Expression Profiling of Peripheral-Blood Mononuclear Cells from Patients with Chronic Hepatitis C Undergoing Interferon Therapy

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Background. Interferon (IFN) is now the standard treatment for chronic hepatitis C (CH-C); however, treatment efficacy is unpredictable before IFN therapy is started.

Methods. We investigated the gene-expression profiles of peripheral-blood mononuclear cells (PBMCs) from patients with CH-C showing different responses to IFN. Gene-expression profiles of PBMCs were analyzed in 21 patients with CH-C treated with IFN alone or in combination with ribavirin as well as in 6 healthy volunteers. Serial changes in the gene-expression profiles of PBMCs from individual patients were evaluated before treatment, 2 weeks after the start of IFN therapy, and 6 months after the completion of IFN therapy.

Results. Interestingly, the gene-expression profiles of PBMCs from patients with CH-C and healthy volunteers differed substantially; early T cell–activation antigen CD69 was significantly up-regulated in patients with CH-C, but immune-related molecules such as chemokine (C-C motif) receptor 2 and interleukin 7 receptor were significantly down-regulated. Selected combinations of expressed genes obtained before treatment and during IFN therapy by use of a fuzzy neural network combined with the SWEEP operator method predicted the outcome of IFN therapy with peak accuracies of 91.0% and 90.2%, respectively.

Conclusions. These findings suggest that the gene-expression profiles of PBMCs from patients with CH-C may be useful biomarkers for IFN therapy.

Although interferon (IFN) is currently the standard treatment for patients with chronic hepatitis C (CH-C), only 30%–40% of patients completely eliminate the virus, even after effective IFN and ribavirin combination therapy [1–3]. The mechanism of viral persistence during IFN treatment remains to be clarified. It has been reported that several clinical factors, such as viral load, genotype, degree of fibrosis, and expression of type I IFN receptors, are useful predictive factors for the outcome of IFN therapy [4–6]; however, precise prediction is not possible at present.

Type I IFN, such as IFN-α and IFN-β, plays an important role in innate immunity against viral infections by suppressing viral replication [7, 8]. However, the biological activities of IFN have not been fully elucidated. In viral infections such as measles, the number of peripheral lymphocytes generally decreases. It has also been reported that infection of dendritic cells and other immunocompetent cells is involved in exacerbated disease states and persistent infection [9]. Hence, it may be possible to assess disease state and severity by examining peripheral-blood mononuclear cells (PBMCs) from infected individuals. PBMCs include lymphocytes and monocytes, which play the most important roles in the immunological response to viral infection.

In the present study, we investigated the gene-expression profiles of PBMCs from patients with CH-C and healthy volunteers by use of cDNA microarray techniques [10–16]. By determining the gene-expression profiles of PBMCs from patients with CH-C receiving IFN therapy, we also clarified the differences in the PBMC gene-expression profiles between patients

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Table 1. Clinical characteristics of patients and responses to interferon (IFN) therapy.

Group, patient (sex, age in years)	ALT level, IU/L	Histology score[a]	Serotype	IFN therapy	Response	Serum HCV RNA level, kIU/mL			PBMC HCV RNA at 2 weeks
						Before	2 weeks	6 months	
Group A									
1 (M, 46)	31	F1/A1	2	Mono	CR	23	<0.5	<0.5	−
2 (F, 47)	40	F1/A1	2	Mono	CR	416	<0.5	<0.5	+
3 (M, 71)	59	F4/A2	1	Mono	CR	42.3	2.2	<0.5	−
4 (M, 55)	19	F4/A2	2	Mono	CR	1.3	<0.5	<0.5	−
5 (M, 54)	30	F2/A1	1	Mono	BR	620	ND	>850	ND
6 (F, 43)	46	F2/A1	1	Mono	BR	160	<0.5	611	+
7 (M, 58)	236	F1-2/A1	NA	Mono	BR	360	<0.5	620	−
8 (M, 60)	114	F3/A2	2	Mono	BR	770	<0.5	2200	−
9 (M, 62)	70	F2/A1	1	Mono	NR	130	130	350	+
10 (M, 42)	59	F2/A1	1	Mono	NR	800	7.2	190	−
11 (F, 62)	138	F2-3/A2	2	Mono	NR	650	183	1400	+
12 (M, 49)	48	F2/A2	2	Mono	NR	330	<0.5	69.5	−
13 (F, 56)	104	F1/A1	1	Mono	NR	751	<0.5	610	−
Group B									
14 (M, 49)	69	F3/A2	1	Combination	CR	>850	ND	<0.5	ND
15 (M, 50)	35	F1/A2	1	Combination	CR	475	<0.5	<0.5	ND
16 (M, 44)	106	F2/A2	1	Combination	NR	325	68.8	82.6	ND
17 (M, 56)	30	F2/A1	1	Combination	CR	91	<0.5	<0.5	ND
18 (F, 39)	47	F1/A1	1	Combination	CR	>850	0.7	<0.5	ND
19 (F, 64)	117	F2/A1	1	Combination	NR	484	0.8	>850	ND
20 (M, 66)	31	F2/A1	1	Combination	NR	>850	390	1300	ND
21 (F, 62)	103	F3/A2	1	Combination	NR	820	270	1200	ND

NOTE. +, positive; −, negative; ALT, alanine aminotransferase; BR, biochemical responder; CR, complete responder; F, female; M, male; NA, not applicable; ND, not detected; NR, nonresponder; PBMC, peripheral-blood mononuclear cell.

[a] Grading and staging of chronic hepatitis were histologically assessed according to the method of Desmet et al. [17], as described in the text.

with CH-C who responded to IFN therapy (complete responders [CRs]) and those who did not (nonresponders [NRs]).

SUBJECTS, MATERIALS, AND METHODS

Patients. Subjects were 21 patients with CH-C and 7 patients who showed no clinical signs of hepatitis at Kanazawa University Hospital, Japan, between 1999 and 2001. To 13 patients with CH-C (group A), 6 million IUs of IFN-α2b was administered every day for 2 weeks and then 3 times weekly for 22 weeks. To 8 patients with CH-C (group B), IFN-α2b was administered in the same fashion, and ribavirin was administered concomitantly (600 mg for ≤60 kg of body weight, 800 mg for >60 and ≤80 kg of body weight, and 1000 mg for >80 kg of body weight). The 6 age- and sex-matched healthy volunteers were seronegative for either hepatitis B surface antigen or hepatitis C virus (HCV) antibody and had liver function values within normal limits. Eight CRs (negative HCV RNA for >6 months), 4 biochemical responders (BRs; normal serum alanine aminotransferase [ALT] levels for >6 months and positive serum HCV RNA), and 9 NRs to IFN therapy were enrolled. After informed consent was obtained from patients, peripheral-blood samples were collected before the start of IFN therapy, at 2 weeks into treatment, and at 6 months after the completion of treatment. PBMCs were then isolated from whole blood and stored in liquid nitrogen until use. Grading and staging of chronic hepatitis were histologically assessed according to the method of Desmet et al. [17]. Clinical characteristics, such as sex, age, ALT levels, degree of histological activity or staging, HCV RNA load and HCV serotype, did not differ significantly among the groups (table 1).

Virological assessment. The amount of HCV RNA was assayed by the Amplicor Monitor Test (Roche Molecular Systems). HCV was classified by a serologic genotyping assay that has been shown to be specific and sensitive for determining HCV genome subtype [18].

Preparation of cDNA microarray slides. Most cDNA clones used in the present study were obtained from IMAGE Consortium libraries through their distributor, Research Genetics, as described elsewhere [19–24]. In addition to these clones, we included clones to monitor IFN signaling. The newly constructed cDNA microarray slide (Kanazawa IFN chip; version 1.0) comprised 400 representative IFN signaling–related

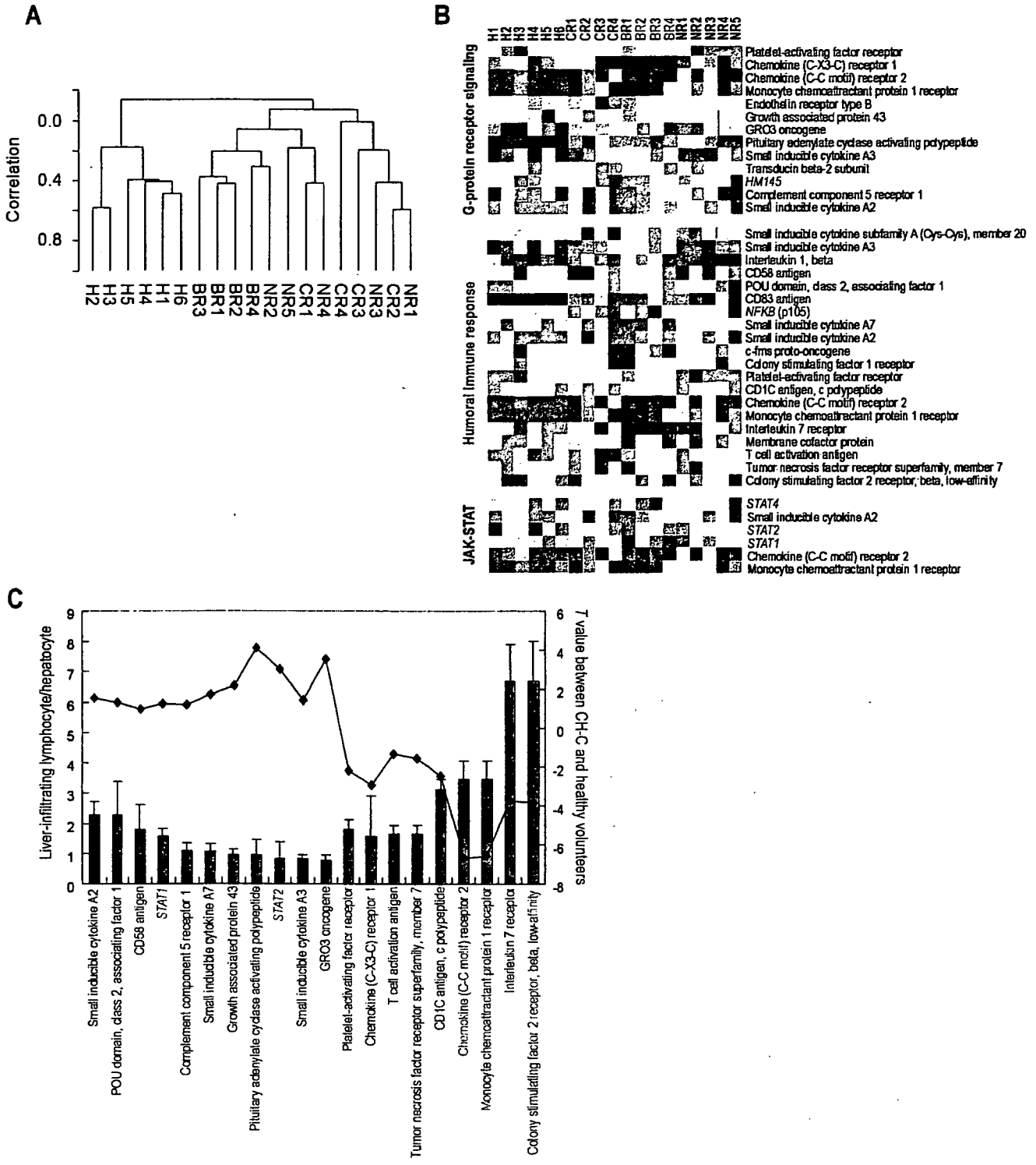


Figure 1. *A,* Hierarchical clustering analysis of gene-expression profiles of peripheral-blood mononuclear cell (PBMC) samples from 13 patients with chronic hepatitis C (CH-C; complete responders [CRs] 1–4, biochemical responders [BRs] 1–4, and nonresponders [NRs] 1–5) and 6 healthy volunteers (H1–H6) among 1305 tested genes before the start of interferon (IFN) therapy, performed using BRB-ArrayTools software. The dendrogram indicates the order in which patients were grouped on the basis of similarities in their gene-expression patterns. *B,* One-way clustering analysis of gene-expression profiles of PBMCs before the start of IFN therapy, using differentially expressed genes in the Janus kinase signal transducer and activation of transcription (JAK-STAT) cascade, humoral immune response, and G protein–coupled receptor protein signaling pathway. Gene cluster data are presented graphically as colored images; red indicates up-regulated genes, and blue indicates down-regulated genes. *C,* Bar graph indicating gene expression in liver-infiltrating lymphocytes relative to that in hepatocytes *(left axis),* and line graph indicating the *T* values for class-prediction analysis between patients with CH-C and healthy volunteers *(right axis).* Genes with increased expression in the liver *(red)* tended to be expressed at lower levels in PBMCs, and genes with decreased expression in the liver *(blue)* tended to be expressed at higher levels in PBMCs.

Table 2. Representative up- or down-regulated genes in patients with chronic hepatitis C, compared with that in healthy volunteers.

Category, gene name	Ratio	*T*	*P*	GenBank accession no.	Gene annotation
Up-regulated					
CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	3.60	4.26	.00125	NM_004233	Defense response
Thrombospondin 1	3.29	5.19	.00014	NM_003246	Endopeptidase inhibitor activity
CD69 antigen (p60, early T cell–activation antigen)	2.87	5.55	.00001	NM_001781	Transmembrane receptor activity
Regulator of G protein signaling 1	2.33	4.31	.00029	NM_002922	Signal transducer activity
Pituitary adenylate cyclase–activating polypeptide	2.01	4.12	.00046	NM_001117	Neuropeptide hormone activity
Nicotinamide N-methyltransferase	1.99	5.29	.00003	NM_006169	Methyltransferase activity
Clone rasi-1 matrix metalloproteinase RASI-1	1.70	4.56	.00019	NM_002429	Hydrolase activity
VASP, exons 4–13	1.68	4.35	.00026	NM_003370	Actin binding
Xeroderma pigmentosum, complementation group A	1.63	3.86	.00085	NM_000380	Damaged DNA binding
Urokinase-type plasminogen activator receptor; GPI-anchored form precursor *(UPAR)*; monocyte-activation antigen Mo3; CD87 antigen	1.53	4.41	.00023	NM_002659	Protein binding
Down-regulated					
Chemokine (C-C motif) receptor 2	0.35	−6.69	.00000	NM_000647	C-C chemokine receptor activity
Interleukin 7 receptor	0.47	−3.69	.00129	NM_002185	Antigen binding
Annexin II (lipocortin II)	0.49	−4.86	.00007	NM_004039	Calcium ion binding
Colony stimulating factor 2 receptor β, low-affinity (granulocyte-macrophage)	0.52	−3.85	.00088	NM_000395	Interleukin 3 receptor activity
Cytoplasmic dynein light chain	0.53	−4.12	.00046	NM_003746	Enzyme inhibitor activity
Ribosomal protein L13a	0.55	−3.94	.00070	X56932	Structural constituent of ribosome
Ikaros/LyF-1 homolog	0.56	−4.30	.00029	NM_006060	DNA binding
Chaperonin-containing TCP1, subunit 4 (Δ)	0.56	−4.60	.00014	NM_006430	Unfolded protein binding
Eosinophil Charcot-Leyden crystal (CLC) protein (lysophospholipase)	0.57	−3.73	.00116	NM_001828	Hydrolase activity
Myeloid cell nuclear differentiation antigen	0.57	−3.66	.00138	M81750	DNA binding
Ribosomal protein S16	0.59	−3.84	.00091	M60854	Structural constituent of ribosome
FK506-binding protein 4 (59 kDa)	0.62	−4.28	.00030	NM_002014	Isomerase activity
Transforming growth factor β receptor IIB	0.62	−3.87	.00082	NM_003242	Type II transforming growth factor β receptor activity
Ribosomal protein L3	0.62	−3.80	.00099	X73460	Structural constituent of ribosome
KIAA0053	0.63	−5.73	.00001	D29642.1	GTPase activator activity
Peptidylprolyl isomerase D (cyclophilin D)	0.65	−4.71	.00011	NM_005038	FK506 binding
Citrate synthase	0.66	−5.54	.00001	NM_004077	Transferase activity
FADD	0.66	−3.72	.00119	NM_003824	Protein binding
C-myc oncogene	0.66	−3.84	.00089	NM_002467	Transcription factor activity
Interferon regulatory factor 2	0.66	−3.60	.00159	NM_002199	RNA polymerase II transcription factor activity
Intercellular adhesion molecule 3	0.66	−4.30	.00029	NM_002162	Protein binding

Table 3. Gene ontology (GO) comparison to discriminate between patients with chronic hepatitis C and healthy volunteers.

GO category	GO description	Genes, no.	*P* LS permutation	*P* KS permutation
7259	JAK-STAT cascade	6	.00167	.17913
6959	Humoral immune response	25	.00303	.03114
7186	G protein–coupled receptor protein signaling pathway	18	.00348	.17617

NOTE. JAK-STAT, Janus kinase signal transducer and activation of transcription; KS, Kolmogorov-Smirnov; LS, least squares.

genes, 200 receptor- and cell adhesion–related genes, 160 apoptosis- and cell cycle–related genes, 150 transcription factors, 120 stress-response genes, and 275 other functional genes.

RNA isolation and antisense RNA amplification. Total RNA from PBMCs was isolated using Micro RNA Isolation Kits (Stratagene), and antisense RNA (aRNA) was amplified as described elsewhere [20, 22, 24]. The quality and degradation of isolated RNA were estimated after electrophoresis using an Agilent 2001 bioanalyzer. The references used for each microarray analysis were aRNA samples prepared from PBMCs obtained from a healthy volunteer. Microarray hybridization was performed as described elsewhere [19–24], and each hybridization was repeated for all samples.

Gene-expression profiles of liver-infiltrating lymphocytes in

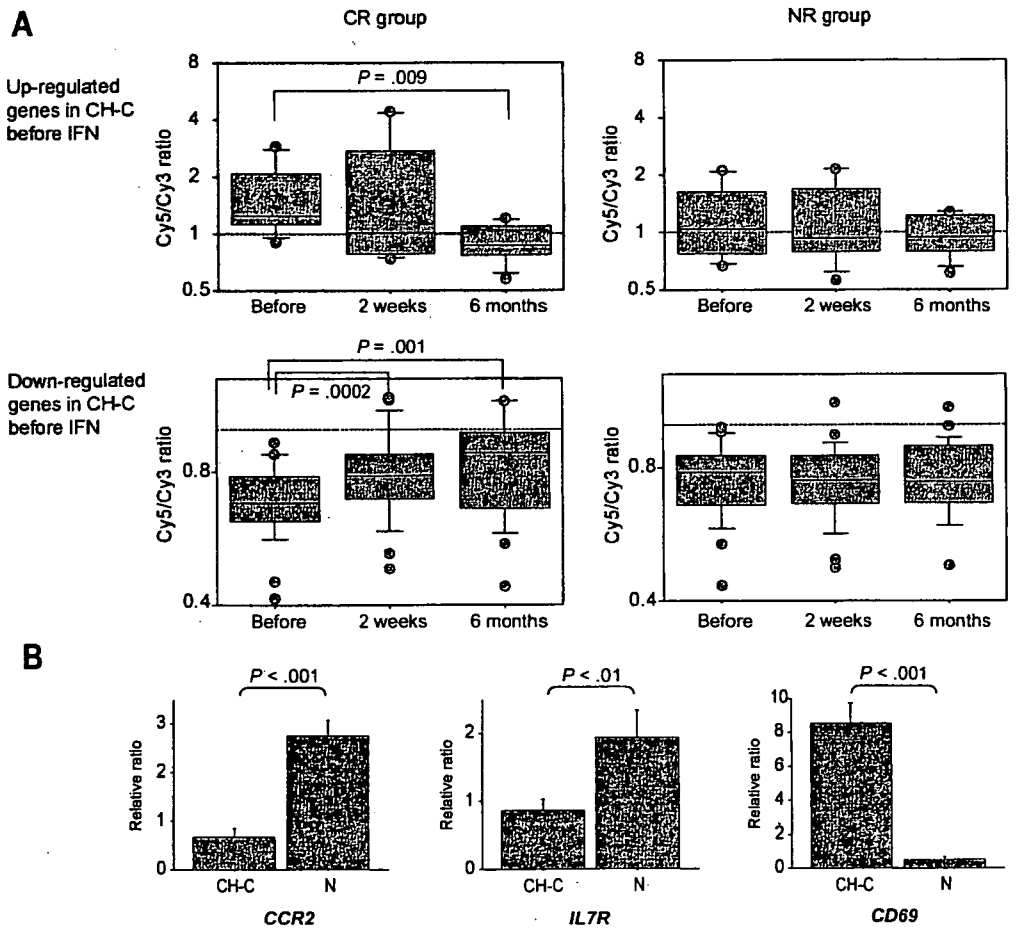


Figure 2. *A*, Changes in gene-expression profiles over the course of interferon (IFN) therapy (as shown in table 2) distinguishing patients with chronic hepatitis C (CH-C) from healthy volunteers before the start of IFN therapy. Box charts show average rates of change in relation to healthy volunteers as index functions. *B*, Real-time polymerase chain reaction data for *CCR2* and *IL7R*, which were down-regulated (as determined on the basis of microarray data) in patients with CH-C before the start of IFN therapy, and *CD69*, which was up-regulated in patients with CH-C.

patients with CH-C were investigated by laser-capture microdissection (LCM). Infiltrated lymphoid cells in the portal area and hepatocytes in liver-biopsy specimens obtained from 8 patients with CH-C were isolated by LCM. After 2 rounds of total RNA amplification, the gene expression in infiltrated lymphoid cells was compared with that in hepatocytes [25]. Optimal conditions for LCM and reproducibility of data were assessed repeatedly [24, 25]. Some of these data were used for the analysis of genes expression.

Image analysis and data processing. Quantitative assessment of signals on the slides was performed using a ScanArray 5000 device (General Scanning), followed by image analysis using QuantArray software (version 2.0; General Scanning).

Hierarchical clustering of gene expression in patients was performed using BRB-ArrayTools software (version 3.3.0; available at: http://linus.nci.nih.gov/BRB-ArrayTools.html). Filtered data were log transformed, normalized, centered, and applied to the average linkage clustering with centered correlation. BRB-ArrayTools include class comparison and class prediction tools based on univariate *F* tests to identify genes differentially expressed between predefined clinical groups. The permutation distribution of the *F* statistic, based on 2000 random permutations, was also used to confirm statistical significance. $P < .05$, as well as >1.5-fold differences in gene expression, were considered to be significant. A gene ontology (GO) comparison tool provides a list that has more genes differentially expressed and is coordinately regulated among predefined clinical groups than expected by chance and enables findings among biologically related genes to reinforce one another. Fisher and Kolmogorov-Smirnov tests were performed for GO comparison ($P < .005$) (BRB-ArrayTools).

Changes in gene expression in patients receiving IFN therapy were classified on the basis of self-organizing maps (GeneCluster software; version 2.0; available at: http://www.broad.mit.edu/cancer/software/genecluster2/gc2.html).

To identify class predictor genes for IFN therapy, projective adaptive resonance theory (PART) was used as a screening method for cDNA microarray data; unlike conventional clustering methods, PART enables the elimination of nonspecific dimensions for clustering from high-dimensional data [28–30]. From the genes extracted by PART, class predictor genes were selected using a fuzzy neural network (FNN) combined with the SWEEP operator method (FNN-SWEEP method). An FNN model with 1 input unit was initially created. Expression data for genes from data sets for patients with CH-C were entered into the FNN model, and the weight parameter was determined by the SWEEP operator method. We repeated this procedure for all genes to construct a model for each gene. The 10 genes with the highest accuracy levels were selected as the "first gene." The parameter increasing method was then applied. Having the first gene fixed, we used a similar method to select the second gene, which gave the highest accuracy in combination with the first gene. Having the first gene and the second gene fixed, we selected the third gene. For validation of this model, we performed leave-one-out cross-validation (LOOCV); we left out 1 test sample and used the remaining 12 samples as training samples. We created 13 such sets. The FNN model was built up for 12 test samples, and the accuracy of training and test samples was calculated.

Real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR). Quantitation of chemokine (C-C motif) receptor 2 *(CCR2)*, *CD69*, and interleukin 7 receptor *(IL7R)* RNA expression was performed using the TaqMan real-time PCR assay (ABI PRISM 7700 Sequence Detection System; PE Applied Biosystems), as described elsewhere [22, 23].

Statistical analysis. All data are expressed as mean ± SE values. One-way analysis of variance by the Bonferroni method or Student's *t* test was used to determine the significance of differences in clinical characteristics between patients in this study. $P < .05$ was considered to be significant.

RESULTS

cDNA microarray analysis of expression profiles of PBMCs from patients with CH-C. We initially compared the PBMC

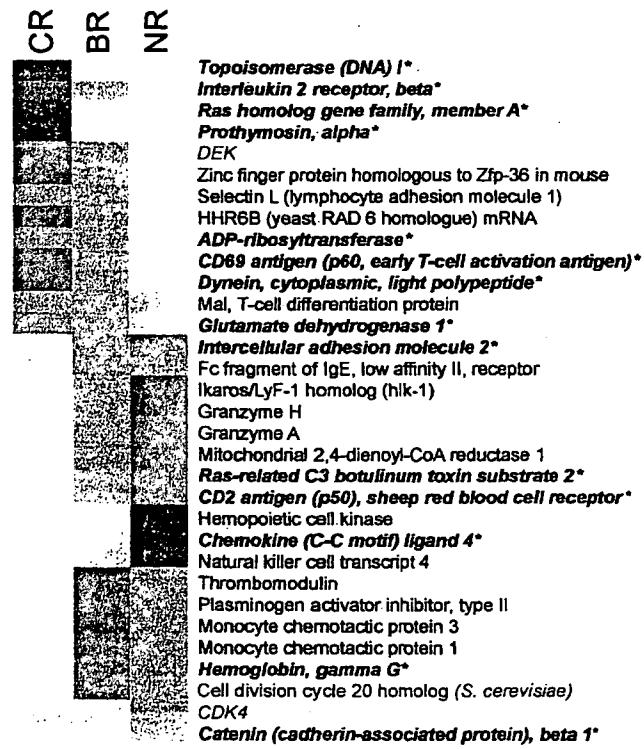


Figure 3. Thirty-two genes screened for gene-expression data before interferon (IFN) therapy by projective adaptive resonance theory. Red indicates up-regulated genes, and blue indicates down-regulated genes. Asterisks indicate genes that are included in table 4. BR, biochemical responder; CR, complete responder; NR, nonresponder.

Table 4. Ten gene combinations selected by the SWEEP operator method for construction of chronic hepatitis C class prediction at the start of interferon (IFN) therapy.

Combination	Input	Gene name	GenBank accession no.	Accuracy, %	
				Training	Test
1	1	***CD2 antigen (p50), sheep red blood cell receptor***[a]	NM_001767	21.2	14.1
	2	Glutamate dehydrogenase 1	NM_005271	72.4	46.2
	3	Dynein, cytoplasmic, light polypeptide	NM_003746	55.8	49.4
2	1	Ras-related C3 botulinum toxin substrate 2	NM_002872	34.6	20.5
	2	Glutamate dehydrogenase 1	NM_005271	81.4	68.6
	3	***Interleukin 2 receptor β***[a]	NM_000878	53.2	43.6
3	1	***Hemoglobin γG***[a]	NM_000184	19.9	16.7
	2	Ras-related C3 botulinum toxin substrate 2	NM_002872	64.7	36.6
	3	Dynein, cytoplasmic, light polypeptide	NM_003746	62.2	58.3
4	1	Intercellular adhesion molecule 2	NM_000873	28.9	26.3
	2	Ras homolog gene family member A	NM_001664	41.7	25.7
	3	Prothymosin α	NM_002823	66.0	47.4
5	1	Topoisomerase (DNA) I	NM_003286	53.9	46.2
	2	Catenin (cadherin-associated protein) β1 (88 kDa)	NM_001904	66.0	57.1
	3	Ras-related C3 botulinum toxin substrate 2	NM_002872	91.0	89.1
6	1	Catenin (cadherin-associated protein) β1 (88 kDa)	NM_001904	44.9	41.0
	2	Topoisomerase (DNA) I	NM_003286	66.0	57.1
	3	Ras-related C3 botulinum toxin substrate 2	NM_002872	91.0	89.1
7	1	Catenin (cadherin-associated protein) β1 (88 kDa)	NM_001904	35.3	31.4
	2	***Interleukin 2 receptor β***[a]	NM_000878	47.4	43.6
	3	ADP-ribosyltransferase (NAD+; poly [ADP-ribose] polymerase)	NM_001618	62.2	60.9
8	1	Chemokine (C-C motif) ligand 4	NM_002984	44.9	41.0
	2	***Interleukin 2 receptor β***[a]	NM_000878	37.8	29.5
	3	Topoisomerase (DNA) I	NM_003286	44.9	34.6
9	1	***Interleukin 2 receptor β***[a]	NM_000878	30.8	30.8
	2	Catenin (cadherin-associated protein) β1 (88 kDa)	NM_001904	47.4	43.6
	3	ADP-ribosyltransferase (NAD+; poly [ADP-ribose] polymerase)	NM_001618	62.2	60.9
10	1	CD69 antigen (p60, early T cell–activation antigen)	NM_001781	42.3	32.1
	2	Prothymosin α	NM_002823	33.3	24.4
	3	Glutamate dehydrogenase 1	NM_005271	39.1	31.4

[a] Genes that present similar expression patterns during IFN and ribavirin combination therapy.

gene-expression profiles of patients with CH-C with those of healthy volunteers. For all 1305 genes, the results of hierarchical clustering analysis, a nonsupervised learning method, confirmed that the gene-expression profiles of PBMCs from the 6 healthy volunteers clearly differed when compared with those of the 13 patients with CH-C (group A) before IFN therapy (figure 1*A*). When the 2 groups were compared by support vector machine, a supervised learning method (BRB-ArrayTools), a total of 48 predictor genes were identified with a significance level of $P < .002$, and it was possible to differentiate the 2 groups with 100% accuracy. Gene parameters (ratio, *T* value, *P* value, description, GenBank accession no., and annotation) are summarized in table 2.

A GO comparison tool (BRB-ArrayTools) identifies more genes that are differentially expressed and are coordinately regulated among predefined clinical groups than expected by chance, thus enabling the finding of biologically related genes to reinforce one another. GO comparison of gene expression between the patients with CH-C and the healthy volunteers revealed significant differences in the Janus kinase signal transducer and activation of transcription (JAK-STAT) cascade, humoral immune response, and G protein–coupled receptor protein signaling pathway ($P < .005$) (table 3). One-way clustering analyses of representative differentially expressed genes are shown in figure 1*B*. These genes were generally activated in PBMCs from patients with CH-C; however, genes such as *CCR2*, monocyte chemoattractant protein 1 receptor, and *IL7R* were significantly down-regulated. The reason for this is not known, but it may reflect infiltration of PBMCs into the liver. The top 20 differentially expressed genes were selected, and gene-expression profiling of these genes in liver-infiltrating lymphocytes was performed (figure 1*C*). Most of the gene-

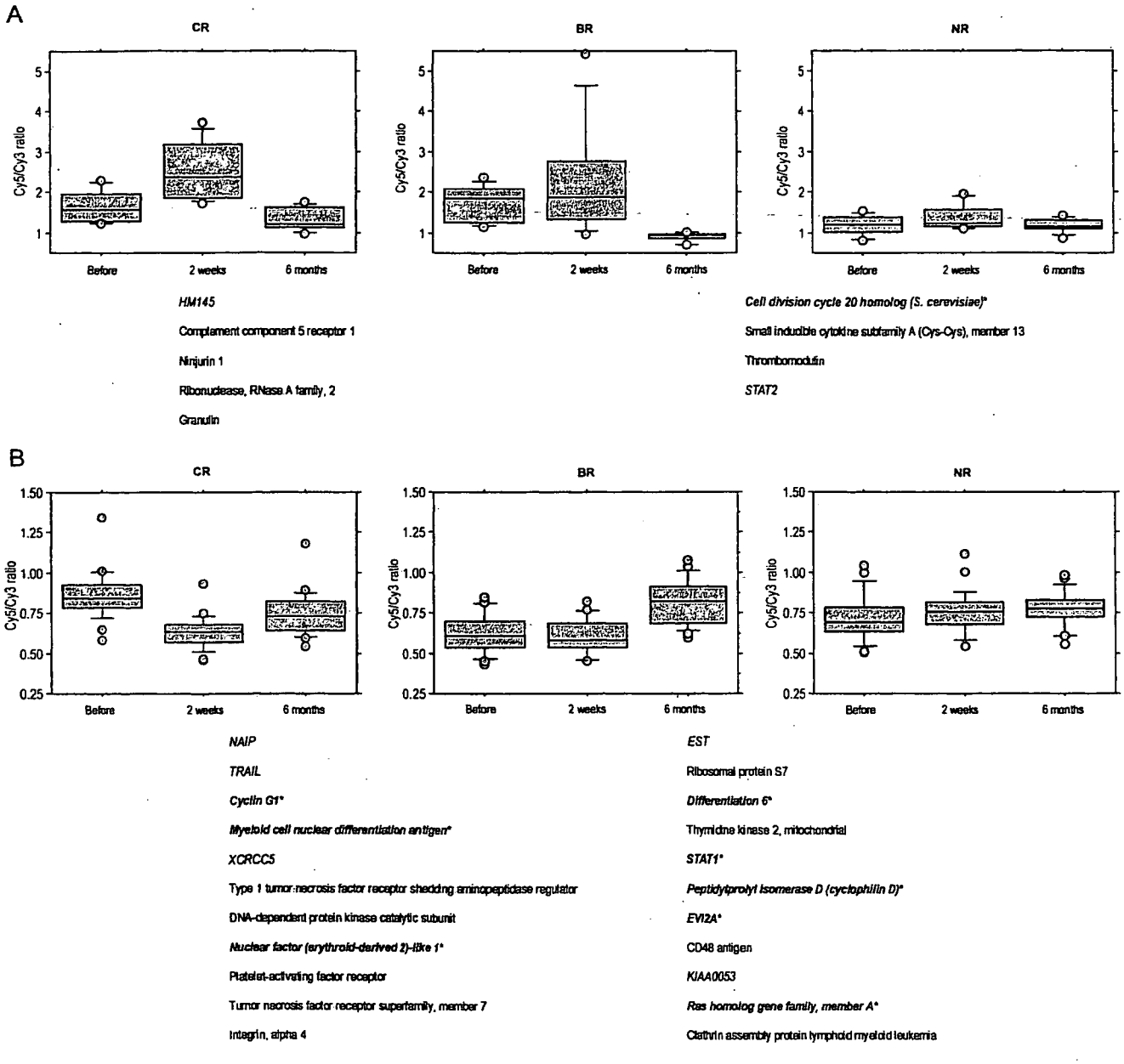
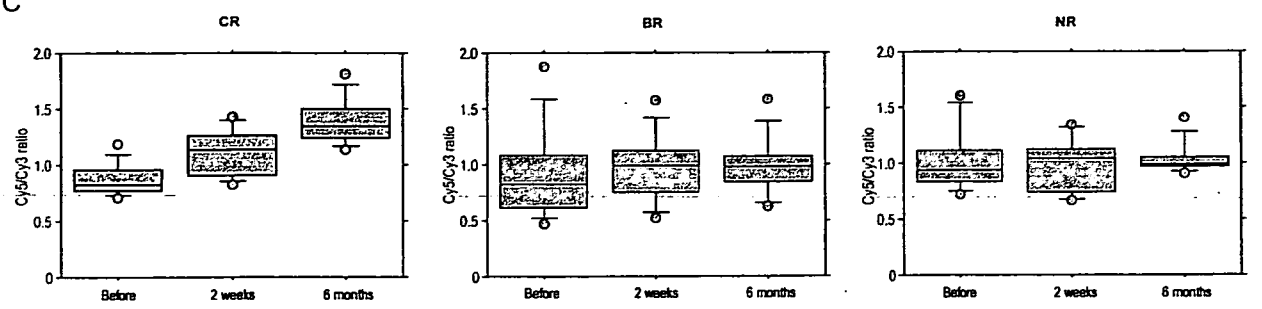


Figure 4. Gene-expression patterns. By use of projective adaptive resonance theory, 86 genes with changes in gene expression before and 2 weeks after the start of interferon (IFN) therapy were selected. For the complete responder (CR) group, changes in the expression of the 86 genes due to IFN therapy were classified into the following 5 patterns, on the basis of self-organizing maps (GeneCluster): up-regulated at 2 weeks after the start of IFN therapy and then down-regulated after the end of IFN therapy *(A)*; down-regulated at 2 weeks after the start of IFN therapy and then up-regulated after the end of IFN therapy *(B)*; up-regulated at 2 weeks after the start of IFN therapy and also up-regulated after the end of IFN therapy *(C)*; up-regulated at 2 weeks after the start of IFN therapy and then returned to normal after the end of IFN therapy *(D)*; and down-regulated at 2 weeks after the start of IFN therapy and also down-regulated after the end of IFN therapy *(E)*. Representative genes are listed under each pattern. Asterisks indicate genes that are included in table 5.

expression ratios for liver-infiltrating lymphocytes showed >1-fold increases compared with hepatocytes, thus indicating that most genes were preferentially expressed in lymphocytes. Interestingly, the genes with increased expression in liver-infiltrating lymphocytes tended to be expressed at lower levels in PBMCs (figure 1*C*).

Serial changes in the differentially expressed genes listed in table 2 during IFN treatment are shown in figure 2*A*. In the

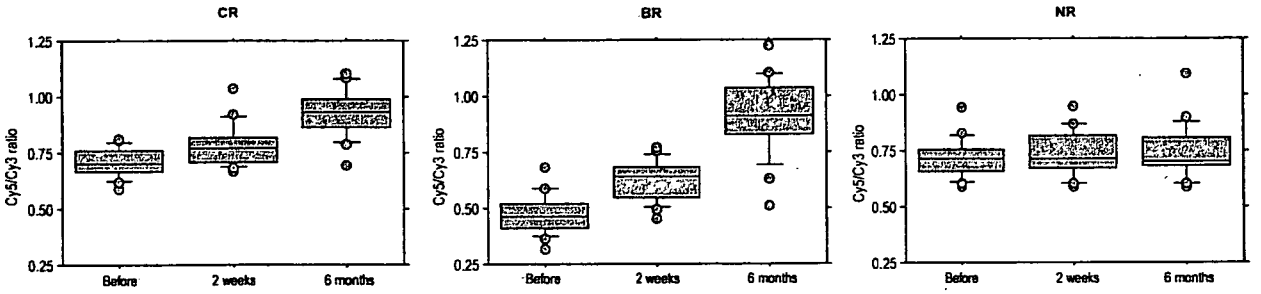
C



Serine (or cysteine) proteinase inhibitor, clade A*	Ribosomal protein S27 (metallopanstimulin 1)	Eukaryotic translation elongation factor 2
Epidermal growth factor receptor	Hemopoietic cell kinase	

Bruton agammaglobulinemia tyrosine kinase	*LITAF*	Hemopoietic cell kinase
GZMA		

D

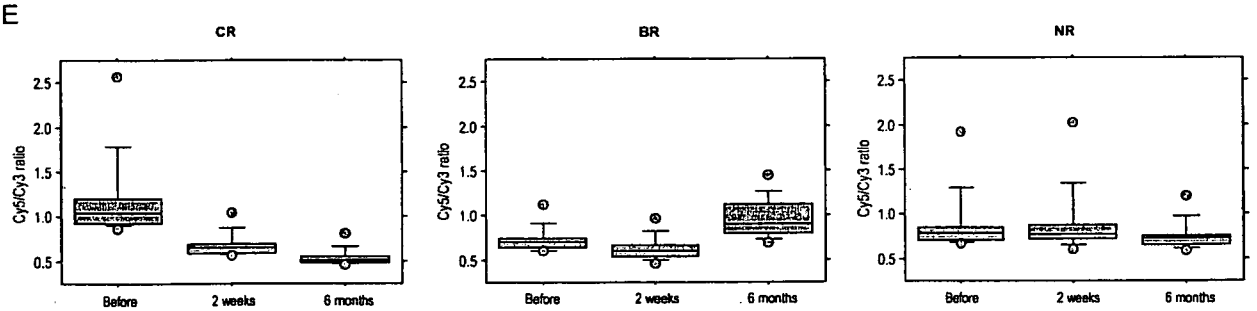


Interleukin 2 receptor beta chain	*ICE*	Ribosomal protein S16
Glutamate dehydrogenase 1	Small nuclear ribonucleoprotein polypeptide N	Mutant lymphocyte-specific protein tyrosine kinase

ADP-ribosyltransferase*	Interleukin enhancer binding factor 1	CD3D antigen, delta polypeptid
Fc fragment of IgE, low affinity II, receptor	Selectin L (lymphocyte adhesion molecule 1)	Ikaros/LyF-1 homolog (hlk-1)

Semaphorin (CD100)	Mitochondrial 2,4-dienoyl-CoA reductase	***Ras-related C3 botulinum toxin substrate 2****
E2L1	Citrate synthase	*ERP72*

E



Baculoviral IAP repeat–containing 2*	***MIHC****	NRAS-related gene
Reticulocalbin 2, EF-hand calcium binding domain	***ERCC5****	Integrin, beta 1

Rho-associated, coiled-coil containing protein kinase 1	***Apoptosis inhibitor 1****	Uracil-DNA glycosylase
Damage-specific DNA binding protein 1 (127kD)	CD69 antigen (p60, early T-cell activation antigen)	

Figure 4. *(Continued.)*

Table 5. Ten gene combinations selected by the SWEEP operator method for the construction of chronic hepatitis C class prediction 2 weeks after the start of interferon (IFN) therapy.

Combination	Input	Gene name	GenBank accession no.	Accuracy, %	
				Training	Test
1	1	*ERCC5*	NM_000123	55.3	45.5
	2	Serine (or cysteine) proteinase inhibitor clade A member 1	NM_000295	85.6	54.5
	3	Ras homolog gene family member A	NM_001664	80.3	70.5
2	1	Baculoviral IAP repeat–containing 2	NM_001166	47.7	41.7
	2	Serine (or cysteine) proteinase inhibitor clade A member 1	NM_000295	80.3	53.8
	3	Ras homolog gene family member A	NM_001664	80.3	70.5
3	1	Cyclin G1	NM_004060	36.6	44.0
	2	Ras-related C3 botulinum toxin substrate 2	NM_002872	79.6	61.4
	3	*EST*		70.5	56.8
4	1	Ecotropic viral integration site 2A	NM_001003927	41.7	25.8
	2	Peptidylprolyl isomerase D (cyclophilin D)	NM_005038	60.6	46.2
	3	Cyclin G1	NM_004060	77.3	67.4
5	1	***Myeloid cell nuclear differentiation antigen***[a]	NM_002432	55.3	25.8
	2	Cyclin G1	NM_004060	85.6	64.4
	3	ADP-ribosyltransferase (NAD+; poly [ADP-ribose] polymerase)	NM_001618	80.3	87.1
6	1	Integrin β1	NM_033666	47.7	19.7
	2	Cyclin G1	NM_004060	80.3	62.9
	3	***STAT1***[a]	NM_139266	80.3	68.2
7	1	Differentiation 6 (septin 2)	NM_004404	28.8	25.8
	2	Cyclin G1	NM_004060	75.0	64.4
	3	Cell division cycle 20 homolog *(S. cerevisiae)*	NM_001255	90.2	87.9
8	1	*MIHC*	NM_001165	28.8	25.0
	2	Cyclin G1	NM_004060	75.0	64.4
	3	Cell division cycle 20 homolog *(S. cerevisiae)*	NM_001255	90.2	87.9
9	1	Apoptosis inhibitor 1 (baculoviral IAP repeat–containing 3)	NM_001165	28.8	25.0
	2	Cyclin G1	NM_004060	75.0	64.4
	3	Cell division cycle 20 homolog *(S. cerevisiae)*	NM_001255	90.2	87.9
10	1	Nuclear factor (erythroid-derived 2)–like 1	NM_003204	25.0	25.8
	2	Cyclin G1	NM_004060	75.0	63.6
	3	ADP-ribosyltransferase (NAD+; poly [ADP-ribose] polymerase)	NM_001618	88.6	81.8

[a] Genes that present similar expression patterns during IFN and ribavirin combination therapy.

CR group, the expression profiles of genes that were either up- or down-regulated before IFN therapy were similar to those of healthy volunteers 6 months after the end of IFN therapy (figure 2*A*, CR group). On the other hand, in the NR group, expression of genes that were either up- or down-regulated before IFN therapy tended to remain up- or down-regulated 6 months after the end of IFN therapy (figure 2*A*, NR group). This suggests that the changes in gene-expression profiles of patients with CH-C before IFN therapy reflect the state of HCV infection.

We performed real-time PCR to corroborate the microarray data. Real-time PCR revealed that *CD69* was up-regulated in patients with CH-C and that *CCR2* and *IL7R* were down-regulated in patients with CH-C (figure 2*B* and table 2).

Relationship between PBMC gene-expression profiles and IFN response. We then analyzed the relationship between PBMC gene-expression profiles before the start of IFN therapy and IFN response. Because the regimen of IFN treatment was different in group A and group B patients, we first focused on group A patients (table 1). In hierarchical clustering analysis using all genes before IFN therapy, no clustering was seen in the CR, BR, or NR groups. Conventional supervised learning methods, such as support vector machine and nearest neighbor (BRB-ArrayTools), could not discriminate between the CR, BR, and NR groups. Therefore, we applied the FNN-SWEEP method to predict the outcome of IFN therapy. Before FNN-

Table 6. Comparison of interferon (IFN)–stimulated gene (ISG) expression induced by IFN.

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

SWEEP analysis, nonspecific genes or genes with errors were eliminated by the PART method. The 32 genes screened by PART are shown in figure 3. Topoisomerase (DNA) I *(TOP1)* and interleukin 2 receptor β *(IL2RB)* were up-regulated in the CR group, hemoglobin γG *(HBG2)* and monocyte chemotactic protein were up-regulated in the BR group, and chemokine (C-C motif) ligand 4 and ras-related C3 botulinum toxin substrate 2 *(RAC2)* were up-regulated in the NR group. Genes selected by PART were subjected to the FNN-SWEEP method to construct a class prediction model. Consequently, we selected 10 gene combinations by the SWEEP operator method for CH-C class prediction before the start of IFN therapy (table 4). The most effective gene combination for the prediction of an IFN response was *TOP1;* catenin (cadherin-associated protein) β1 (88 kDa); and *RAC2.* The accuracy of the training and test sets were high, at 91.0% and 89.1%, respectively.

Changes in gene-expression profiles over the course of IFN therapy. We next focused on the changes in gene-expression profiles over the course of IFN therapy and their relationship with IFN response. Using PART, 86 genes with changes in expression between before and 2 weeks after the start of IFN therapy were selected. To investigate the relationship between the 86 genes with changes due to IFN therapy and the efficacy of IFN therapy, changes in the expression of the 86 genes were determined for the CR group. On the basis of self-organizing maps, changes in gene expression in the CR group were classified into the following 5 patterns (figure 4): pattern A, up-regulated 2 weeks after the start of IFN therapy and then down-regulated after the end of IFN therapy; pattern B, down-regulated 2 weeks after the start of IFN therapy and then up-regulated after the end of IFN therapy; pattern C, up-regulated 2 weeks after the start of IFN therapy and also up-regulated after the end of IFN therapy; pattern D, up-regulated at 2 weeks after the start of IFN therapy and then returned to normal after the end of IFN therapy; and pattern E, down-regulated at 2 weeks after the start of IFN therapy and also down-regulated after the end of IFN therapy. Patterns A and B represent gene groups with temporary changes during IFN therapy, whereas patterns C, D, and E represent gene groups with changes after the end of IFN therapy and are thought to be attributable to viral eradication or normalization of hepatic function. Interestingly, very little change was seen in the patterns for the NR group. Therefore, changes in gene expression are also useful in predicting therapeutic efficacy. From the 86 genes isolated by PART, the SWEEP operator method was used to identify 10 gene combinations, and therapeutic efficacy was predicted according to the FNN-SWEEP method (table 5). The results showed that the accuracy for gene combinations 7, 8, and 9 was high, at 90.2%. LOOCV confirmed the high accuracy (87.9%) of prediction using these gene combinations. These combinations included the following genes that are important for predicting therapeutic efficacy: *CDC20* was classified as belonging to pattern A; cyclin G1 and differentiation 6 were as belonging to pattern B; and *MIHC* and apoptosis inhibitor 1 were as belonging to pattern E (figure 4).

IFN and ribavirin combination therapy. We then investigated the usefulness of the above-mentioned genes in predicting the efficacy of IFN and ribavirin combination therapy. It has been shown that concurrent ribavirin administration improves the rate of CR. In addition, the changes in gene expression during combination therapy are due not only to IFN but also to ribavirin. Thus, the results for monotherapy may not be applicable to combination therapy. However, changes in the expression of several genes—CD2 antigen (p50), *IL2RB, HBG2,* myeloid cell nuclear differentiation antigen *(MNDA),* and *STAT1*—were shown to be extremely useful for distinguishing CR from NR in IFN and ribavirin combination therapy (tables 4 and 5).

DISCUSSION

HCV load, genotype, and fibrosis have been listed as factors that influence the effectiveness of IFN therapy [4, 5], but these factors are not sufficient, and other predictive factors are needed. Unlike liver-biopsy specimens, PBMCs can be easily collected, and collection can be repeated as necessary. We analyzed the gene-expression profiles of PBMCs in patients with CH-C by use of cDNA microarrays under the hypothesis that gene expression in PBMCs is indicative of IFN efficacy and CH-C disease state. In addition, changes in the gene-expression profiles of PBMCs were analyzed during the course of IFN therapy to clarify the relationship between gene-expression profiles of PBMCs and IFN response.

Interestingly, the gene-expression profiles of PBMCs from patients with CH-C and from healthy volunteers were different, and this was confirmed by hierarchical clustering analysis and supervised learning analysis using support vector machine. When patients with CH-C and healthy volunteers were compared, gene expression in the JAK-STAT cascade, humoral immune response, and G protein–coupled receptor protein signaling pathway differed markedly. In most patients with CH-C, expression of these genes is activated, and HCV infection is thought to bring about changes in the gene expression in PBMCs. Several chemokine- and cytokine-related genes, such as *CCR2* and *IL7R,* were down-regulated. Although the reason for this was not clear, expression of these genes in liver-infiltrating lymphocytes was up-regulated. Therefore, the down-regulation of immune-related genes may represent increased levels of liver-infiltrating lymphocytes accompanying hepatitis. Interestingly, when the chronological changes in PBMC gene-expression profiles were analyzed for the CR group, the profiles at 6 months after the end of therapy were similar to those of healthy volunteers. Therefore, the changes in gene-expression

profiles before IFN therapy were due to HCV infection. On the other hand, the gene-expression profiles of the NR group before IFN therapy were similar to those at 6 months after the end of IFN therapy (figure 2*A*).

Unfortunately, it was not possible to differentiate between CR, BR, and NR patients on the basis of gene-expression profiles of PBMCs by use of nonsupervised learning methods, such as hierarchical clustering, before IFN therapy. Therefore, we used FNN theory for CH-C class prediction. The most attractive feature of FNN is that causality between input and output variables can be described very accurately as explicit if-then roles obtained from the constructed model. For the purpose of analyzing numerous genes in a short time, FNN combined with the SWEEP operator method was developed (FNN-SWEEP method) and has been shown to be a precise, simple tool for predicting patient survival on the basis of microarray data [28, 29]. In addition, by first filtering genes by use of PART, the accuracy of the FNN-SWEEP method was further increased [30]. In the present study, a total of 32 genes were identified by PART on the basis of genetic changes before therapy, and, in the CR group, expression of genes such as *TOP1*, *IL2RB*, prothymosin α (*PTMA*), and ADP-ribosyltransferase was up-regulated, thus indicating active cellular proliferation. In the NR group, the expression of genes indicating activated cytotoxic T cells—such as granzyme, CD2 antigen, *RAC2*, and natural killer cell transcript 4—was up-regulated. Because these genes were up-regulated by IFN therapy in the CR group, they were thought to be up-regulated before therapy in the NR group. Lempicki et al. reported elevated expression of endogenous IFN/innate immune response genes in PBMCs from NR patients coinfected with HCV and HIV [31]. This suggests that, in many NR patients, few immune effector cells are active or that these effector cells cannot infiltrate the liver and remain in the peripheral blood.

To further investigate the above-mentioned points, changes in the gene-expression profiles of PBMCs were determined during the course of IFN therapy. On the basis of expression profiles before and 2 weeks after the start of IFN therapy, 86 genes were selected. These genes did not include as many IFN-α–stimulated genes as were noted in liver [25–27] (table 6), but they included valuable immune regulatory genes.

On the basis of self-organizing maps, changes in gene expression in the CR group were then classified into 5 patterns (figure 4). These gene groups represent genes with temporary changes due to IFN therapy and those with changes after the end of IFN therapy. Gene groups with changes after the end of IFN therapy are thought to be involved in viral eradication or the normalization of hepatic function. Interestingly, little change was seen in any of the patterns in the NR group. In efficacy prediction by the FNN-SWEEP method, the accuracy for the gene combinations 7, 8, and 9 was high, at 90.2%, thus suggesting that changes in gene expression 2 weeks after the start of IFN therapy are also useful in predicting therapeutic efficacy.

We also investigated whether these genes are useful in predicting the efficacy of IFN and ribavirin combination therapy. Changes in gene expression during combination therapy were due not only to IFN but also to ribavirin, and the results for monotherapy could not simply be applied to combination therapy. However, changes in the expression of several genes—CD2 antigen (p50), *IL2RB*, *HBG2*, *MNDA*, and *STAT1*—were shown to be extremely useful for distinguishing CR from NR in IFN and ribavirin combination therapy.

Unfortunately, because the number of subjects in the present study was small, the genes that were identified as predictors for IFN monotherapy were not necessarily predictors for IFN and ribavirin combination therapy. However, the present study was the first to show that responses to IFN therapy could be predicted on the basis of changes in gene expression by PBMCs, and further investigations in greater numbers of patients are required.

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Analysis of hepatitis C virus-specific CD8+ T-cells with HLA-A*24 tetramers during phlebotomy and interferon therapy for chronic hepatitis C

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Abstract. Hepatitis C virus (HCV)-specific, HLA class I-restricted, CD8-positive (CD8+) T lymphocytes are thought to contribute to viral clearance as well as liver disease in chronic hepatitis C. For the patients who do not respond to interferon (IFN) therapy, phlebotomy can be used as a tool to reduce inflammation and lower transaminase levels; however, the immunological aspects have not been clearly defined. In this study, we evaluated the HCV-specific CD8+ T-cell responses during phlebotomy and IFN therapy using HLA-A*24 tetramers in 6 Japanese patients with chronic hepatitis C. During phlebotomy, 4 of the 6 cases achieved a biochemical response, but there was no clear correlation between its efficacy and HCV viral loads or changes in frequencies or activation status of tetramer-positive T-cells. In contrast, the frequencies of tetramer-positive cells and the proportions of T-cells expressing activation marker HLA-DR were higher in sustained viral responders than in transient responders to IFN therapy. Furthermore, expression of the activation marker was enhanced in the initial period of IFN therapy. The results suggest that the immunological aspects of phlebotomy obviously differ from those of IFN therapy and these differences may provide clues as to a therapeutic strategy of their combination for patients who do not respond to IFN monotherapy.

Introduction

Interferon (IFN) treatment is a radical therapy for the elimination of hepatitis C virus (HCV), but many patients do not respond to it; so called 'non-responders'. There have been recent advances in treatment, such as combination therapy with Peginterferon α-2a or α-2b and ribavirin (1-4). To date, there are no therapies for HCV elimination with a sufficiently high success rate and low rate of adverse events.

For non-responders to IFN therapy, secondary treatment is needed to lower serum transaminase levels, slow the progression of fibrosis and reduce the occurrence of hepatocellular carcinoma (5). Phlebotomy is one of the treatments used to reduce inflammation and lower serum transaminase levels (6).

Several studies have examined the correlation between HCV and iron levels. Smith *et al* reported that the progression of fibrosis is faster in patients with chronic hepatitis C with congenital hemochromatosis than in those with normal iron levels (7). Fontana *et al* reported that combination therapy with IFN and phlebotomy may be more effective than IFN monotherapy in patients with chronic hepatitis C (8). We reported the hemosiderin deposition may be a predictive parameter for the efficacy of IFN therapy (9). Mandishona *et al* reported that excess iron may promote the occurrence of hepatocellular carcinoma (10).

The mechanism by which phlebotomy decreases transaminase levels is thought to involve a decrease in the toxic effects of superoxide produced by iron excess (11). However, no studies have been reported regarding the immunological effects of phlebotomy that may be important in mitigating liver injury.

HCV-specific CD8+ cytotoxic T lymphocytes (CTLs) contribute to viral clearance in acute, self-limited hepatitis C as well as to liver cell injury in the more frequent cases with chronic hepatitis C (12-16). In a study using HLA-A*24 tetramer, we previously showed that a close correlation exists between the HCV-specific CD8+ T-cell profile and hepatic fibrosis in HCV-infected Japanese patients, most of whom are HLA-A*24 positive (17). In this study, we analyzed HCV-specific CD8+ T-cell responses during phlebotomy and IFN therapy and observed a correlation between changes in the HCV-specific CD8+ T-cell profile and the therapeutic effects of each treatment.

Materials and methods

Patients. Patients with chronic hepatitis C presented at Kanazawa University Hospital between June 2000 and June 2001 were included in this study. Their selection and diagnosis were based on the following criteria: 1) age from 20 to 70

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Key words: chronic hepatitis C, phlebotomy, interferon therapy, tetramers, HLA-A*24

Table I. Clinical characteristics of patients.

Patients	Age (years)	Sex (M/F)	HCV Serogroup	HCV-RNA (KIU/ml)	ALT (IU/l)	HAI Stage (F)	HAI Grade (A)	ALT change during phlebotomy	Response to IFN therapy
1	47	F	2	69	40	1	1	Not decreased	SVR
2	60	M	2	88	104	3	2	Decreased	TVR
3	43	M	1	>500	97	3	2	Decreased	TVR
4	65	M	1	1.9	80	1	1	Decreased	SVR
5	55	M	2	0.7	39	3	2	Decreased	SVR
6	51	M	2	>500	110	1	1	Not decreased	TVR

Serum HCV RNA was quantified with the Amplicore HCV Monitor ver.3. HAI, histological activitiy index; SVR, sustained viral responder; TVR, transient viral responder.

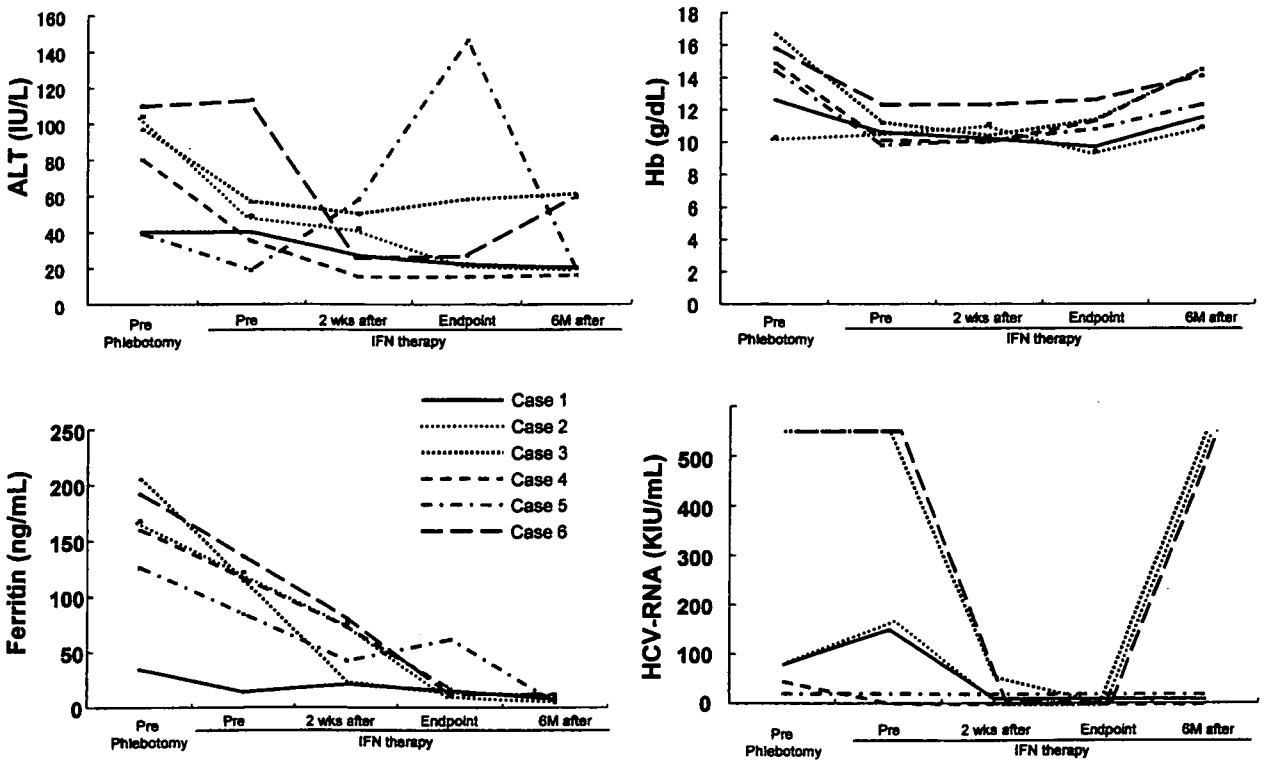

Figure 1. Trends over time for alanine aminotransferase (ALT), hemoglobin, ferritin and HCV-RNA levels during phlebotomy and interferon (IFN) therapy in patients with chronic hepatitis C. Phlebotomy was performed by removing 200 to 400 ml blood per session, once or twice weekly. The sessions were continued until achieving the target levels of hemoglobin (10 g/dl) and/or ferritin (10 ng/ml). After achieving the target levels, IFN-α-2b was administered at a dose of 6 to 10 MU per day for 2 weeks and then 3 times a week for 22 weeks. Each line indicates a single patient.

years; 2) elevated serum aminotransferase (ALT; >50 IU/l) at least once within 1 year; 3) hemoglobin greater than 13.0 g/dl in males or greater than 11.0 g/dl in females; 4) no liver cirrhosis; and 5) HLA-A*24 positive. All cases provided written informed consent.

Phlebotomy and IFN therapy. Phlebotomy was performed by removing 200 to 400 ml blood per session, once or twice weekly. The sessions were continued until achieving the target level of hemoglobin (10 g/dl) and/or ferritin (10 ng/ml). After achieving the target level, IFN-α-2b was administered at a dose of 6 to 10 MU per day for 2 weeks and then 3 times a week for 22 weeks.

Complete blood cell count, liver function tests, HCV-RNA determinations and T-cell analysis were performed pre-phlebotomy, just before IFN administration, 2 weeks after IFN therapy, immediately after IFN therapy and 6 months after IFN administration.

Patients whose transaminases decreased during phlebotomy, were recorded as biochemical responders and the others as non-responders. With respect to the HCV-RNA level, patients whose HCV-RNA levels were undetectable both at the end-points of IFN therapy and even at 6 months after the IFN therapy completion were designated as sustained viral responders (SVR) and those whose HCV-RNA were undetectable at the end point of IFN therapy but reappeared 6 months after the end of IFN therapy were designated as transient viral responders (TVR) (1).

*Synthesis of HLA-A*2402-peptide tetramers.* Five peptides were selected to synthesize HLA-A*2402-peptide tetramers (17): HCV E2 717-725 (EYVLLLFLL), NS3 1292-1300

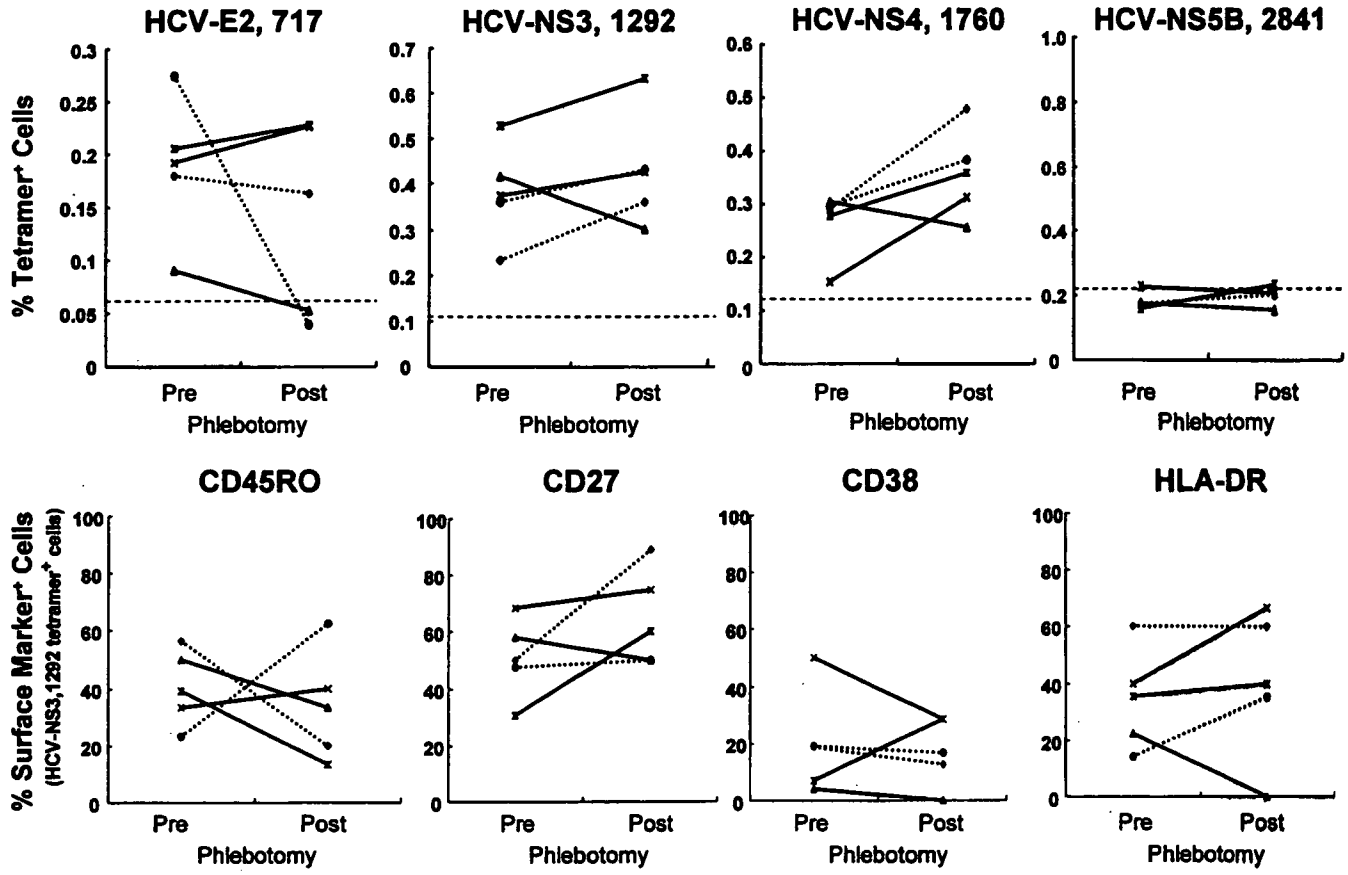

Figure 2. Changes in frequencies of four types of HLA-A*24 tetramer-positive cells and surface markers of tetramer HCV-NS3, 1292-positive cells during phlebotomy. Solid lines represent biochemical responders to phlebotomy and broken lines represent biochemical non-responders (subjects whose ALT did not decrease during phlebotomy). Horizontal broken lines indicate the cut-off value for each HLA tetramer, as mentioned in Materials and methods. There was no correlation between the biochemical effects of phlebotomy and the frequencies of tetramer-positive T-cells or the phenotypes of tetramer-positive cells.

(TYSTYGKFL), NS4 1760-1768 (FWAKHMWNF), NS5B 2841-2849 (RMILMTHFF) and NS5B 2870-2878 (CYSIEPLDL). Three of them, E2 717-725, NS3 1292-1300 and NS5B 2870-2878 were selected because they have been reported to bind to HLA-A*24 with good affinity (IC_{50} <500 nM) in a direct peptide binding assay (18). The other two peptides were chosen because they were conserved within the reported major HCV genotypes 1a and 1b sequences (19-21).

The cut-off values for positive staining with the tetramers was 2 SD above the mean for all control subjects studied previously (17): 0.064% for tetramer HCV-E2.717, 0.11% for tetramer HCV-NS3.1292, 0.12% for tetramer HCV-NS4 1760, 0.22% for tetramer HCV-NS5B.2841 and 0.10% for tetramer HCV-NS5B.2870.

Tetramer staining and flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by separation using Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation. Freshly isolated PBMCs were stained with tetrameric complexes and antibodies and were then analyzed. The following monoclonal antibodies (mAbs) were used; anti-CD8-Cy-Chrome (CyC) (HIT8a), anti-CD-4-Allophycocyanin (APC) (SK3), anti-CD14-APC (MΦP9), anti-CD19-APC (SJ25C1), anti-CD45RA-FITC (HI100), anti-CD27-FITC (M-T271), anti-CD38-RITC (HIT2) and anti-HLA-DR-FITC (L243) (BD PharMingen, Sand Diego, CA). Freshly isolated cells ($1x10^6$) were washed, resuspended in 200 μl PBS without calcium and phosphate, and stained with 40 μg/ml of tetrameric complexes for 30 min at room temperature. Subsequently, antibodies against cell surface proteins were added and incubated for an additional 30 min at room temperature. Cells were washed, fixed with 1% formalin/PBS, and analyzed on a FACSCalibur™ flow cytometer. Data were analyzed with CELLQuest™ software (Becton Dickinson, San Jose, CA).

Results

Clinical course. The 6 cases studied included 5 males and 1 female (Table I). They ranged in age from 43 to 60 years. Of the patients, 4 had serogroup 2 HCV and 2 had serogroup 1 HCV. Serogroup 1 HCV is known to be more common than serogroup 2 in Japan (22).

Phlebotomies were performed in all cases without significant adverse events over a period of 7 to 20 days. Eventually, the total volume of blood removed was from 600 to 2800 ml (mean = 1600 ml). In 4 of the 6 cases, transaminase levels decreased during phlebotomy, but there was no effect of phlebotomy on HCV viral loads (Fig. 1).

IFN treatments were associated with lower HCV viral loads. At the endpoints of the treatments, HCV-RNA disappeared from sera of all 6 cases. Six months after IFN therapy, HCV reappeared in 3 cases (transient viral responder,

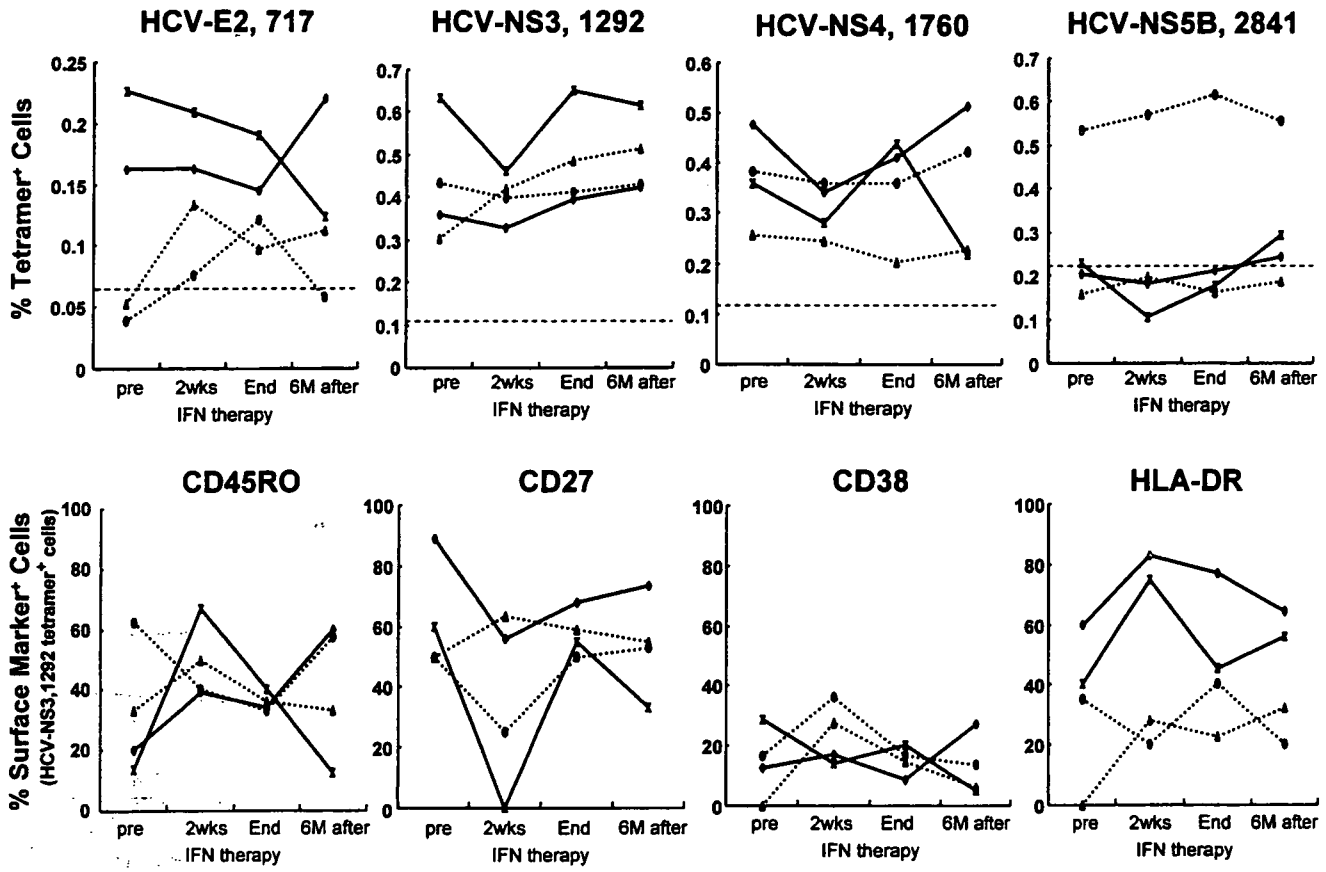

Figure 3. Changes in frequencies of four types of HLA-A*24 tetramer-positive cells and surface markers of tetramer HCV-NS3, 1292-positive cells during IFN therapy. Solid lines represent viral responders to IFN therapy (sustained viral responders, SVRs) and the broken lines represent transient viral responders (TVRs) whose HCV reappeared within 6 months after the end of IFN therapy. Horizontal broken lines in the upper four panels indicate the cut-off values for HLA tetramers, as mentioned in Materials and methods. The HLA-DR positive rates among tetramer (HCV-NS3, 1292)-positive cells were higher in SVRs than in TVRs at the start of IFN treatment and further increased after 2 weeks of IFN therapy.

TVR) and in the other 3 cases HCV remained below the detection limit (sustained viral responder, SVR) (Fig. 1).

HCV-specific CD8+ T-cell responses during phlebotomy. During phlebotomy, we analyzed HCV-specific CD8+ T-cell responses in 5 cases (patients 1, 3, 4, 5, and 6); and among them, 3 cases were biochemical responders to phlebotomy and the other 2 cases were non-responders (Table I and Fig. 2). The numbers of HLA-A*24 tetramer-positive T-cells were above the cut-off levels for all 3 of the tetramers, HCV-E2.717, HCV-NS3.1292, and HCV-NS4.1760. There was no correlation between the biochemical effects of phlebotomy and the frequencies of tetramer-positive T-cells. We also analyzed the phenotypes of tetramer-positive cells by staining CD45RO, CD27, CD38 and HLA-DR, but we did not observe a correlation between the biochemical effects and the phenotypes. The data indicate that phlebotomy displayed its therapeutic effects for the patients with chronic hepatitis C without affecting the frequencies and phenotypes of HCV-specific CD8+ T-cell responses.

HCV-specific CD8+ T-cell responses during IFN therapy. During and after IFN therapy, we analyzed HCV-specific CD8+ T-cell responses in 2 sustained responders (patients 1 and 5) and 2 transient responders (patients 3 and 6) (Table I and Fig. 3). The three different tetramer-positive T-cells, HCV-E2.717, HCV-NS3. 1292 and HCV-NS4.1760 were also detectable at levels above the cut-off during IFN therapy. The frequencies of HCV-E2.717 positive cells were higher in SVR patients than in TVR patients before, during and 6 months after IFN therapy. Interestingly, activation marker HLA-DR positive rates among tetramer-positive cells were higher in SVR patients than in TVR patients before IFN treatment and increased further after 2 weeks of IFN therapy and beyond. These results demonstrate that IFN therapy exerted its beneficial effects on the patients with high frequencies of the HCV-specific CD8+ T-cells and enhanced expression of the activation markers, suggesting that the efficacy of IFN therapy for chronic hepatitis C may be mediated by the virus-specific T-cell dependent immunity.

Discussion

For patients with chronic hepatitis C who do not respond to IFN therapy, other treatments to reduce inflammation and decrease transaminase levels are indicated to slow the progression of fibrosis and to lower the incidence of hepatocellular carcinoma. Phlebotomy is one of the therapies used to decrease the inflammation in the liver. In this study, we analyzed HCV-specific CD8+ T-cell responses in 6 patients with chronic hepatitis C treated by phlebotomy followed by IFN therapy. HLA-A*24 tetramer HCV-E2.717ÅAHCV-

NS3.1292 and HCV-NS4.1760 positive T-cells were detected at levels above the cut-off values. During phlebotomy, there was no correlation between the effectiveness of treatments and virological and immunological parameters, such as HCV viral loads, frequencies of tetramer-positive cells and their phenotypes of activation status, although 4 of the 6 cases achieved biochemical improvement. During IFN therapy, interestingly, the frequencies of HCV-E2.717 positive cells were higher in SVR patients than in TVR patients. Additionally, proportions of HLA-DR positive cells among tetramer-positive cells were higher in SVRs than in TVRs at the start of treatment; the proportions increased after 2 weeks of IFN administration and remained elevated during the follow-up periods.

Phlebotomy is thought to be effective by correcting iron excess in chronic hepatitis C. The previous studies have reported that the progression of fibrosis is faster in chronic hepatitis C patients with congenital hemochromatosis (7), that combination therapy with IFN and phlebotomy may be more effective than IFN monotherapy (8), that hemosiderin deposition may be a predictive factor for IFN efficacy (9) and that dietary iron overload may be a risk factor for hepatocellular carcinoma (10). Inhibition of the toxic effects of superoxide or excess iron has been postulated as a mechanism underlying the therapeutic effects of phlebotomy (11); however, the possible involvement of immunological mechanisms had not been addressed.

Recently developed HLA-class I peptide tetramers, consisting of fluorescently-tagged tetrameric complexes of HLA heavy chains folded around epitope peptides, allow the sensitive and precise enumeration of T lymphocytes with specific T-cell antigen receptors (TCR) (23,24). With regard to HCV infection, this technology revealed that epitope-specific $CD8^+$ T lymphocytes are not only detectable in *in vitro* expanded $CD8^+$ T lymphocytes but also in freshly isolated PBMCs at more than 10-fold higher frequencies than those previously reported (25). Furthermore, the technology has facilitated the phenotypic, functional and molecular analysis of virus-specific immune responses at the single cell level (25). Additionally, by means of tetramers, the relative frequencies of T lymphocytes specific for different epitopes were observed to change during the course of viral infection (26). We have reported the frequency, phenotype and clinical significance of HCV-specific $CD8^+$ T lymphocytes using five different HLA-A*24 tetramers in HCV-infected Japanese patients (17).

Manfras *et al* reported that increased oligoclonality of circulating $CD8^+$ T-cells in chronic HCV infection was an indicator of a poor clinical reponse to IFN-α therapy; that IFN-α therapy enhanced the differentiation of $CD8^+$ T-cells towards a late differentiation phenotype ($CD28^-$ $CD57^+$); and that in cases of virus elimination, there was disappearance of expanded, terminally-differentiated $CD8^+$ cells (27). In our study, we found that the HLA-DR positive $CD8^+$ T-cells increased after 2 weeks of IFN therapy. On the other hand, during phlebotomy, there was no correlation between the improvement of liver function parameters and the frequencies of tetramer-positive cells or changes in the levels of activation markers. These findings may indicate that the mechanisms of phlebotomy and IFN therapy differ immunologically.

This is the first study to observe the alteration of HCV-specific T-cells, not only during IFN therapy, but also during the phlebotomy and the findings suggest that there may be important differences in their immunological aspects. The use of a combination of therapies which have different but complimentary mechanisms may be more beneficial for the treatment of chronic hepatitis C.

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Intrahepatic interleukin-8 production during disease progression of chronic hepatitis C

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Abstract

The current study was designed to investigate the contribution of chemokines to the pathogenesis of chronic hepatitis C and hepatocellular carcinoma (HCC) by measuring the production of IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 alpha (MIP-1α). A solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) was established to quantitate serum concentrations of the chemokines. Expression of chemokines in liver tissues was evaluated immunohistochemically using specific monoclonal antibodies. As the severity of chronic hepatitis escalated, serum IL-8 levels increased progressively. Moreover, in the hepatocellular carcinoma (HCC) patients, IL-8 concentrations were positively correlated with the macroscopic staging of HCC, and inversely correlated with the duration of the survival periods. The results demonstrate that IL-8 production may be augmented upon the malignant transformation of hepatocytes in chronic hepatitis C.

Keywords: Interleukin-8; Monocyte chemoattractant protein-1; Macrophage inflammatory protein-1 alpha; Sandwich enzyme-linked immunosorbent assay; Chronic hepatitis C; Hepatocellular carcinoma

1. Introduction

Chemokines are known not only to mediate the recruitment of inflammatory cells such as neutrophils or lymphocytes, but also to regulate the balance of helper T cells (Th1/Th2) as well as the activation of antigen-presenting dendritic cells, and thus to be deeply involved in immune responses. Moreover, chemokine-mediated cellular responses are known to be involved in neovascularization nd fibrosis, and since chemokines have growth factor activity, their association with malignant transformation has been suggested [1,2].

Recent findings that the core and nonstructural 5A (NS5A) proteins of hepatitis C virus (HCV) induce the expression of interleukin (IL)-8 gene *in vitro* have suggested that chemokines may be

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