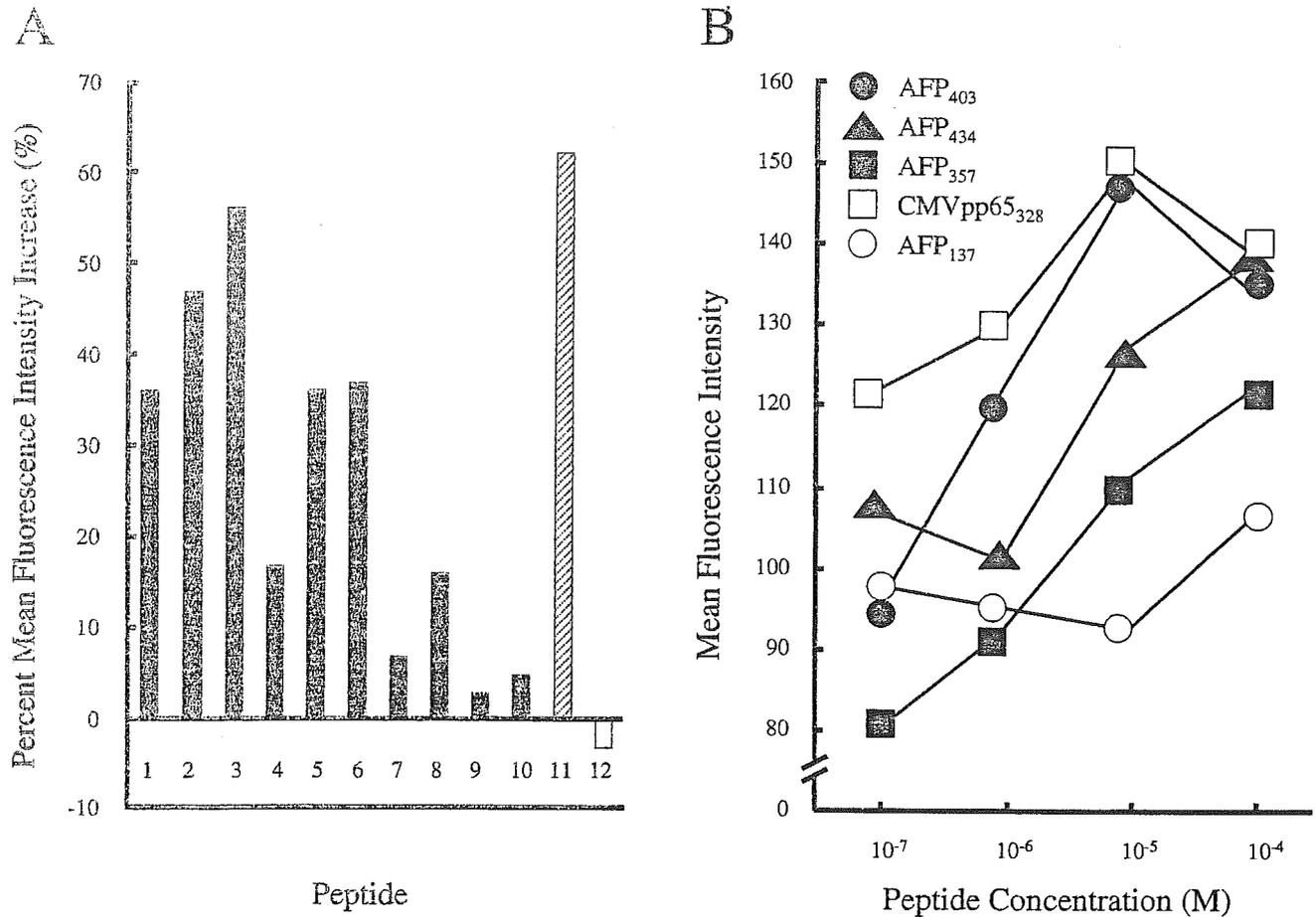
FIGURE 1 — MHC binding affinity. (a) TAP-deficient T2-A24 cells were cultured for 16 hr at 26°C to enhance the expression of peptide-receptive cell surface molecules. They were incubated with individual peptides at 10 μg/ml at 37°C for 2 hr, washed and stained with anti-HLA-A24 MAb, anti-mouse immunoglobulin conjugated FITC and 1 μg/ml of propidium iodide. The data are expressed as the %mean fluorescence intensity (MFI) increase for live, propidium iodide-negative cells. 1, AFP_{403}; 2, AFP_{424}; 3, AFP_{434}; 4, AFP_{357}; 5, AFP_{150}; 6, AFP_{504}; 7, AFP_{591}; 8, AFP_{414}; 9, AFP_7; 10, AFP_{322}; 11, $CMVpp65_{328}$; 12, AFP_{137}. (b) The MHC binding affinity of representative peptides is shown at various concentrations. The data are expressed as MFI for live, propidium iodide-negative cells.

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" ($\leq$2cm) for 10 cases or "large" (>2cm) for 28 cases, and tumor multiplicity was categorized as "multiple" ($\geq$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),[26] 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 AFP_{591}, AFP_7 and AFP_{322} (Fig. 1a). Peptide $CMVpp65_{328}$, which is identified as a strong binder of the HLA-A*2402 molecule,[22] increased HLA-A24 expression, but peptide AFP_{137}, which is HLA-A2 restricted,[14] 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

1 patient, and 21 of 38 patients (55%) responded to at least 1 of the analyzed AFP-derived peptides.

An overview of all responses is shown in Figure 2a. Single AFP epitope-specific IFN-γ producing cells were detected in 5 (13.2%), 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 AFP_{403}, AFP_{424}, AFP_{434}, AFP_{357}, AFP_{150}, AFP_{504} and AFP_{414}, respectively. Peptides AFP_{591}, AFP_7 and AFP_{322} were not recognized by any patient. Among the peptides, AFP_{591}, AFP_7 and AFP_{322} displayed a relatively low binding affinity for the HLA-A*2402 molecule compared with the other peptides (Fig. 1a). In contrast, peptides AFP_{403}, AFP_{434} and AFP_{357}, those with a high binding affinity for the HLA-A*2402 molecule, were recognized by 5, 8 and 7 patients, respectively. 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 × 10^5 PBMCs. Most patients, however, displayed between 10 and 60 specific cells per 3 × 10^5 PBMCs. The frequency of positive T-cell responses was lower than that of peptides $EBVlm_{287}$ and $CMVpp65_{328}$, which were derived from EBV latent membrane or CMV pp65 protein, respectively, and are strongly immunogenic. All the patients who showed positive T-cell responses against $EBVlm_{287}$ or $CMVpp65_{328}$ were sero-positive for EBV or CMV, respectively. No patient exhibited positive T-cell responses against peptide $HIVenv_{584}$ 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 AFP_{403}, AFP_{424}, AFP_{434}, AFP_{357}, AFP_{150}, AFP_{504} and AFP_{414} 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 AFP_{403}, AFP_{424}, AFP_{434}, AFP_{357} and AFP_{414} lysed the peptide-pulsed C1R-A*2402 cells as shown in Figure 3. On the other hand, other peptides, including those that showed binding affinity for the HLA-A24 molecule, failed to induce CTLs specific for the corresponding peptide. Thus, peptides AFP_{403}, AFP_{424}, AFP_{434}, AFP_{357} and AFP_{414} contained a HLA-A24 restricted AFP epitope.

Lysis of hepatoma cell lines by AFP peptide-specific CTL lines

We next determined whether AFP-derived peptide-induced CTLs showed cytotoxicity against hepatoma cell lines that produced AFP. As shown in Figure 4, peptides AFP_{403}- and AFP_{357}-specific CTLs showed cytotoxicity against HepG2, which expressed HLA-A*2402 and produced AFP, but not against HLE or HuH7, which lacked HLA-A*2402 expression or production of AFP.[27] These results indicate that the CTLs generated from PBMCs of HCC patients were able to kill hepatoma cells, and that the cytotoxicity was restricted by HLA-A24 and specific for AFP.

Furthermore, to confirm that the cytotoxicity was mediated by $CD8^+$ T cells and restricted HLA-A24, we examined the T-cell responses against peptide-pulsed C1R-A24 or HepG2 cells incubated with specific MAb. Anti-CD8 Mb and anti-HLA-A24 MAb efficiently inhibited the specific response of peptide AFP_{357}-induced CTLs against both cell types (Figs. 5a and 5b). Also,

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 AFP_{403}, AFP_{424}, AFP_{434}, AFP_{357} or AFP_{414}-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 AFP_{403}, AFP_{424}, AFP_{434}, AFP_{357} or AFP_{414} 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

FIGURE 2 – Direct ex vivo analysis (IFN-γ ELISPOT assay) of peripheral blood T-cell responses to AFP-derived peptides (solid bars) or control peptides (open bars) in HCC patients (a) and normal donors (b). Only significant IFN-γ responses to at least 1 of the 13 tested peptides are included in the figure. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen. The peptide sequences are described in Table II. The data for AFP_{591}, AFP_7 and AFP_{322} are excluded because there was no positive T-cell response. * denotes 177 specific spots; **, 68 specific spots and ***, 92 specific spots.

A

B
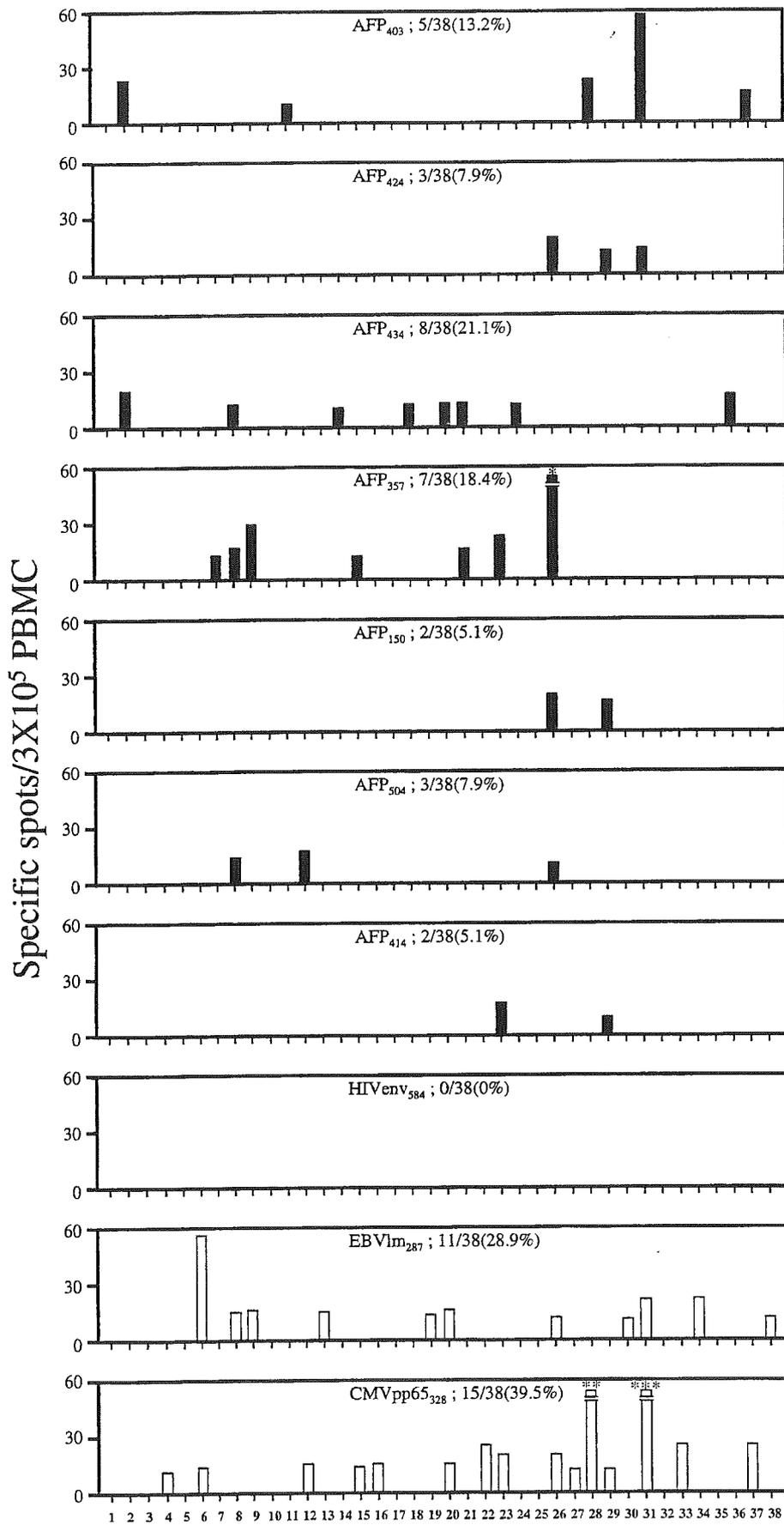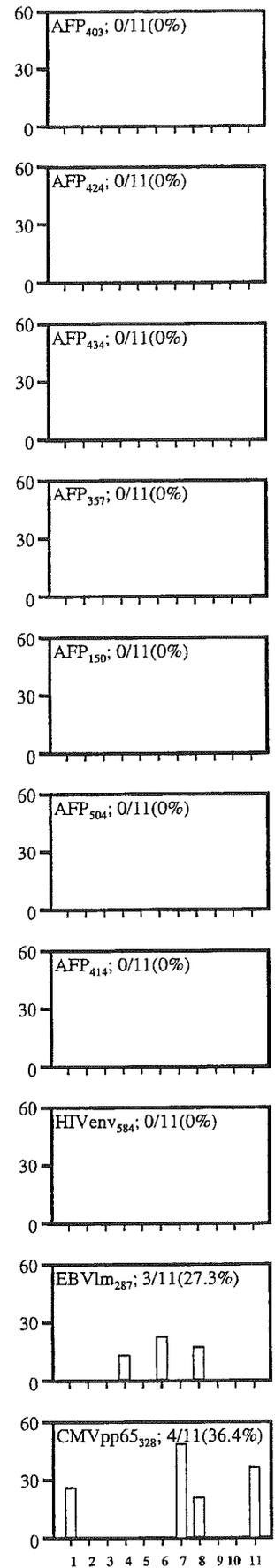
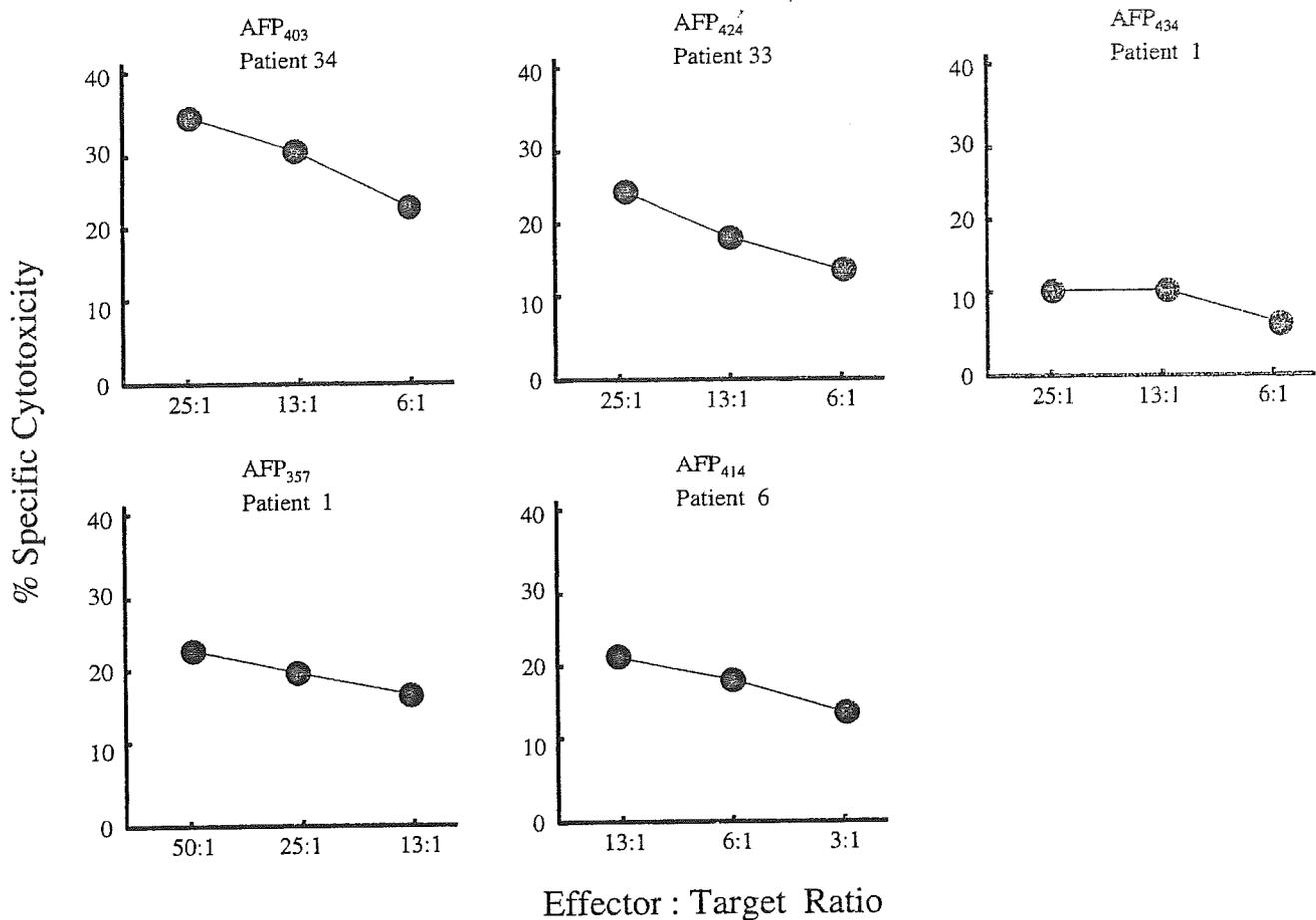
Patients with HCC

Normal Donors

FIGURE 2.

FIGURE 3 – Cytotoxicity of AFP-specific T-cell lines in patients with HCC. The cytotoxicity of the T-cell lines was determined by a standard 6 hr cytotoxicity assay at various effector to target (E/T) ratios against C1R-A*2402 cells pulsed with 1of the AFP-derived peptides listed in Table II. The number of patients corresponds to the numbers as shown in Table I. The data are indicated as the percent specific cytotoxicity, which is calculated as follows: (cytotoxicity in the presence of specific peptide) – (cytotoxicity in the absence of peptide).

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[28] 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.[29,30] Furthermore, the expression of AFP in cancerous tissue is related to the biological malignancy of HCC.[31] Recent studies reported that AFP-specific T-cell clones are not deleted during ontogeny and that AFP is recognized by murine[16] and human T cells[13-15] and serves as a tumor rejection antigen in a murine tumor model.[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,[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-γ 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 × 10⁶ 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 EBVlm287 or CMVpp65328 were sero-positive

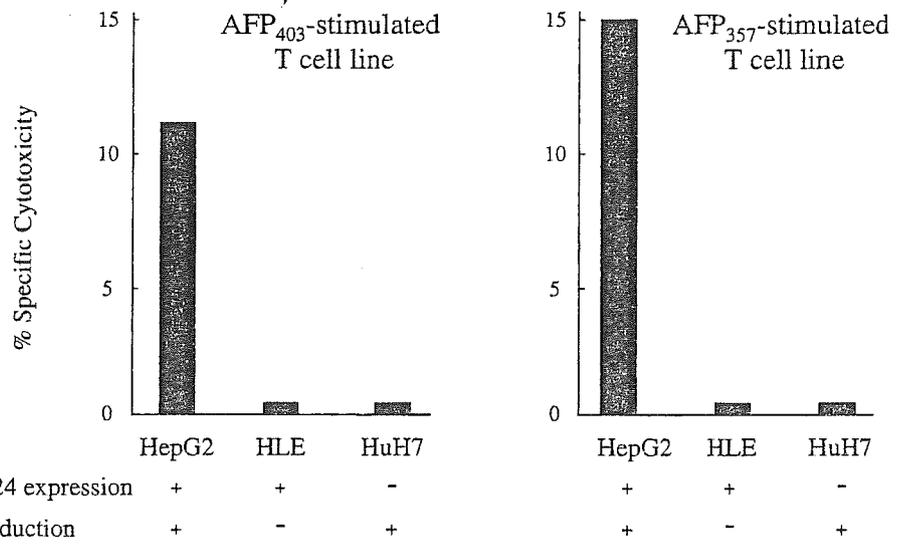| Target	HepG2	HLE	HuH7		HepG2	HLE	HuH7
HLA-A24 expression	+	+	-		+	+	-
AFP production	+	-	+		+	-	+

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 cytotoxicity was determined by a standard 6 hr cytotoxic assay (E/T ratio of 50:1).
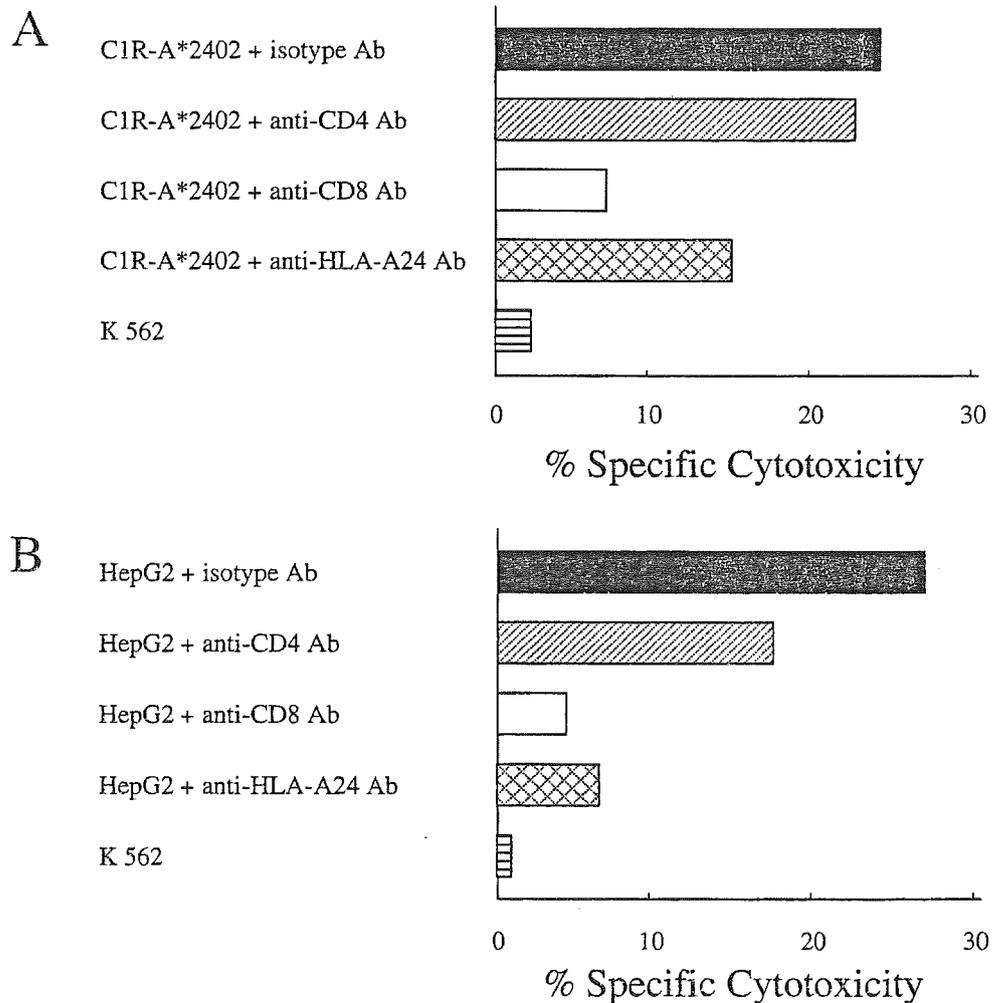

FIGURE 5 – Inhibition of cytotoxicity of AFP-specific T-cell lines by specific antibodies. T-cell lines were generated from PBMC of HCC patients by stimulation with AFP$_{357}$. Inhibition of cytotoxicity was determined using a standard 6 hr cytotoxicity assay against C1R-A*2402 cells pulsed with AFP$_{357}$ (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.

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 associated antigen epitope, the number of specific T cells for tyrosinase, MelanA/MART-1, gp100 or CEA in patients with melanoma or colorectal cancer was found to be 11–130 per 1 × 10^6 PBMCs.[34,35] In addition, single AFP epitope-specific IFN-γ producing cells were detected in 5.1–21.1% of the patients for peptides AFP$_{403}$, AFP$_{424}$, AFP$_{434}$, AFP$_{357}$ or AFP$_{414}$. These rates are similar to previously reported epitopes for tyrosinase, MelanA/MART-1, gp100, Her-2/neu and CEA.[34-39] Comparing the present

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)[1]	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)	2/16	11/9	0.006
(N0/N1)	18/0	19/1	NS
(M0/M1)	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.
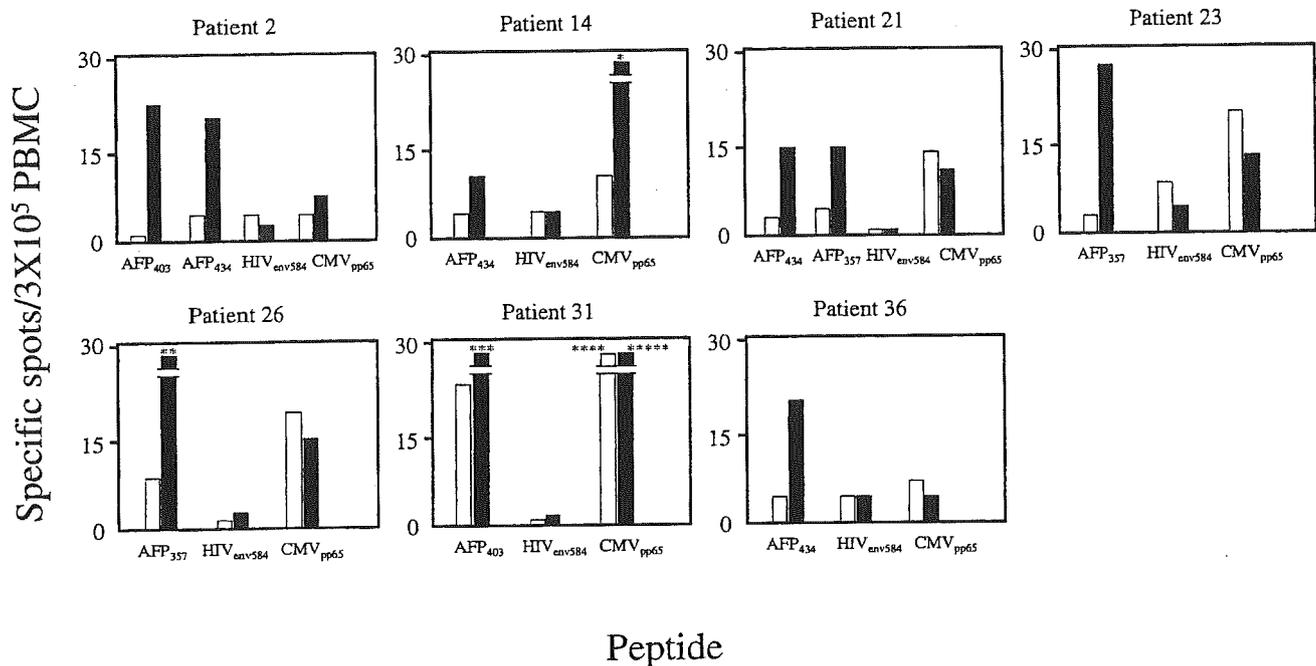[1]Data expressed as mean ± SD.

FIGURE 6 – The induction of AFP-specific T-cell responses in HCC patients after treatment of HCC. Direct *ex vivo* analysis (IFN-γ 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_{403}, AFP_{424}, AFP_{434}, AFP_{357}, or AFP_{414} were included in the figure. A significant change in the IFN-γ 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-γ 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.

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-γ 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 mole-

cule, her-2/neu or CEA was also reported among patients with advanced colorectal cancer.[34,35]

Other factors, including serum AFP levels, histology of the nontumor 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.[40] 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

TABLE IV – CHARACTERISTICS OF PATIENTS STUDIED FOR T-CELL RESPONSIVENESS AFTER HCC TREATMENT

	Patients with increasing T-cell responsiveness	Patients without increasing T-cell responsiveness	p-value
No. of patients	7	10	
Age (years)[1]	69.1 ± 7.0	69.9 ± 7.1	NS
Sex (M/F)	6/1	8/2	NS
AFP level (≤20/>20)	1/6	3/7	NS
Diff. degree of HCC (well/moderate or poor/ND)	3/3/1	4/3/3	NS
Tumor multiplicity (multiple/solitary)	5/2	8/2	NS
Vascular invasion (+/−)	2/5	5/5	NS
TNM factor			
(T1–T2/T3–T4)	6/1	4/6	NS
(N0/N1)	7/0	10/0	NS
(M0/M1)	6/1	8/2	NS
TNM stage (I,II/III,IV)	6/1	3/7	0.049
Histology of nontumor liver (LC/chronic hepatitis)	7/0	10/0	NS
Liver function (Child A/B/C)	3/4/0	7/3/0	NS
Etiology (HCV/HBV/others)	7/0/0	9/1/0	NS
Positive T-cell responses before treatment (+/−)	2/5	7/3	NS
Treatments (PEIT/RF/TAE/chemotherapy)	1/2/4/0	1/0/8/1	NS

NS, no statistical significance; ND, not determined.
[1]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.[31] 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.

Also, 17 HCV infected and 3 HBV infected patients had AFP-specific T cells in the peripheral blood. AFP-specific CTLs could also be expanded in patients with HCV infection by in vitro peptide stimulation. Furthermore, the frequency of T cells reactive toward a single AFP epitope was equal or higher than that for a single HCV epitope.[41] 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.[42–44]

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[45,46] 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, anticancer 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.

Acknowledgements

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cDNA microarray analysis of autoimmune hepatitis, primary biliary cirrhosis and consecutive disease manifestation

Masao Honda, Hiroshi Kawai, Yukihiro Shirota, Taro Yamashita, Toshinari Takamura, Shuichi Kaneko*

Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Takara-Machi 13-1, Kanazawa 920-8641, Japan

Abstract

While autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) usually have distinct clinical manifestations, some patients present with features of both conditions. Using cDNA microarrays, we analyzed and compared gene expression profiles in 8 patients with AIH, 9 with PBC, 8 with chronic hepatitis C (CHC), 8 with non-alcoholic steatohepatitis (NASH) and 9 with normal livers. We subsequently applied this method to a tissue sample from a 61-year-old woman with overlapping features of both AIH and PBC. A 61-year-old woman was admitted to our hospital for evaluation of elevated serum alkaline phosphatase. A liver biopsy showed accumulation of mononuclear cells around the bile duct cells, a feature characteristic of chronic non-suppurative destructive cholangitis (CNSDC). Three years later, her serum alanine aminotransferase (ALT) level had increased, and a liver biopsy demonstrated evidence of a severe form of hepatitis. A cDNA microarray analysis of both biopsies identified the molecular events associated with her altered histology. The expression profile of this patient, which was originally different from that of the other PBC patients, changed to an AIH pattern. Our results suggest that this patient has characteristics of both AIH and PBC.
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Keywords: AIH; PBC; cDNA microarray

Autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) are both considered as autoimmune diseases of the liver. AIH is characterized clinically by elevated serum transaminase levels, hyper-gammaglobulinemia, and the presence of anti-nuclear antibodies (ANA) in serum [1]. In contrast, PBC is characterized by the sustained elevation of bile duct enzymes, the presence of anti-mitochondrial antibodies (AMA) in serum [2] and the occurrence of chronic non-suppurative destructive cholangitis (CNSDC) in the liver [3]. While there are usually distinct differences in the clinical manifestations of these two diseases, some patients share features of both, and AIH and PBC are sometimes referred to as overlapping diseases [4–7].

Recently developed cDNA microarray technology provides a means to simultaneously analyze the expression levels of hundreds to thousands of genes [8,9]. This method may also be useful in determining appropriate therapeutic strategies for individual patients [10]. We have applied cDNA microarray analysis to tissue samples from a patient with overlapping features of AIH and PBC in order to investigate the expression profiling of these diseases.

Table 1
Patients' characteristics

Clinical diagnosis	Patient number	Age (year)	Sex (M:F)	ALT (IU/l)	ALP (IU/l)	Albumin (g/dl)	T-bilirubin (mg/dl)	Prothrombin time (s)	AMA(+) number
Normal	9	66.5 ± 5.23	7:2	18.4 ± 2.61	259 ± 23.2	4.25 ± 0.14	0.50 ± 0.07	NA	0
Autoimmune hepatitis	8	49.0 ± 3.59	2:6	113.3 ± 25.4[a]	351.3 ± 69.3	3.95 ± 0.24	0.96 ± 0.28	11.9 ± 0.45	0
Primary biliary cirrhosis	9	57.2 ± 2.08	2:7	50.8 ± 6.07	1059 ± 382[b]	4.19 ± 0.19	1.06 ± 0.21	11.7 ± 0.28	6
Chronic hepatitis C	8	56.6 ± 6.33	6:2	54.0 ± 13.6	214 ± 16.1	4.17 ± 0.28	0.71 ± 0.14	11.5 ± 0.35	0
Non-alcoholic steatohepatitis	8	45.0 ± 5.43	4:4	102.8 ± 12.5	214 ± 16.1	4.57 ± 0.12	0.87 ± 0.09	11.5 ± 0.28	0

NA: not applicable.
[a] Autoimmune hepatitis vs. normal ($p < 0.05$).
[b] Primary biliary cirrhosis vs. normal ($p < 0.05$).

1. Patients and methods

1.1. Patients and tissue samples

Liver biopsies were obtained from 9 patients with normal liver, 8 with AIH, 9 with PBC, 8 with chronic hepatitis C (CHC) and 8 with non-alcoholic steatohepatitis (NASH), all of whom had been admitted to Kanazawa University Hospital between 1997 and 2002 (Table 1). Biopsy samples were immediately frozen and stored in liquid nitrogen until use.

Tissue samples were assessed histologically by standard methods [11,12] (Table 2). AIH was diagnosed according to the criteria of the International Autoimmune Hepatitis Group [1]; all 8 patients were found to have type I AIH (score > 15). PBC was diagnosed by the elevation of alkaline phosphatase (ALP) levels and seropositivity for anti-mitochondrial antibody (AMA, PDH-E2), as well as representative histological features of bile duct injury, such as CNSDC. While 3 PBC patients were seronegative for AMA (Table 1), liver specimens from these patients revealed the histological features of CNSDC. All 8 patients with CHC were positive for HCV-RNA. The clinical and pathological stages of NASH were assessed as described by Sanyal [13].

1.2. Antisense RNA amplification and labeling

Total RNA was isolated from each biopsy specimen and subjected to antisense RNA amplification (aRNA) as described [14–16]. The isolated RNA was evaluated by electrophoresis in an Agilent 2001 bioanalyzer. As a reference for each microarray analysis, we utilized aRNA prepared from the normal liver of 1 patient. Each test sample aRNA was fluorescently labeled with Cy5, and the reference aRNA was labeled with Cy3, and these were used for microarray hybridization as described [14–16]. Each hybridization was performed at least twice.

1.3. cDNA microarray and image analysis

For cDNA microarray analysis, we prepared microarray slides containing 1080 cDNA clones and quantitatively assessed gene expression as described [14–16].

1.4. Quantitative real time detection (RTD)-PCR

Quantitative real time detection (RTD)-PCR was performed using TaqMan Universal Master Mix (PE Applied Biosystems, CA).

1.5. Statistical analysis

Hierarchical clustering and principal component analysis of gene expression were performed by BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html), which contains a class comparison tool based on univariate F tests to find genes differentially expressed in predefined clinical groups. The permutation distribution of the F statistic, based on 2000 random permutations, was also used to confirm statistical significance. Statistical significance was defined as a p-value less than 0.005.

2. Results

2.1. Hierarchical clustering of AIH and PBC patients

To compare the expression profiles in AIH and PBC, we performed hierarchical clustering of all 1080 genes in liver biopsies of 8 AIH and 9 PBC patients, all of whom had been selected because they did not have advanced liver disease (Table 1). The patients were clustered into two groups, one for AIH and the other for PBC, with a few exceptions (Fig. 1a, $p < 0.05$). Interestingly, 1 of

Table 2
Histological assessment

PBC	Number	AIH	Number	CHC	Number	NASH	Number
Stage I	5	F1A1	3	F1A1	2	F3A2	3
Stage II	4	F1A3	1	F2A1	4	F3A3	4
		F1A2	1	F2A2	2	F3A4	1
		F2A1	2				
		F2A2	1				

PBC: primary biliary cirrhosis; AIH: autoimmune hepatitis; CHC: chronic hepatitis C; and NASH: non-alcoholic steatohepatitis.
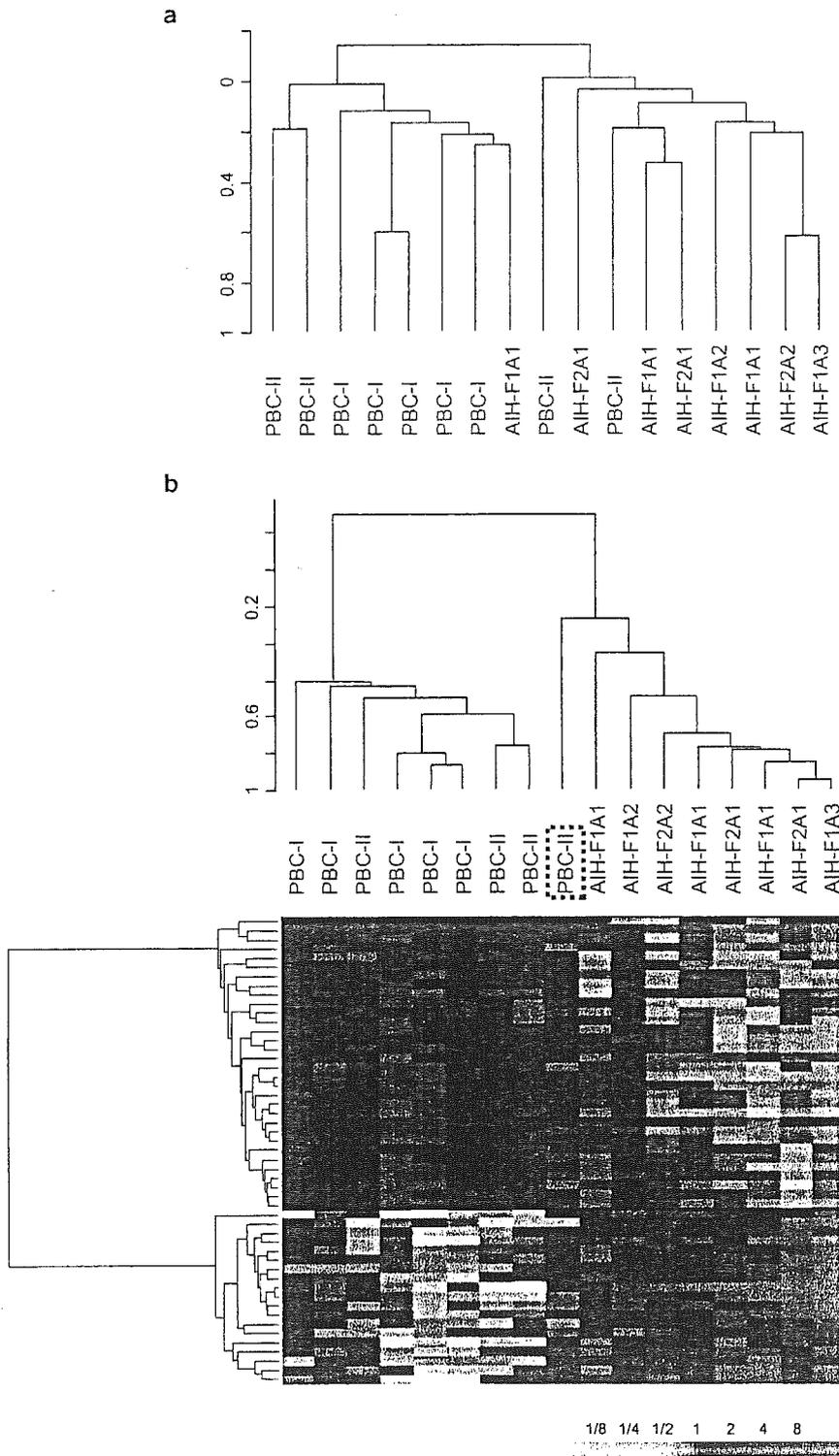
Fig. 1. a: Hierarchical clustering analysis of 1080 genes in AIH (red) and PBC (blue) patients. The dendrogram shows the grouping of patients based on similarities in their gene expression patterns. Histological findings for each patient are shown under individual roots. b: Hierarchical clustering analysis of AIH and PBC patients using 63 selected, differentially expressed genes ($p < 0.005$). All 8 AIH patients and 8 of 9 PBC patients were clearly separated, implying that these genes were characteristic of the expression profiling of each disease.

the AIH patients was clustered into the PBC group, while 2 PBC patients were clustered into the AIH group.

2.2. Gene expression differentiating AIH from PBC

We performed hierarchical clustering of 63 genes differentially expressed in AIH and PBC biopsies ($p < 0.005$; Table 3). All patients, except for 1 PBC patient, were clearly separated into two groups, indicating that the genes selected were characteristic of the

expression profiling of AIH and PBC. In AIH, transcripts of MHC class II, STAT1, and caspase 1 genes, all of which are induced by interferon (IFN)-γ or related to the induction of apoptosis, were consistently expressed at higher levels than that in PBC. In PBC, however, we observed differential up-regulation of endothelin 3 and bone morphogenic protein 4 genes, which are representative of vascular endothelium and were recently characterized as potential angiogenic factors. In PBC, we also noted up-regulation of the expression of

Table 3
The representative genes that were differentially expressed in AIH and PBC

| Parametric p-value	Fold difference	Description	GB accession number
Up-regulated in AIH relative to PBC			
0.00004	5.16	MHC class II DR	NM_021983
0.00242	2.90	Zinc finger protein 336	NM_022482
0.00055	2.47	c-fms Proto-oncogene	NM_005211
0.00346	2.21	Zinc finger protein 91 (HPF7, HTF10)	NM_003430
0.00600	2.14	Vascular cell adhesion molecule 1 (VCAM 1)	NM_080682
0.00537	2.08	STAT1	NM_139266
0.00038	2.05	S100 calcium-binding protein A9 (calgranulin B)	NM_002965
0.00024	2.03	Rho GDP dissociation inhibitor (GDI) beta	NM_001175
0.00003	1.97	Human alpha-tubulin	NM_006082
0.00263	1.96	Ras-related C3 botulinum toxin substrate 2	NM_002872
0.00048	1.95	Vimentin	NM_003380
0.00205	1.93	Heme oxygenase-2	NM_002134
0.00078	1.85	Hematopoietic cell-specific Lyn substrate 1	NM_005335
0.00116	1.85	Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	NM_003336
0.00141	1.79	Inhibitor of DMA binding 3, dominant negative helix—loop—helix protein	NM_002167
0.00178	1.79	MHC class IB	NM_005514
0.00379	1.75	RPB5	NM_134447
0.00045	1.75	Interleukin-1 receptor-associated kinase 1	NM_001569
0.00135	1.74	Granulin	NM_002087
0.00118	1.65	Ras homolog gene family, member G (rho G)	NM_001665
0.00101	1.65	14-3-3n protein	NM_003405
0.00449	1.64	Non-metastatic cells 1, protein (NM23A) expressed in	NM_000269
0.00117	1.64	Interferon, alpha-inducible protein (clone IFI-6-16)	NM_022872
0.00125	1.60	Caspase 1	NM_033292
Up-regulated in PBC relative to AIH			
0.0041	2.38	Acyl-coenzyme A dehydrogenase, short/branched chain	NM_001609
0.0003	1.73	Interleukin-7	NM_000880
0.0006	1.63	Endothelin 3	NM_000114
0.0020	1.59	Human CB1 cannabinoid receptor (CNR1) gene	NM_016083
0.0005	1.58	SMARCA3	NM_139048
0.0012	1.56	Human HGF agonist/antagonist	NM_000601
0.0015	1.56	Integrin, beta 8	NM_002214
0.0040	1.56	Catenin (cadherin-associated protein), beta 1 (88 kDa)	NM_001904
0.0006	1.54	Cell division cycle 25C	NM_022809
0.0021	1.54	CART	NM_004291
0.0002	1.52	Fibroblast growth factor 2 (basic)	NM_002006
0.0016	1.52	Cadherin 3, P-cadherin (placental)	NM_001793
0.0058	1.52	Desmogiein 2	NM_001943
0.0044	1.52	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	NM_002422
0.0014	1.51	Bone morphogenetic protein 4	NM_130850
0.0030	1.51	CD1C antigen, c polypeptide	NM_001765
0.0050	1.51	Fetal liver non-specific cross-reactive antigen-2 (FL-NCA-2)	NM_021016
0.0035	1.50	A disintegrin and metalloproteinase domain 17	NM_021832
0.0027	1.50	Wingless-type MMTV integration site family member 2	NM_003391
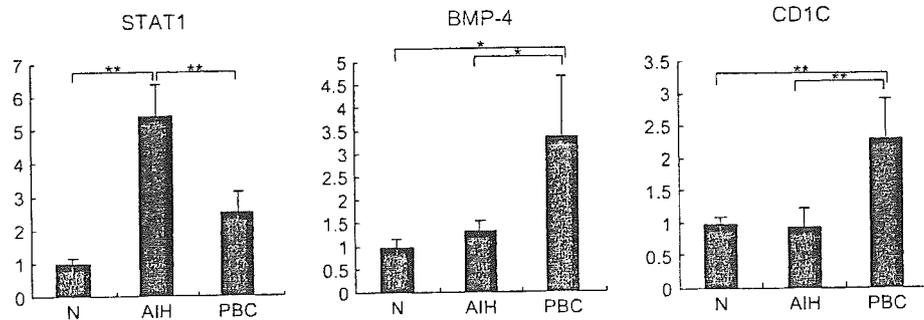
Fig. 2. Quantitative real time detection (RTD)-PCR. $*p < 0.05$, $**p < 0.01$.

genes encoding several cell adhesion molecules, such as CD1C, a group 1 member of the CD1 family of major histocompatibility complex (MHC)-like glycoproteins, and pregnancy specific beta 1 glycoprotein (PSG-1), which belongs to the carcinoembryonic antigen (CEA) gene family. Differential expression of some of these genes was confirmed by RTD-PCR (Fig. 2).

2.3. Gene expression analysis of AIH, PBC, CHC and NASH patients

To examine these differences were also found in the analysis of various liver diseases, we performed hierarchical clustering of all 1080 genes in liver biopsies of 8 AIH, 9 PBC, 8 CHC and 8 NASH patients. Interestingly, the patients were clustered into four groups, AIH, PBC, CHC and NASH, with a few exceptions (data not shown) indicating the different gene expression pattern according to the different etiology of liver diseases. Two hundred and one genes were differentially expressed among these diseases ($p < 0.005$). Principal component analysis of gene expression of these patients using differentially expressed genes showed the three dimensional scattering for each patient (Fig. 3). Interestingly, only the same PBC patient was separated from other PBC patients. The representative genes that were up-regulated in AIH or PBC patients relative to other diseases are shown in Table 4. Several genes such as STAT1 and interferon, alpha-inducible protein (clone IFI-6-16), HGF and CD1C, etc. (Table 3), were induced in CHC and NASH patients. Thus genes listed in Table 4 were more specific in AIH and PBC patients.

2.4. Clinical and molecular characterizations of an overlapping patient

Based on hierarchical clustering and principal component analysis, we found that 1 PBC patient was clustered into the AIH group. The clinical course of this patient (no. 220) is illustrated in Fig. 4. In April 1999, she was admitted to the hospital for evaluation of elevated serum ALP. She was positive for AMA (PDH-E2)

(80-fold), and her AIH score was less than that required for diagnosis of possible or definite AIH (Table 5). A liver biopsy revealed accumulation of mononuclear cells around the bile duct cells, a feature typical of CNSDC (Fig. 5a). Ursodeoxycholic acid (UDCA) was prescribed, but she did not take this medication. In April 2002, she was again admitted to the hospital for the evaluation of elevated serum alanine aminotransferase (ALT). After admission, her serum ALT level increased to 407 IU/ml, while her ALP level gradually decreased (Fig. 4). Her serum ANA and gamma-globulin were higher, while her serum IgM was lower, than that at her previous admission. A liver biopsy demonstrated evidence of a severe form of hepatitis, including marked lobular cell
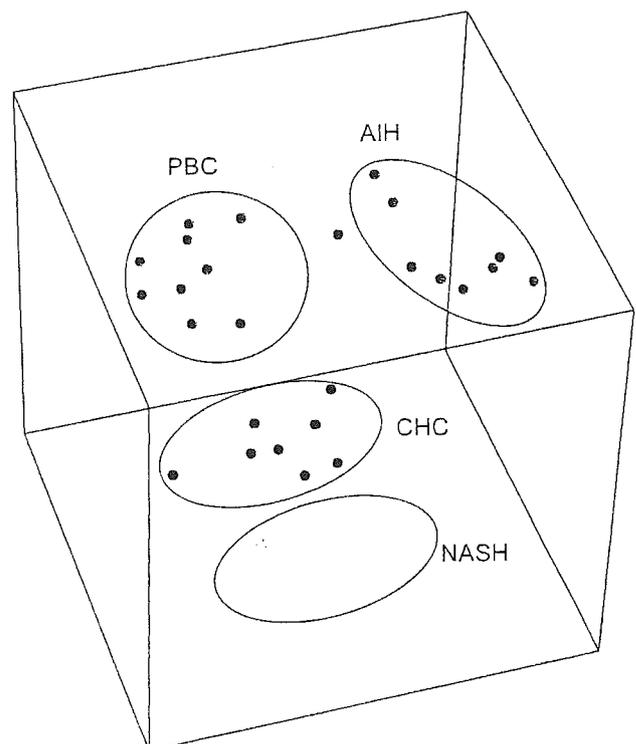

Fig. 3. Principal component analysis of gene expression, showing the three dimensional scattering for each patient with AIH, PBC, CHC and NASH. One PBC patient was separately scattered from other PBC patients.

Table 4
The representative genes that were up-regulated in AIH or PBC patients relative to other diseases

Parametric p-value	Fold AIH/PBC	AIH/CHC	AIH/NASH	Description	GB accession number
Up-regulated in AIH relative to PBC, CHC and NASH					
0.00001	5.16	5.42	5.13	MHC class II DR	NM_021983
0.00001	3.17	2.01	1.94	MHC class II DR (derived from Riken)	NM_002123
0.00010	2.90	2.35	1.80	Zinc finger protein 336	NM_022482
0.00000	2.82	1.53	1.55	Small inducible cytokine A4 (homologous to mouse Mip-1b)	NM_002984
0.00005	2.48	2.44	2.38	Natural killer cell transcript 4	NM_004221
0.00000	2.25	3.83	3.60	MIHC	NM_001165
0.00027	2.17	2.28	1.86	Major histocompatibility complex, class I, C	NM_002116
0.00003	2.03	1.66	2.11	Rho GDP dissociation inhibitor (GDI) beta	NM_001175
0.00118	2.02	1.70	3.79	Nicotinamide N-methyltransferase	NM_006169
0.00035	1.94	2.00	2.51	X-ray repair complementing defective repair in Chinese hamster cells 1	NM_006297
0.00063	1.87	1.72	1.91	Integrin, beta 2	NM_000211
0.00023	1.76	2.63	1.60	Cyclin A	NM_001237
0.00001	1.75	3.32	1.66	Interleukin-1 receptor-associated kinase 1	NM_001569
0.00001	1.74	2.56	1.72	Granulin	NM_002087
0.00004	1.65	1.55	1.61	Ras homolog gene family, member G (rho G)	NM_001665
0.00056	1.60	1.71	1.81	Caspase 1, apoptosis-related cysteine protease	NM_033292
0.00267	1.55	1.50	1.74	Chemokine (C–C motif) receptor 5	NM_000579
0.00014	1.53	2.75	1.72	Cytochrome p450 reductase	NM_000941
0.00000	1.45	2.72	5.82	TRAF1	NM_133484
0.00047	1.43	1.88	1.60	Perforin 1 (preforming protein)	NM_005041
Up-regulated in PBC relative to AIH, CHC and NASH					
0.00489	2.38	1.67	1.98	Acyl-coenzyme A dehydrogenase, short/branched chain	NM_001609
0.00273	1.73	1.71	1.43	Interleukin-7	NM_000880
0.00000	1.63	3.12	3.83	Endothelin 3	NM_000114
0.00008	1.59	1.93	1.91	Human CB1 cannabinoid receptor (CNR1) gene	NM_016083
0.00094	1.56	1.82	1.83	Integrin, beta 8	NM_002214
0.00081	1.56	3.20	2.72	Colony stimulating factor 2 (granulocyte–macrophage)	NM_000758
0.00000	1.52	1.81	2.99	Cadherin 3, P-cadherin (placental)	NM_001793
0.00023	1.39	2.70	1.87	c-myb cDNA clone lambda-LMC8	NM_005375
0.00072	1.38	1.70	1.81	Cellular retinoic acid-binding protein 2	NM_001878
0.00265	1.38	1.86	1.77	Protein kinase, mitogen-activated 4 (MAP kinase 4; p63)	NM_002747
0.00045	1.35	1.44	2.71	Transcription factor AREB6	NM_030751
0.00199	1.35	1.43	1.66	STAT4	NM_003151
0.00007	1.35	1.99	1.98	Inhibin, beta A (activin A, activin AB alpha polypeptide)	NM_002192
0.00422	1.33	1.42	1.46	RNase A family, 2 (liver, eosinophil-derived neurotoxin)	NM_002934

infiltration and interface hepatitis (Fig. 5b). Her AIH score was 15, and she was diagnosed with AIH overlapping with PBC, stage III. A combination of UDCA and steroid was prescribed (Fig. 4, Table 5).

We subjected the liver biopsies of this patient to cDNA microarray analysis to identify the molecular events associated with the altered histological findings. The expression profiling of this patient, which was originally differed from that of the other PBC patients (Fig. 6, 4.27.99), subsequently changed to an AIH pattern (Fig. 6, 6.11.02).

3. Discussion

Despite extensive cytokine profiling of the liver, the pathogenesis of AIH and PBC has not been completely elucidated. Moreover, diagnosis of these diseases is sometimes difficult, since some patients present with features of both. We have applied cDNA microarray analysis to patients with AIH and PBC to determine the expression profiling of these diseases.
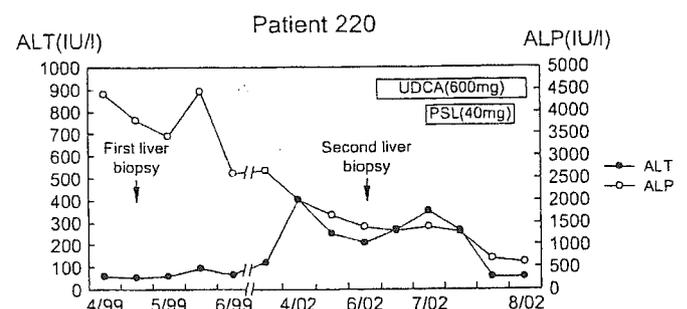

Fig. 4. Clinical course of patient no. 220. This patient, originally diagnosed with PBC, later developed AIH. Serum concentrations of ALP and ALT are shown.

Table 5
Biochemical data of a PBC patient (no. 220) who later developed AIH

Date	ALT (<50 IU/l)	ALP (<324 IU/l)	ANA (<20)	AMA (<20)	Gamma-globulin (g/dl)	IgM (mg/dl)	AIH score	Medication
April 1999	79	4922	20	80	3.1	772	7	–
June 2002	407	2035	80	80	3.7	327	15	UDCA + steroid

Hierarchical clustering of all 1080 analyzed genes demonstrated differences in gene expression patterns between AIH and PBC (Fig. 1a, $p < 0.05$). As references, we analyzed patients with CHC, NASH and normal liver. We identified genes that were specifically expressed in AIH or PBC ($p < 0.005$; Tables 3 and 4). We demonstrated here that different genes from those described in previous reports were up-regulated in AIH and PBC, although many similar genes, as previous reports, were also up-regulated [17,18]. One of the PBC patients, a 61-year-old female with stage II disease, developed AIH two years later.

When we subjected the liver biopsies of this patient to cDNA microarray analysis to identify the molecular events associated with the altered histology, we found that her original expression pattern differed from that of the other PBC patients, later changed to an AIH pattern. These findings suggest that overlapping patients might be grouped into a disease category separated from AIH and PBC, although more number of these patients should be carefully analyzed for the conclusions.

In conclusion, we have used cDNA microarray analysis to identify genes differentially expressed in AIH and PBC, showing the diagnostic relevance of this technique. Further studies with more overlapping patients would provide a more profound understanding of this disease.
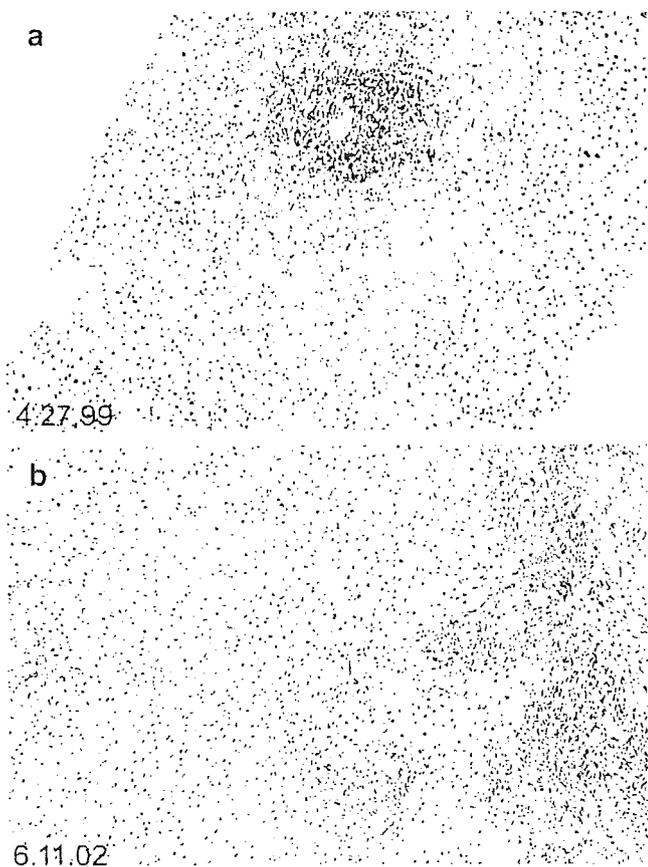

Fig. 5. Histopathological findings for patient no. 220. (a) The first liver biopsy, performed April 27, 1999, showing accumulation of mononuclear cells around the bile ducts, a finding typical of CNSDC. (b) The second liver biopsy, performed June 11, 2002, showing a more marked lobular cell infiltration and interface hepatitis.
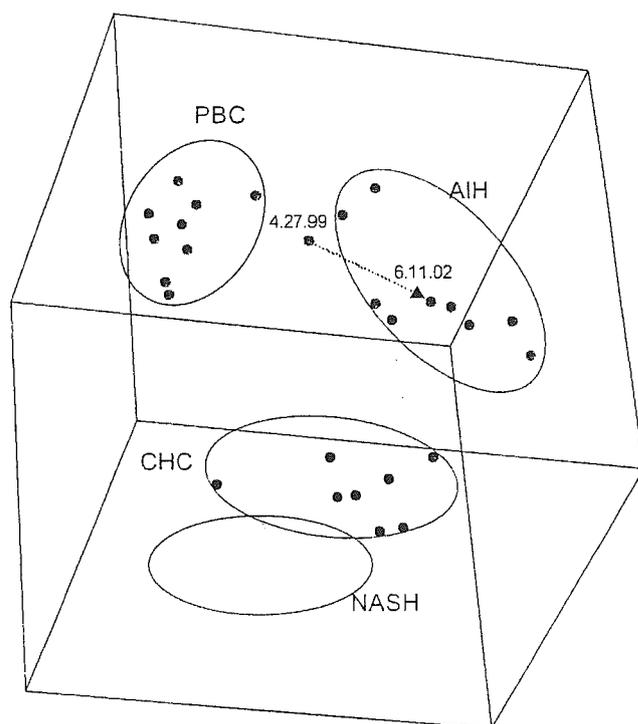

Fig. 6. Principal component analysis of gene expression of AIH, PBC, CHC and NASH. The expression profiling of 1 PBC patient, which was originally differed from that of the other PBC patients (4.27.99), subsequently changed to an AIH pattern (6.11.02).

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