Pretreatment Prediction of Interferon-Alfa Efficacy in Chronic Hepatitis C Patients

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Background & Aims: Interferon has been used widely to treat patients with chronic hepatitis C infections. Prediction of interferon efficacy before treatment has been performed mainly by using viral information, such as viral load and genotype. This information has allowed the successful prediction of sustained responders (SR) and non-SRs, which includes transient responders (TR) and nonresponders (NR). In the current study we examined whether liver messenger RNA expression profiles also can be used to predict interferon efficacy. **Methods:** RNA was isolated from 69 liver biopsy samples from patients receiving interferon monotherapy and was analyzed on a complementary DNA microarray. Of these 69 samples, 31 were used to develop an algorithm for predicting interferon efficacy, and 38 were used to validate the precision of the algorithm. We also applied our methodology to the prediction of the efficacy of interferon/ribavirin combination therapy using an additional 56 biopsy samples. **Results:** Our microarray analysis combined with the algorithm was 94% successful at predicting SR/TR and NR patients. A validation study confirmed that this algorithm can predict interferon efficacy with 95% accuracy and a *P* value of less than .00001. Similarly, we obtained a 93% prediction efficacy and a *P* value of less than .0001 for patients receiving combination therapy. **Conclusions:** By using only host data from the complementary DNA microarray we are able to successfully predict SR/TR and NR patients for interferon therapy. Therefore, this technique can help determine the appropriate treatment for hepatitis C patients.

Chronic hepatitis C is one of the major causes of chronic liver disease and can lead to cirrhosis and hepatocellular carcinoma. Interferon is the only effective drug for chronic hepatitis C patients, although better efficacy can be attained with modification of the regimen including the amount of interferon, the duration of treatment, and the use of a combination of pegylated-interferon and ribavirin.

Many studies have identified factors that can help predict the efficacy of interferon therapy such as hepatitis C virus (HCV) genotype[1] and viral loads.[2] Methods based on viral information are able to identify sustained responders (SR). However, this method places transient responders (TR) and nonresponders (NR) in the same category. Follow-up data clearly indicate that interferon treatment of patients in the TR group can lead to a reduction in the probability of tumor development compared with the NR group.[3,4] This suggests that the NR patients should be separated out first and that the TR group should be handled separately as an SR-like group. Furthermore, host factors may help the prediction of NR clinical outcome before treatment. Several candidates have been suggested that may be used to predict this effect including body mass index,[5] γ-glutamyltransferase/alanine transaminase levels,[6] the messenger RNA expression levels of the interferon receptor,[7,8] interferon-γ and tumor necrosis factor-α levels,[9] and the Th1/Th2 ratio[10]; however, there is no definitive evidence that any of these is a single dominant factor. Therefore, additional studies must be performed to identify host factors that can predict the efficacy of interferon therapy because complex changes in these host parameters may reflect variations in hepatic gene expression.

Complementary DNA (cDNA) microarrays can provide an enormous amount of data for identifying clusters

Abbreviations used in this paper: cDNA, complementary DNA; HCV, hepatitis C virus; MD, Mahalanobis distance; NR, nonresponder; SR, sustained responder; SSDB, standard space database; TR, transient responder.

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of predictive factors. For example, we previously have used custom-made cDNA microarrays to dissect gene expression patterns and to differentiate between patients infected with HCV and hepatitis B virus.[11,12] Other oligo-DNA chip approaches have proven to be very effective for identifying sets of genes expressed in vitro in response to interferon.[13] However, these approaches have not been useful for determining which treatment regimen should be used for each patient. In the current study we developed a cDNA microarray and a data analysis algorithm that can predict whether a patient will be an NR for interferon therapy based only on host messenger RNA expression and without the use of viral data.

Materials and Methods

Patients and Biopsy Samples

From 1993 to 2001, we collected liver biopsy samples from 99 HCV patients undergoing interferon monotherapy at Kyushu University Hospital. In addition, between 1999 and 2002, an additional 4 samples were collected from patients undergoing interferon monotherapy at Kanazawa University Hospital and 5 samples from Kyoto Prefectural University Hospital as part of a validation study. These patients received the standard 6-month protocol for interferon-alfa treatment. Thus, all patients received more than 468 MU of interferon-alfa monotherapy. Finally, between 2002 and 2003, 56 patients at Kanazawa University Hospital and Tokyo Metropolitan Komagome Hospital were treated with a 6-month regimen of interferon-alfa combined with 600–800 mg/day of ribavirin. Informed consent was obtained from all patients in accordance with the Helsinki protocol. Liver samples were obtained from these patients by biopsy procedure with a 14- or 16-gauge needle. The samples were snap-frozen in liquid nitrogen and stored at −80°C until use for RNA extraction. The viral genotype in pretreatment serum samples from these patients was determined as described previously,[14] the viral RNA copy number was tested using the HCV Amplicore kit (Roche Japan, Tokyo, Japan), and the viral serotype was assayed using an F-HCV-Gr enzyme-linked immunosorbent assay kit (Sysmex, Kobe, Japan). The patients were categorized into 3 groups: SR (patients with an absence of serum HCV RNA both during therapy and 6 months after the completion of therapy), NR (patients persistently positive for serum HCV RNA during therapy), and TR (patients negative for serum HCV RNA at the end of interferon treatment but positive after cessation of therapy).

RNA Extraction, Complementary DNA Microarray, Data Collection, and Data Mining

The total RNA extraction procedure from biopsy samples and the low-density cDNA microarray together with a unique artificial reference RNA (Genomessage; JGS, Tokyo, Japan) used in these studies were described in our previous report.[15]

Results

Selection of Liver Biopsy Samples

RNA degradation is one of the main factors causing variability in data from cDNA microarrays. Because some of the biopsy samples used in this study were stored for more than 8 years, we examined the quality of the extracted RNA by microcapillary electrophoresis. Enough RNA (>2 μg) was obtained from only 69 of the 108 samples from patients treated with interferon monotherapy. We randomly divided these 69 samples into 2 groups of 31 and 38 samples for training and validation of the prediction algorithm, respectively. Based on the 28S/18S ratio, the RNA quality of these 69 samples was good. Of the 69 total patients, 47 were men and 22 were women, and the average age was 49 ± 12 years (range, 21–71 y). Table 1 summarizes the values of alanine transaminase, γ-glutamyltransferase, viral load, and genotypes for the 31 samples used for developing the prediction algorithm. Similarly, qualified RNA extracted from all 56 samples that had been obtained from patients receiving combination therapy were divided randomly into 2 groups of 33 and 23 samples. Of these patients, 46 were men and 10 were women, and the average age was 54 ± 8 years (range, 39–71 y). The characteristics of the group of 33 patients for establishment of SSDB are summarized in Table 2.

Development of the Complementary DNA Microarray

To develop the cDNA microarray for the current study we first performed a serial analysis of gene expression on data from normal and hepatitis B and C patients for approximately 2000 genes. For this serial analysis of gene expression study we analyzed the results from our previous microarray analysis combined with publicly available data.[16,17] During this initial screening phase we tried to choose genes that could distinguish between hepatitis and normal samples. In addition, to focus on genes with meaningful signal levels, we omitted those with a low-frequency expressed tag in serial analysis of gene expression. These approaches are consistent with those of Chang et al[18] who, for statistical calculation, selected only the strong intensity signals from their GeneChip (Affymetrix; Santa Clara, CA) data. Furthermore, we omitted most sequences representing expressed sequence tags in the serial analysis of gene expression data. Finally, we selected genes whose functions have been well established. For example, 26 interferon-related genes were selected for the microarray. We also tried to select genes that have been reported previously to predict interferon efficacy, including interferon-alfa/β receptor,

Table 1. Characteristics of the Core Patients Used for the SSDB and Training

Number	Age	Sex	Genotype	Viral load (*KIU/mL*)	Histology/stage and activity	ALT (*IU/L*)	γ-GT (*IU/L*)	Clinical outcome	Use
1	23	F	1b	4.4	F1A1	90	32	SR	SSDB
2	31	M	2a	23	F1A1	29	11	SR	SSDB
3	34	F	2a	3.5	F1A1	32	199	SR	SSDB
4	40	M	2a	100	F1A2	233	68	SR	SSDB
5	41	M	1b	110	F1A2	182	117	SR	SSDB
6	48	M	2a	2.2	F2A2	189	37	SR	SSDB
7	50	M	2b	3.7	F1A3	267	114	SR	SSDB
8	54	F	2a	2.3	F1A2	41	31	SR	SSDB
9	55	M	2a	2.4	F1A1	301	85	SR	SSDB
10	58	M	1b	50	F1A2	36	59	SR	SSDB
11	60	M	2b	50	F1A1	149	150	SR	SSDB
12	66	M	2a	1.8	F3A2	286	104	SR	SSDB
13	66	M	1b	140	F1A1	88	31	SR	SSDB
14	21	M	1a, 1b	480	F1A1	34	32	NR	Training
15	27	M	1b	520	F1A1	62	39	NR	Training
16	31	F	2a	20	F1A1	63	36	SR	Training
17	35	M	2a	5.9	F1A1	72	34	SR	Training
18	37	F	1b	650	F1A1	219	58	NR	Training
19	37	M	1b	150	F0A1	79	74	TR	Training
20	37	M	2b	250	F1A1	225	29	TR	Training
21	40	M	2a	16	F1A2	211	129	SR	Training
22	42	M	1b	900	F3A2	86	139	TR	Training
23	49	M	1b	540	F1A1	100	30	TR	Training
24	51	F	1b	480	F1A1	80	34	NR	Training
25	52	M	1b	50	F1A2	96	79	SR	Training
26	53	M	1b	520	F4A2	97	90	NR	Training
27	57	M	1b	130	F1A2	61	37	TR	Training
28	57	M	2a	120	F1A2	164	53	SR	Training
29	59	F	1b	230	F3A2	70	38	NR	Training
30	59	M	2b	32	F1A1	162	119	NR	Training
31	62	F	1b	91	F1A2	90	34	NR	Training

ALT, alanine transaminase; γ-GT, γ-glutamyltransferase.

tumor necrosis factor-α.[7–9] In addition, we incorporated clinical information to help select genes; specifically, we included iron transporter–related genes, such as transferrin and the transferrin receptor, because iron depletion has been shown to enhance the efficacy of hepatitis C treatment.[19] Finally, we included some genes (eg, housekeeping genes) as controls for the microarray. Together, 295 genes were selected originally for the low-density cDNA microarray. Furthermore, after we developed the low-density cDNA microarray, interferon-stimulated genes were analyzed systematically by using a different microarray.[20] Another 452 genes that were derived mainly from interferon-, tumor necrosis factor–, and extracellular matrix–related genes were added to a new cDNA microarray that included a total of 747 genes.

Establishment of the Algorithm for Predicting Interferon Efficacy Based on Complementary DNA Microarray Data

As previously described,[15] we used a series of steps to make a reasonable prediction, including establishing a standard space database (SSDB), selecting characteristic parameters to differentiate groups of interest, setting variance–covariance, calculating the variance–covariance matrix, selecting a correlation/gene network, and, finally, calculating the Mahalanobis distance (MD) (the distance from the center of gravity can be determined for a new test sample using the SSDB), leading to a single parameter as a scale from multiple parameters. Thus, the SSDB dataset was selected from the SR patients that had clear clinical outcomes (13 members). This SSDB was trained by expanding it to different datasets, including SR/TR (10 members) and NR (8 members) data, to find genes that are expressed differentially between the 2 groups. The prediction probability of this stage was as follows: SR/TR (10 of 10; 100%), NR (7 of 8; 88%), with a P value of less than .0005.

By using the new prediction algorithm we assessed the accuracy of prediction using the 38 validation samples (31 SR/TR and 7 NR). We calculated the MD and scaled MD from this established dataset for each patient to determine the distance from the established SSDB center of gravity. At this point the calculation does not incor-

Table 4. Gene List Highlighted as Differentially Expressed in Combination Therapy

GenBank number	Gene name	*F*-test	*t*-test
X66362	PCTAIRE-3 for serine/threonine protein kinase	.007	.000
U90551	Histone (H2A II; histone 2A-like protein)	.054	.002
Y00285	Insulin-like growth factor II receptor	.027	.002
X03884	CD3 epsilon (T3 epsilon chain [20K] of T-cell receptor)	.045	.007
U12779	MAP kinase-activated protein kinase 2	.143	.008
Z33642	Leukocyte surface protein V7; immunoglobulin superfamily, member 2	.086	.010
U49837	LIM protein (cystein-rich protein 3)	.001	.011
M77349	BIGH3, TGF-β–induced product, TGF-β–induced 68 kilodalton	.141	.013
L16499	Orphan homeobox protein; hematopoietically expressed homeobox	.017	.013
X78817	ρ-GAP hematopoietic protein C1; ρ guanosine triphosphatase–activating protein 4	.070	.016
AF159442	Phospholipid scramblase 3	.000	.017
J04164	Interferon-inducible transmembrane protein 9–27	.199	.018
L41351	Serine protease 8 (prostatin)	.033	.019
U62437	Nicotinic acetylcholine receptor β2 subunit prexursor	.052	.020
X58072	GATA binding protein 3; transacting T-cell–specific transcription factor	.132	.027
X53414	L-alanine: glyoxylate transaminase	.021	.030
Y00052	Cyclophilin A (peptidylprolyl isomerase A; T-cell cyclophilin)	.152	.034
BC004490	Fos	.002	.035
U03397	Tumor necrosis factor–receptor superfamily, member 9	.140	.035
Z47087	Pol V elongation factor-like protein; S-phase kinase-associated protein 1A	.190	.047
M14758	Multiple drug resistance protein 1; P-glycoprotein	.066	.047
U61397	Ubiquitin-homology domain protein PIC1 (sentrin)	.021	.050
U16031	Interleukin-4–induced transcription factor, signal transducer and activator of transcription 6	.194	.050
BC032130	Asialoglycoprotein receptor 1	.032	.057
X05610	Type IV collagen alpha (2)	.046	.059
D23661	Ribosomal protein L37	.035	.066
X69150	Ribosomal protein S18	.013	.068
M15400	Retinoblastoma susceptibility	.040	.104
NM_001012	Ribosomal protein S8	.037	.133
M31627	X-box binding protein-1	.005	.198

NOTE. Both *F*-test and *t*-test values less than .2 are listed.

related genes, the SSDB includes genes related to immune response, stress, metal transport, and lipid metabolism. The inclusion of genes controlled by the interferon signal cascade and related to the immune response is not surprising. In addition, genes associated with lipid metabolism are not unexpected because HCV has a high affinity for lipids.[21,22] Furthermore, lipoprotein receptors were reported as HCV receptor candidates.[23,24] In fact, the involvement of lipid metabolism–related genes is described in depth in a study of HCV clearance in the chimpanzee by GeneChip analysis.[25] Therefore, the lipid metabolism–related genes that we included in our analysis could be targets for future study and therapeutic intervention. Finally, the presence of iron transport–related genes in the SSDB corresponds with the use of blood depletion therapies to reduce liver inflammation in hepatitis patients. It also may be of interest to study how genes in the SSDB, including additional metal-related genes such as metallothioneins, play a role in interferon efficacy.

These findings suggest that the TR patients have an anti-HCV interferon response similar to that of the SR patients. Indeed, it is possible that these TR patients may have become SR patients if interferon treatment was administered for more than 6 months because there is a significant effect of treatment duration in the efficacy of interferon treatment for chronic hepatitis C.[26] This observation is consistent with a study of chimpanzee HCV cases based on oligo-chip data.[25] Furthermore, the fact that we could predict the NR group without any viral information suggests that, in these cases, the host has an unfavorable response to the interferon treatment, which also suggests that, as in the SR group, there is an interaction between the host and the virus. Understanding the host response to interferon in NR patients could provide interesting targets for the development of new treatments for HCV.

In conclusion, we have established a low-density cDNA microarray for predicting interferon efficacy in chronic hepatitis C patients. Based only on host messenger RNA expression profiles from pretreatment biopsy samples, we can categorize patients successfully into SR/TR and NR groups with over 90% accuracy.

References

1. Yoshioka K, Kakumu S, Wakita T, et al. Detection of hepatitis C virus by polymerase chain reaction and response to interferon-

alpha therapy: relationship to genotypes of hepatitis C virus. Hepatology 1992;16:293–299.
2. Lau JY, Davis GL, Kniffen J, et al. Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet 1993;341: 1501–1504.
3. International Interferon-alpha Hepatocellular Carcinoma Study Group. Effect of interferon-alpha on progression of cirrhosis to hepatocellular carcinoma: a retrospective cohort study. Lancet 1998;351:1535–1539.
4. Kasahara A, Hayashi N, Mochizuki K, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. Hepatology 1998;27:1394–1402.
5. Bressler BL, Guindi M, Tomlinson G, et al. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. Hepatology 2003;38:639–644.
6. Mihm S, Monazahian M, Grethe S, et al. Ratio of serum gamma-GT/ALT rather than ISDR variability is predictive for initial virological response to IFN-alpha in chronic HCV infection. J Med Virol 1999;58:227–234.
7. Yatsuhashi H, Yamasaki K, Aritomi T, et al. Quantitative analysis of interferon alpha/beta receptor mRNA in the liver of patients with chronic hepatitis C: correlation with serum hepatitis C virus-RNA levels and response to treatment with interferon. J Gastroenterol Hepatol 1997;12:460–467.
8. Mizukoshi E, Kaneko S, Yanagi M, et al. Expression of interferon alpha/beta receptor in the liver of chronic hepatitis C patients. J Med Virol 1998;56:217–223.
9. Dumoulin FL, Wennrich U, Nischalke HD, et al. Intrahepatic mRNA levels of interferon gamma and tumor necrosis factor alpha and response to antiviral treatment of chronic hepatitis C. J Hum Virol 2001;4:195–199.
10. Sobue S, Nomura T, Ishikawa T, et al. Th1/Th2 cytokine profiles and their relationship to clinical features in patients with chronic hepatitis C virus infection. J Gastroenterol 2001;36:544–551.
11. Honda M, Kaneko S, Kawai H, et al. Differential gene expression between chronic hepatitis B and C hepatic lesion. Gastroenterology 2001;120:955–966.
12. Shirota Y, Kaneko S, Honda M, et al. Identification of differentially expressed genes in hepatocellular carcinoma with cDNA microarrays. Hepatology 2001;33:832–840.
13. Der SD, Zhou A, Williams BR, et al. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A 1998;95: 15623–15628.
14. Okamoto H, Sugiyama Y, Okada S, et al. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 1992;73:673–679.
15. Daiba A, Inaba N, Ando S, et al. A low-density cDNA microarray with a unique reference RNA: pattern recognition analysis for IFN efficacy prediction to HCV as a model. Biochem Biophys Res Commun 2004;315:1088–1096.
16. Yamashita T, Hashimoto S, Kaneko S, et al. Comprehensive gene expression profile of a normal human liver. Biochem Biophys Res Commun 2000;269:110–116.
17. Yamashita T, Kaneko S, Hashimoto S, et al. Serial analysis of gene expression in chronic hepatitis C and hepatocellular carcinoma. Biochem Biophys Res Commun 2001;282:647–654.
18. Chang JC, Wooten EC, Tsimelzon A, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. Lancet 2003;362:362–369.
19. Fontana RJ, Israel J, LeClair P, et al. Iron reduction before and during interferon therapy of chronic hepatitis C: results of a multicenter, randomized, controlled trial. Hepatology 2000;31: 730–736.
20. de Veer MJ, Holko M, Frevel M, et al. Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol 2001;69:912–920.
21. Andre P, Komurian-Pradel F, Deforges S, et al. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J Virol 2002;76:6919–6928.
22. Kono Y, Hayashida K, Tanaka H, et al. High-density lipoprotein binding rate differs greatly between genotypes 1b and 2a/2b of hepatitis C virus. J Med Virol 2003;70:42–48.
23. Agnello V, Abel G, Elfahal M, et al. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci U S A 1999;96:12766–12771.
24. Scarselli E, Ansuini H, Cerino R, et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J 2002;21:5017–5025.
25. Su AI, Pezacki JP, Wodicka L, et al. Genomic analysis of the host response to hepatitis C virus infection. Proc Natl Acad Sci U S A 2002;99:15669–15674.
26. Poynard T, Leroy V, Cohard M, et al. Meta-analysis of interferon randomized trials in the treatment of viral hepatitis C: effects of dose and duration. Hepatology 1996;24:778–789.

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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 × 10^6 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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Key words: immune response; epitope; CD8; HLA-A24; hepatitis

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 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 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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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 900*g* for 22 min at room temperature, PBMCs were harvested from the interphase, resuspended in PBS and centrifuged again at 600*g*. Each cell pellet was resuspended in PBS, centrifuged at 300*g* 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).

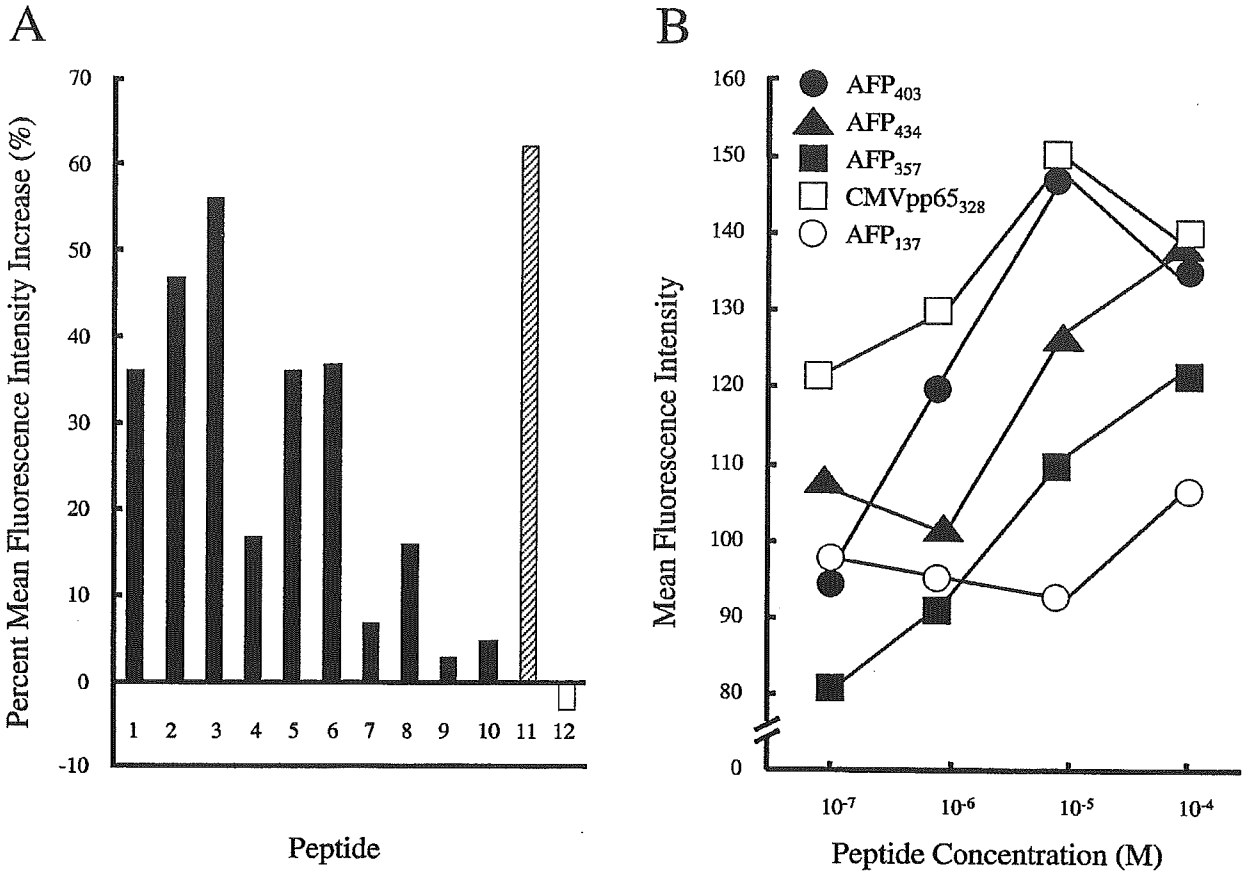

FIGURE 1 – MHC binding affinity. (*a*) TAP-deficient T2-A24 cells were cultured for 16 hr at 26°C to enhance the expression of peptide-receptive cell surface molecules. They were incubated with individual peptides at 10 μg/ml at 37°C for 2 hr, washed and stained with anti-HLA-A24 MAb, anti-mouse immunoglobulin 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" (≦2cm) for 10 cases or "large" (>2cm) 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),[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. 1*a*). 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 1*b*, 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 2*a*. 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. 1*a*). 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 2*a*. 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. 2*b*), 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. 2*b*). 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. 5*a* and 5*b*). Also, CTLs incubated without any Ab did not show cytotoxicity against K562 that did not express HLA molecules (Figs. 4*a* and 4*b*). 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 1of 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.

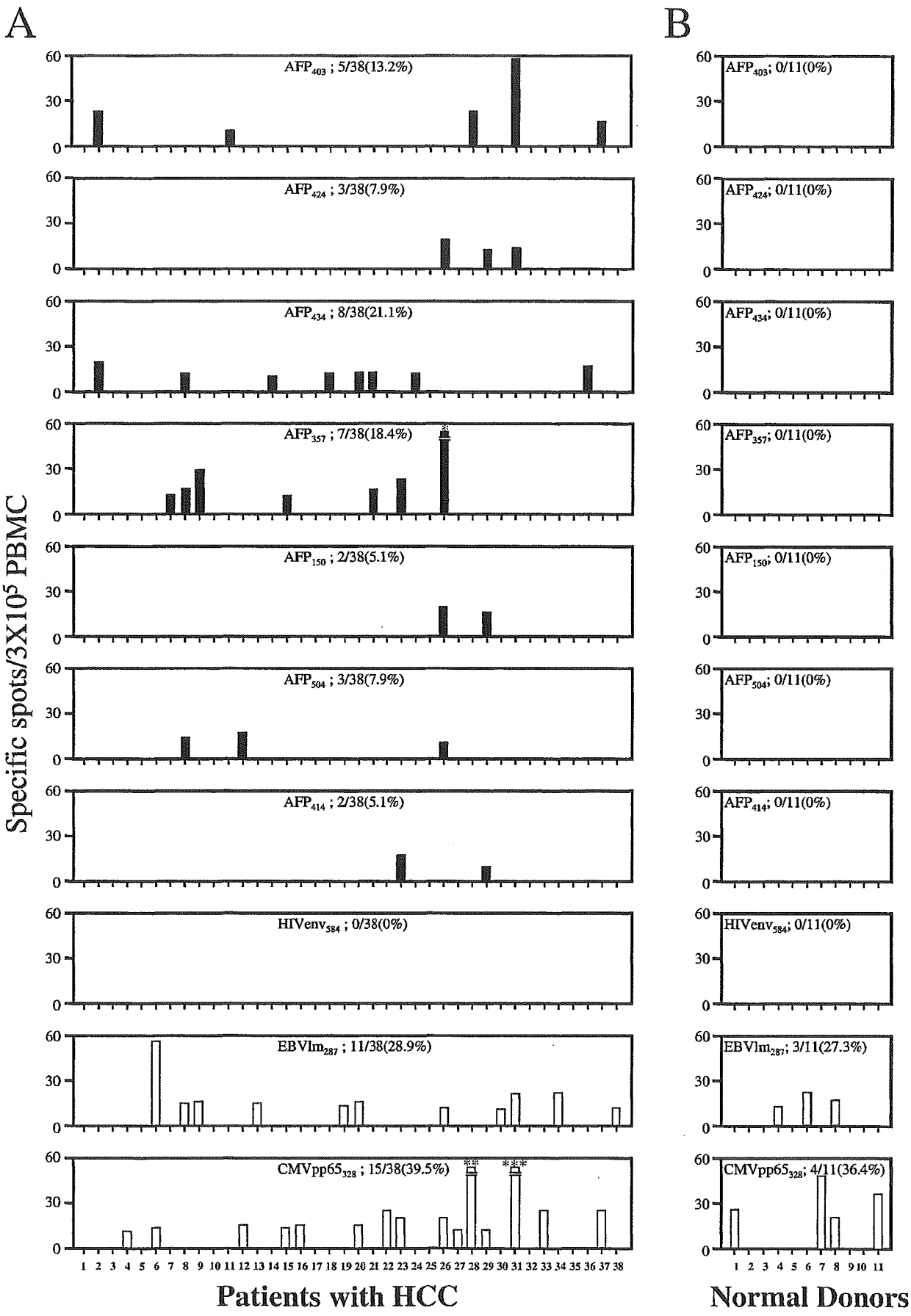
A
B
Specific spots/3X10^5 PBMC
AFP$_{403}$; 5/38(13.2%)
AFP$_{424}$; 3/38(7.9%)
AFP$_{434}$; 8/38(21.1%)
AFP$_{357}$; 7/38(18.4%)
AFP$_{150}$; 2/38(5.1%)
AFP$_{504}$; 3/38(7.9%)
AFP$_{414}$; 2/38(5.1%)
HIVenv$_{584}$; 0/38(0%)
EBVlm$_{287}$; 11/38(28.9%)
CMVpp65$_{328}$; 15/38(39.5%)
AFP$_{403}$; 0/11(0%)
AFP$_{424}$; 0/11(0%)
AFP$_{434}$; 0/11(0%)
AFP$_{357}$; 0/11(0%)
AFP$_{150}$; 0/11(0%)
AFP$_{504}$; 0/11(0%)
AFP$_{414}$; 0/11(0%)
HIVenv$_{584}$; 0/11(0%)
EBVlm$_{287}$; 3/11(27.3%)
CMVpp65$_{328}$; 4/11(36.4%)
60
30
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
1 2 3 4 5 6 7 8 9 10 11
Patients with HCC
Normal Donors

FIGURE 2.

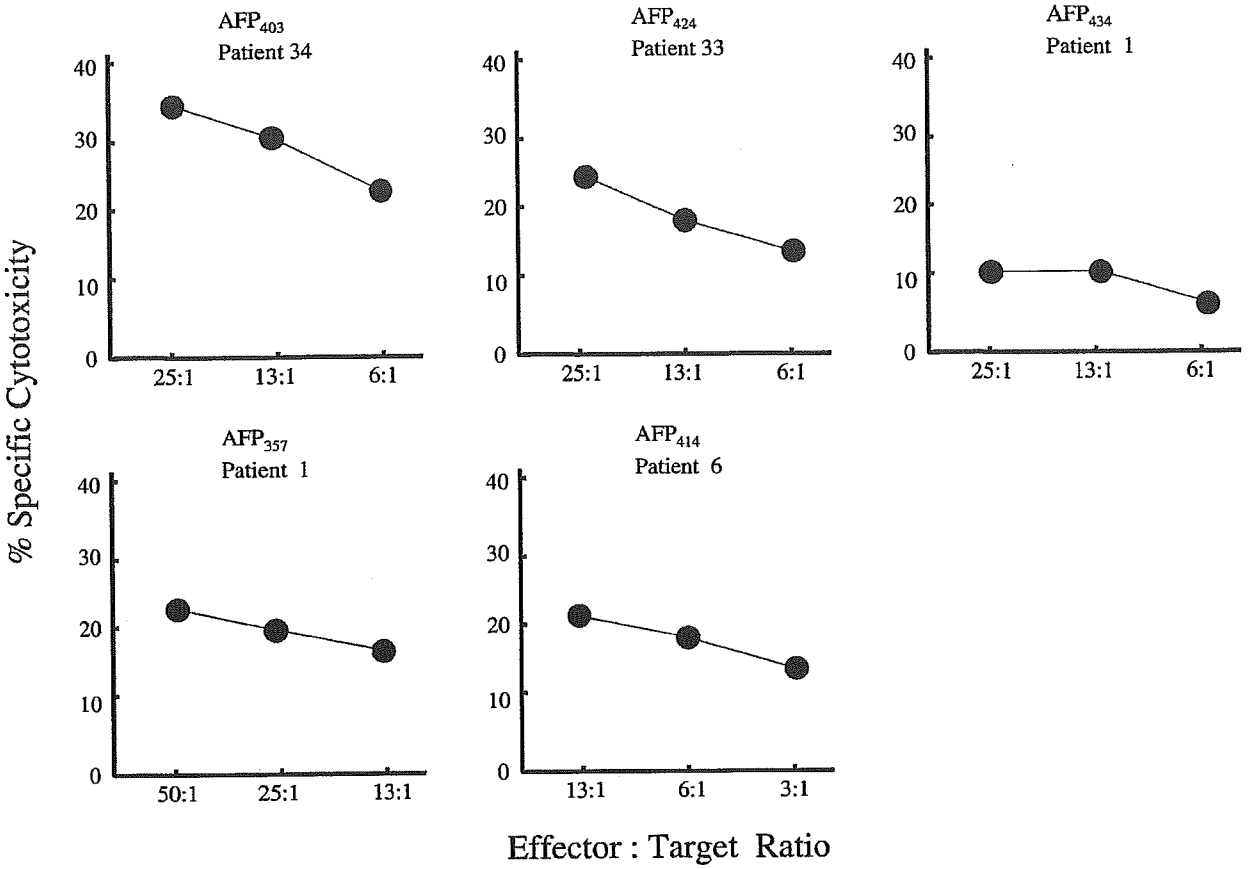

FIGURE 3 – Cytotoxicity of AFP-specific T-cell lines in patients with HCC. The cytotoxicity of the T-cell lines was determined by a standard 6 hr cytotoxicity assay at various effector to target (E/T) ratios against C1R-A*2402 cells pulsed with 1of the AFP-derived peptides listed in Table II. The number 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 AFP_{403}, AFP_{424}, AFP_{434}, AFP_{357} and AFP_{414}) 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 1×10^6 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 $EBVlm_{287}$ or $CMVpp65_{328}$ were sero-positive

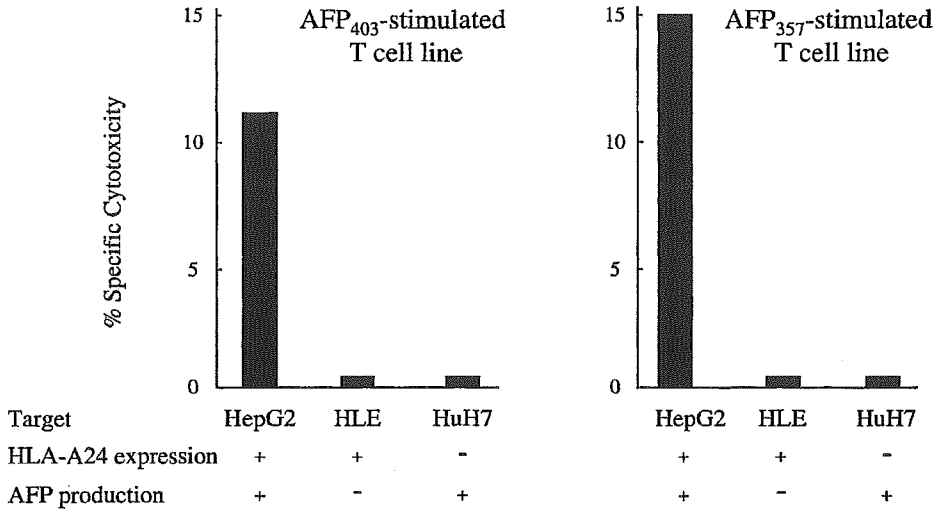

FIGURE 4 – Cytotoxicity of AFP-specific T-cell lines on cancer cell lines that do or do not express HLA-A*2402 or AFP. The cytotoxicity was determined by a standard 6 hr cytotoxic assay (E/T ratio of 50:1).

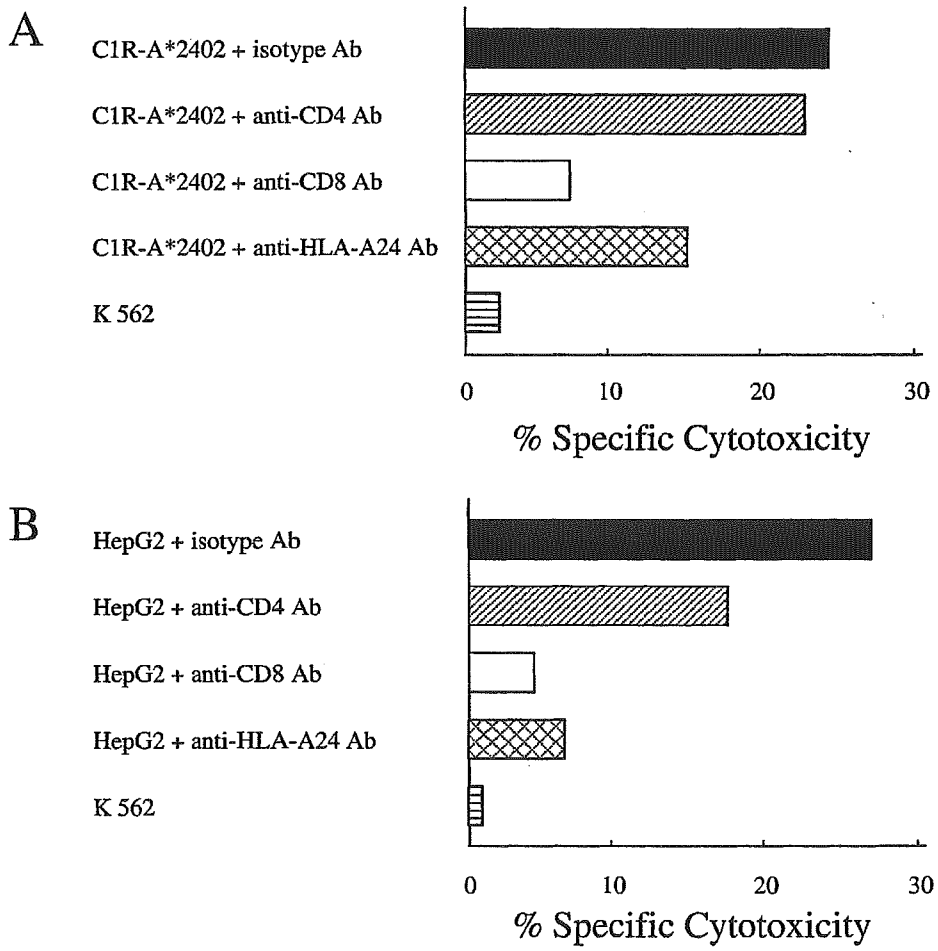

FIGURE 5 – Inhibition of cytotoxicity of AFP-specific T-cell lines by specific antibodies. T-cell lines were generated from PBMC of HCC patients by stimulation with AFP_{357}. Inhibition of cytotoxicity was determined using a standard 6 hr cytotoxicity assay against C1R-A*2402 cells pulsed with AFP_{357}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

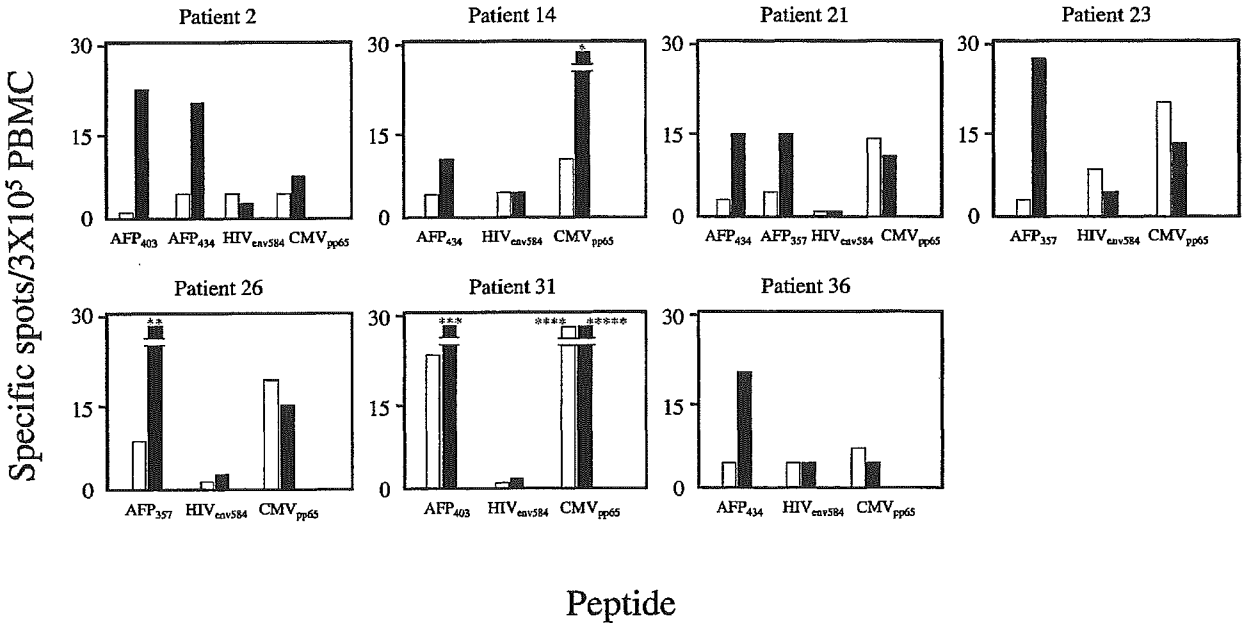

FIGURE 6 – The induction of AFP-specific T-cell responses in HCC patients after treatment of HCC. Direct *ex vivo* analysis (IFN-γ ELISPOT assay) of peripheral blood T-cell responses to AFP-, HIV- or CMV-derived peptides were performed before (open bar) and after (solid bar) HCC treatment. Only patients 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 molecule, 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.

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References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001;94:153–6.
2. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745–50.
3. Deuffic S, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. Lancet 1998;351:214–5.
4. The Liver Cancer Study Group of Japan. Predictive factors for long term prognosis after partial hepatectomy for patients with hepatocellular carcinoma in Japan. Cancer 1994;74:2772–80.
5. Livraghi T, Giorgio A, Marin G, Salmi A, de Sio I, Bolondi L, Pompili M, Brunello F, Lazzaroni S, Torzilli S. Hepatocellular carcinoma and cirrhosis in 746 patients: long-term results of percutaneous ethanol injection. Radiology 1995;197:101–8.
6. Curley SA, Izzo F, Ellis LM, Nicolas Vauthey J, Vallone P. Radiofrequency ablation of hepatocellular cancer in 110 patients with cirrhosis. Ann Surg 2000;232:381–91.
7. Lin DY, Liaw YF, Lee TY, Lai CM. Hepatic arterial embolization in patients with unresectable hepatocellular carcinoma-a randomized controlled trial. Gastroenterology 1988;94:453–6.

8. Urabe T, Kaneko S, Matsushita E, Unoura M, Kobayashi K. Clinical pilot study of intrahepatic arterial chemotherapy with methotrexate, 5-fluorouracil, cisplatin and subcutaneous interferon-α-2b for patients with locally advanced hepatocellular carcinoma. Oncology 1998;55: 39–47.
9. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. N Engl J Med 1996;334:693–9.
10. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudley ME, Schwarz SL, Spiess PJ, Wunderlich JR, Parkhurst MR, *et al.* Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 1998;4:321–7.
11. Ribas A, Butterfield LH, Glaspy JA, Economou JS. Current developments in cancer vaccines and cellular immunotherapy. J Clin Oncol 2003;21:2415–32.
12. Kirkwood JM, Lotze M, Yasko JM. Current cancer therapeutics. Philadelphia: Current Medicine, 1994. 344p.
13. Butterfield LH, Koh A, Meng W, Vollmer CM, Ribas A, Dissette V, Lee E, Glaspy JA, McBride WH, Economou JS. Generation of human T-cell responses to an HLA-A2.1-restricted peptide epitope derived from α-fetoprotein. Cancer Res 1999;59:3134–42.
14. Butterfield LH, Meng WS, Koh A, Vollmer CM, Ribas A, Dissette VB, Faull K, Glaspy JA, McBride WH, Economou JS. T cell responses to HLA-A*0201-restricted peptides derived from human α-fetoprotein. J Immunol 2001;166:5300–8.
15. Butterfield LH, Ribas A, Meng WS, Dissette VB, Amarnani S, Vu HT, Seja E, Todd K, Glaspy JA, McBride WH, Economou JS. T-cell responses to HLA-A*0201 immunodominant peptides derived from α-fetoprotein in patients with hepatocellular cancer. Clin Cancer Res 2003;9:5902–8.
16. Grimm CF, Ortmann D, Mohr L, Michalak S, Krohne TU, Meckel S, Eisele S, Encke J, Blum HE, Geissler M. Mouse α-fetoprotein-specific DNA-based immunotherapy of hepatocellular carcinoma leads to tumor regression in mice. Gastroenterology 2000;119:1104–12.
17. Araki T, Itai Y, Furui S, Tasaka A. Dynamic CT densitometry of hepatic tumors. AJR Am J Roentgenol 1980;135:1037–43.
18. Liver Cancer Study Group of Japan, eds. General rules for the clinical and pathological study of primary liver cancer, English 2nd ed. Tokyo: Kanehara, 1997. 30p.
19. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–20.
20. Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, Takiguchi M. Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1. J Immunol 1997;159:6242–52.
21. Lee SP, Tierney RJ, Thomas WA, Brooks JM, Rickinson AB. Conserved CTL epitopes within EBV latent membrane protein 2: a potential target for CTL-based tumor therapy. J Immunol 1997;158: 3325–34.
22. Kuzushima K, Hayashi N, Kimura H, Tsurumi T. Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood 2001;98:1872–81.
23. Oiso M, Eura M, Katsura F, Takiguchi M, Sobao Y, Masuyama K, Nakashima M, Itoh K, Ishikawa T. A newly identified MAGE-3-derived epitope recognized by HLA-A24-restricted cytotoxic T lymphocytes. Int J Cancer 1999;81:387–94.
24. Wedemeyer H, Mizukoshi E, Davis AR, Bennink JR, Rehermann B. Cross-reactivity between hepatitis C virus and Influenza A virus determinant-specific cytotoxic T cells. J Virol 2001;75:11392–400.
25. Mizukoshi E, Nascimbeni M, Blaustein JB, Mihalik K, Rice CM, Liang TJ, Feinstone SM, Rehermann B. Molecular and immunological significance of chimpanzee major histocompatibility complex haplotypes for hepatitis C virus immune response and vaccination studies. J Virol 2002;76:6093–103.
26. Sobin LH, Wittekind C, eds. TNM classification of malignant tumors, 6th ed. New York: Wiley-Liss, 2002. 81p.
27. Kawai HF, Kaneko S, Honda M, Shirota Y, Kobayashi K. α-Fetoprotein-producing hepatoma cell lines share common expression profiles of genes in various categories demonstrated by cDNA microarray analysis. Hepatology 2001;33:676–91.
28. Taketa K. α-Fetoprotein: reevaluation in hepatology. Hepatology 1990; 12:1420–32.
29. Taketa K, Ichikawa E, Yamamoto T, Kato H, Matsuura S, Taga H, Hirai H. Datura stramonium agglutinin-reactive α-fetoprotein isoforms in hepatocellular carcinoma and other tumors. Tumour Biol 1990; 11:220–8.
30. Taketa K, Sekiya C, Namiki M, Akamatsu K, Ohta Y, Endo Y, Kosaka K. Lectin-reactive profiles of α-fetoprotein characterizing hepatocellular carcinoma and related conditions. Gastroenterology 1990;99: 508–18.
31. Fujioka M, Nakashima Y, Nakashima O, Kojiro M. Immunohistologic study on the expressions of α-fetoprotein and protein induced by vitamin K absence or antagonist II in surgically resected small hepatocellular carcinoma. Hepatology 2001;34:1128–34.
32. Imanishi T, Akaza T, Kimura A, Tokunaga K, Gojobori T. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. Oxford: Oxford Scientific Publications, 1992.1065–220.
33. Tokunaga K, Ishikawa Y, Ogawa A, Wang H, Mitsunaga S, Moriyama S, Lin L, Bannai M, Watanabe Y, Kashiwase K, Tanaka H, Akaza T, *et al.* Sequence-based association analysis of HLA class I and II alleles in Japanese supports conservation of common haplotypes. Immunogenetics 1997;46:199–205.
34. Dhodapkar MV, Young JW, Chapman PB, Cox WI, Fonteneau JF, Amigorena S, Houghton AN, Steinman RM, Bhardwaj N. Paucity of functional T-cell memory to melanoma antigens in healthy donors and melanoma patients. Clin Cancer Res 2000;6:4831–8.
35. Nagorsen D, Keilholz U, Rivoltini L, Schmittel A, Letsch A, Asemissen AM, Berger G, Buhr HJ, Thiel E, Scheibenbogen C. Natural T-cell response against MHC class I epitopes of epithelial cell adhesion molecule, her-2/neu, and carcinoembryonic antigen in patients with colorectal cancer. Cancer Res 2000;60:4850–4.
36. Scheibenbogen C, Lee KH, Stevanovic S, Witzens M, Willhauck M, Waldmann V, Naeher H, Rammensee HG, Keilholz U. Analysis of the T cell response to tumor and viral peptide antigens by an IFN-γ-ELISPOT assay. Int J Cancer 1997;71:932–6.
37. Nagorsen D, Scheibenbogen C, Schaller G, Leigh B, Schmittel A, Letsch A, Thiel E, Keilholz U. Differences in T-cell immunity toward tumor-associated antigens in colorectal cancer and breast cancer patients. Int J Cancer 2003;105:221–5.
38. Griffioen M, Borghi M, Schrier PI, Osanto S. Detection and quantification of CD8(+) T cells specific for HLA-A*0201-binding melanoma and viral peptides by the IFN-γ-ELISPOT assay. Int J Cancer 2001;93:549–55.
39. Rentzsch C, Kayser S, Stumm S, Watermann I, Walter S, Stevanovic S, Wallwiener D, Guckel B. Evaluation of pre-existent immunity in patients with primary breast cancer: molecular and cellular assays to quantify antigen-specific T lymphocytes in peripheral blood mononuclear cells. Clin Cancer Res 2003;9:4376–86.
40. Ritter M, Ali MY, Grimm CF, Weth R, Mohr L, Bocher WO, Endrulat K, Wedemeyer H, Blum HE, Geissler M. Immunoregulation of dendritic and T cells by α-fetoprotein in patients with hepatocellular carcinoma. J Hepatol 2004;41:999–1007.
41. Nakamoto Y, Kaneko S, Takizawa H, Kikumoto Y, Takano M, Himeda Y, Kobayashi K. Analysis of the CD8-positive T cell response in Japanese patients with chronic hepatitis C using HLA-A*2402 peptide tetramers. J Med Virol 2003;70:51–61.
42. Kanto T, Hayashi N, Takehara T, Tatsumi T, Kuzushita N, Ito A, Sasaki Y, Kasahara A, Hori M. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J Immunol 1999;162:5584–91.
43. Auffermann-Gretzinger S, Keeffe EB, Levy S. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. Blood 2001;97:3171–6.
44. Beckebaum S, Cicinnati VR, Zhang X, Ferencik S, Frilling A, Grosse-Wilde H, Broelsch CE, Gerken G. Hepatitis B virus-induced defect of monocyte-derived dendritic cells leads to impaired T helper type 1 response in vitro: mechanisms for viral immune escape. Immunology 2003;109:487–95.
45. Zhang J, Dong B, Liang P, Yu X, Su L, Yu D, Ji X, Yu G. Significance of changes in local immunity in patients with hepatocellular carcinoma after percutaneous microwave coagulation therapy. Chin Med J 2002;115:1367–71.
46. Dong BW, Zhang J, Liang P, Yu XL, Su L, Yu DJ, Ji XL, Yu G. Sequential pathological and immunologic analysis of percutaneous microwave coagulation therapy of hepatocellular carcinoma. Int J Hyperthermia 2003;19:119–33.

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

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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.

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.

Abbreviations: cDNA, complementary DNA; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; aRNA, antisense RNA.

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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.

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.

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

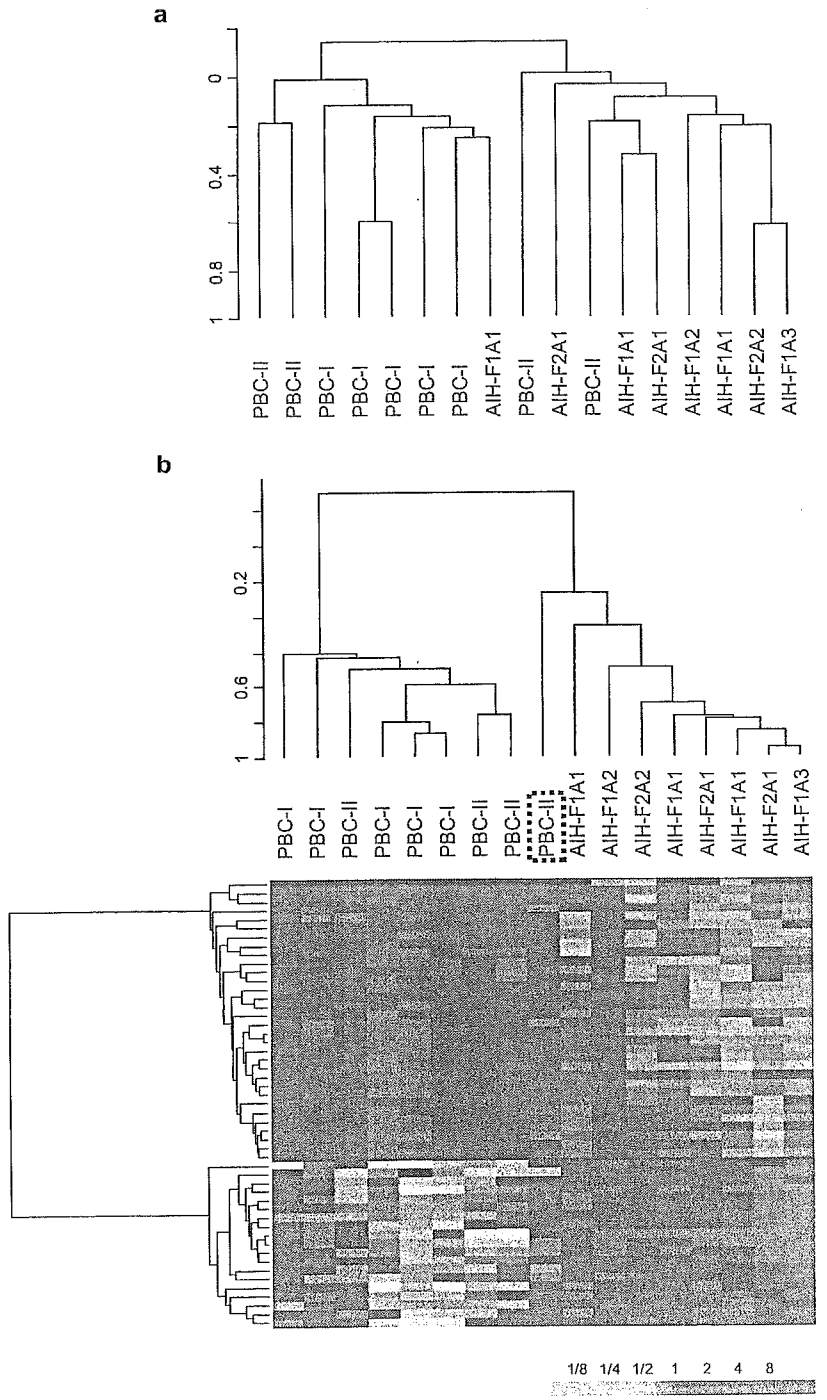

Fig. 1. a: Hierarchical clustering analysis of 1080 genes in AIH (red) and PBC (blue) patients. The dendrogram shows the grouping of patients based on similarities in their gene expression patterns. Histological findings for each patient are shown under individual roots. b: Hierarchical clustering analysis 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	Desmoglein 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