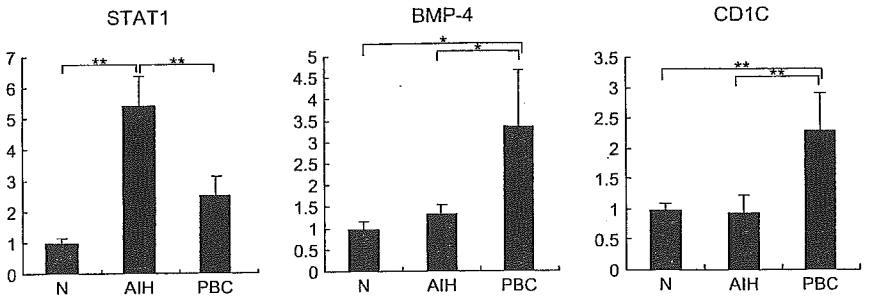

Fig. 2. Quantitative real time detection (RTD)-PCR. $*p < 0.05$, $**p < 0.01$.

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

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

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

2.4. Clinical and molecular characterizations of an overlapping patient

Based on hierarchical clustering and principal component analysis, we found that 1 PBC patient was clustered into the AIH group. The clinical course of this patient (no. 220) is illustrated in Fig. 4. In April 1999, she was admitted to the hospital for evaluation of elevated serum ALP. She was positive for AMA (PDH-E2) (80-fold), and her AIH score was less than that required for diagnosis of possible or definite AIH (Table 5). A liver biopsy revealed accumulation of mononuclear cells around the bile duct cells, a feature typical of CNSDC (Fig. 5a). Ursodeoxycholic acid (UDCA) was prescribed, but she did not take this medication. In April 2002, she was again admitted to the hospital for the evaluation of elevated serum alanine aminotransferase (ALT). After admission, her serum ALT level increased to 407 IU/ml, while her ALP level gradually decreased (Fig. 4). Her serum ANA and gamma-globulin were higher, while her serum IgM was lower, than that at her previous admission. A liver biopsy demonstrated evidence of a severe form of hepatitis, including marked lobular cell

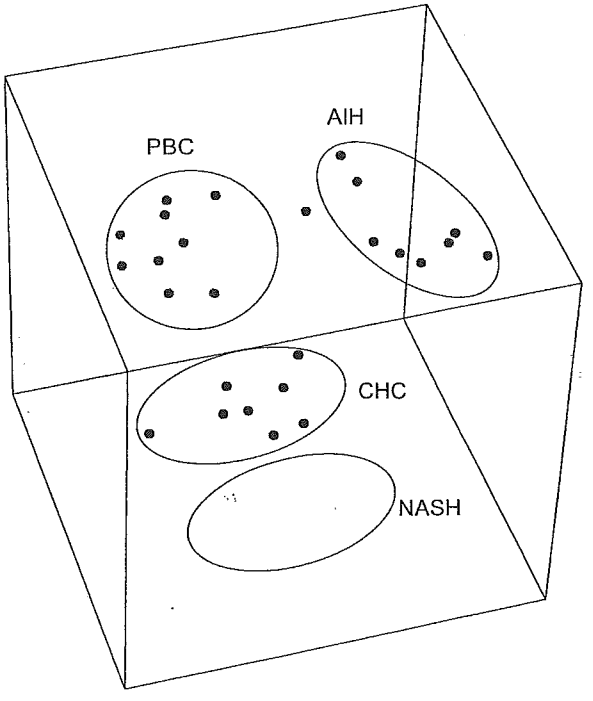

Fig. 3. Principal component analysis of gene expression, showing the three dimensional scattering for each patient with AIH, PBC, CHC and NASH. One PBC patient was separately scattered from other PBC patients.

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

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

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

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

3. Discussion

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

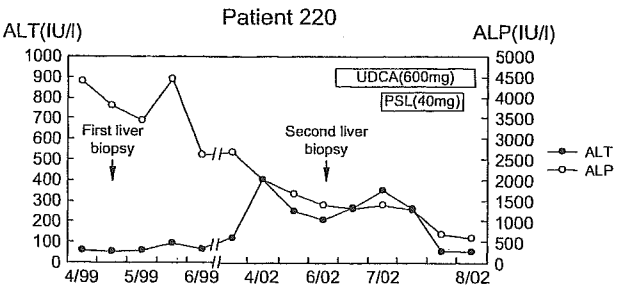

Fig. 4. Clinical course of patient no. 220. This patient, originally diagnosed with PBC, later developed AIH. Serum concentrations of ALP and ALT are shown.

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

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

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

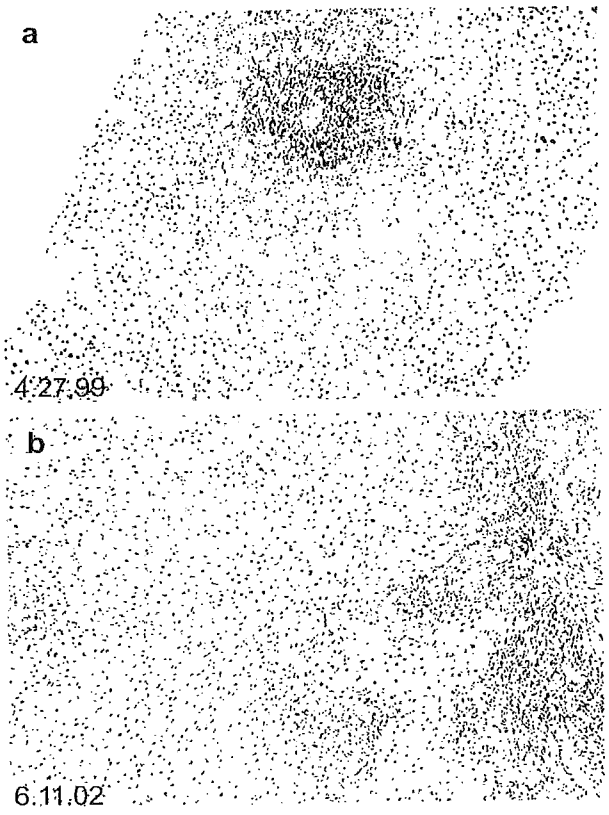

Fig. 5. Histopathological findings for patient no. 220. (a) The first liver biopsy, performed April 27, 1999, showing accumulation of mononuclear cells around the bile ducts, a finding typical of CNSDC. (b) The second liver biopsy, performed June 11, 2002, showing a more marked lobular cell infiltration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

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

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

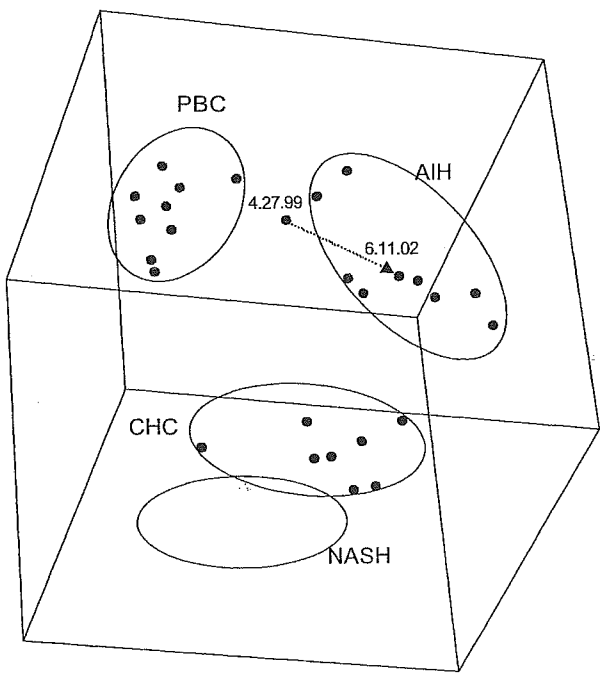

Fig. 6. Principal component analysis of gene expression of AIH, PBC, CHC and NASH. The expression profiling of 1 PBC patient, which was originally differed from that of the other PBC patients (4.27.99), subsequently changed to an AIH pattern (6.11.02).

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Differential Gene Expression Profiles in Stage I Primary Biliary Cirrhosis

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OBJECTIVES: Primary biliary cirrhosis (PBC) is a progressive disease. However, little is understood about the molecular mechanisms underlying its features.

METHODS: We analyzed gene expression profiles of liver biopsy samples from 16 patients with PBC, seven with autoimmune hepatitis, eight with chronic hepatitis C, and eight normal control livers. In addition to whole liver samples, we selectively analyzed chronic nonsuppurative destructive cholangitis (CNSDC) lesions by laser capture microdissection.

RESULTS: Hierarchical clustering analysis using only early-stage liver disease demonstrated 85 genes were upregulated in stage I PBC specifically. Surprisingly, the expression of these genes was not maintained in advanced-stage PBC, while other gene clusters were upregulated. Expression analysis of CNSDC lesions in stage I PBC showed the presence of active inflammatory changes, characterized by the significant elevation of interferon-gamma and the development and maturation of lymphocytes. Expression of these genes was diminished in lymphoid cells aggregation in stage III PBC, and genes reflecting hepatocyte damage were upregulated with disease progression.

CONCLUSION: Gene expression patterns in stage I PBC are different from others. There are distinct changes in molecular pathology from early- to late-stage PBC, which might be a clue to reveal the etiology and progression of PBC.

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic autoimmune liver disease of middle-aged women that is characterized by the sustained elevation of bile duct enzymes, antimitochondrial antibodies (AMA) in serum (1), and chronic nonsuppurative destructive cholangitis (CNSDC) of the liver (2). Although the disease is progressive and a significant proportion of patients eventually develop cirrhosis, little is understood about the molecular mechanisms underlying its features.

Hepatic lesions of PBC can be histologically classified into four stages: *stage I*, florid bile duct lesions; *stage II*, ductular proliferation; *stage III*, scarring; and *stage IV*, cirrhosis, respectively (3). It has been reported that the expression of interferon (IFN)-gamma in infiltrated lymphocytes in CNSDC lesions was rather higher in early-stage PBC than in late-stage PBC (4). Moreover, the frequency of PDC-E2-specific CD8+ T cells in peripheral blood was significantly higher in earlier stage PBC as compared with late-stage PBC (5). Thus, it could be speculated that pathophysiological events in early- and late-stage PBC may be different, and essential findings related to inflammatory lesions of bile duct epithelium in initial-stage PBC would be lost in the cirrhotic stage of liver in late-stage PBC. However, so far, there have been few detailed reports clarifying the differences in molecular events between early- and late-stage PBC.

For revealing the underlying molecular pathogenesis of PBC and preventing the disease progression, it is important to analyze early-stage PBC hepatic lesions. However, molecular biological approaches for the study of CNSDC lesions sometimes have been hampered because the affected lesions were small compared with intact hepatocytes. New advanced technologies need to be developed to overcome this problem.

Complementary DNA (cDNA) microarray technology provides a means of simultaneously analyzing the expression levels of hundreds or thousands of genes. Genome-based expression profiling provides useful information about the molecular pathogenesis of various diseases, as well as on disease progression and prognosis (6) (7–9). In the present study, we analyzed PBC hepatic lesions with varying severity and compared these data with autoimmune hepatitis (AIH), chronic hepatitis C (CH-C), and normal liver. Furthermore, we selectively analyzed gene expression in lymphoid cell aggregations in CNSDC lesions in early- and late-stage PBC using the laser capture microdissection (LCM) technique.

Table 1. Patients' Characteristics

Clinical Diagnosis	Patient No.	Age (yr)	Sex (M:F)	ALT (IU/L)	ALP (IU/I)	ANA (+). No.	AMA (+). No.
Normal	9	66.5 ± 5.23	7:2	18.4 ± 2.61[b]	259 ± 23.2	0	0
Primary biliary cirrhosis (stage I)	6	61.0 ± 2.33	1:5	47.8 ± 7.82	533 ± 70.1	4	5
Primary biliary cirrhosis (stage ≥ II)	10	59.6 ± 2.76	2:8	49.0 ± 6.57	1372 ± 353[c]	4	7
Autoimmune hepatitis	7	50.3 ± 4.02[a]	3:4	48.9 ± 9.95	291 ± 45.5	7	0
Chronic hepatitis C	8	56.6 ± 6.33	6:2	54.0 ± 13.6	214 ± 16.1	0	0

[a]Autoimmune hepatitis versus normal ($p < 0.05$).
[b]Normal versus primary biliary cirrhosis (stage ≥ II), autoimmune hepatitis, chronic hepatitis C ($p < 0.05$).
[c]Primary biliary cirrhosis (stage ≥ II) versus normal, autoimmune hepatitis, chronic hepatitis C ($p < 0.05$).

PATIENTS AND METHODS

Patients and Tissue Samples

We obtained liver biopsy specimens from 16 patients with various stages of PBC. For the controls, we used biopsy specimens from seven patients with AIH and nine with CH-C. All of the patients were admitted to Kanazawa University Hospital between 1997 and 2002 (Table 1). The biopsy specimens were immediately frozen in liquid nitrogen and stored in liquid nitrogen. We obtained nine normal liver samples at surgery from noncancerous regions of the livers from patients with metastatic tumors who manifested no clinical signs of hepatitis. These patients were all seronegative for viral markers and autoantibodies, and their liver function parameters were within normal limits. All samples were histologically assessed as described by Scheuer (3) and Desmet *et al.* (10) (Table 2). All patients were historically confirmed they had not been medicated with ursodeoxycholic acid (UDCA) or steroids for at least 1 yr. PBC was diagnosed by elevated ductal enzyme levels and seropositivity for antimitochondrial antibody (AMA), as well as by the presence of representative histological features of bile duct injury such as CNSDC. While four PBC patients were seronegative for AMA (Table 1), liver specimens from these patients revealed elevated ductal enzyme levels and the histological features of CNSDC. Patients were diagnosed with AIH according to the criteria of the International Autoimmune Hepatitis Group (11); all seven of them had type I autoimmune hepatitis (score >15). All eight patients with CH-C were positive for HCV-RNA.

cDNA Microarray Slides

We used microarray slides containing 1,080 cDNA clones as described (12–14). Genes to be spotted were selected according to the list of the Clontech atlas membrane array and were obtained from I.M.A.G.E. Consortium libraries through its distributor, Research Genetics, Inc. (Huntsville, AL). We confirmed the identity of each clone in our laboratory.

Table 2. Histological Assesment

PBC	No. of Patients	AIH	No. of Patients	CH-C	No. of Patients
Stage I	6	F1A1	5	F1A1	2
Stage II	5	F1A2	1	F2A1	4
Stage III	3	F2A1	1	F2A2	2
Stage IV	2				

PBC, primary biliary cirrhosis; AIH, autoimmune hepatitis; CH-C, chronic hepatitis C.

RNA Isolation, Antisense RNA Amplification, and Hybridization on cDNA Microarray Slides

Total RNAs isolated from liver biopsy specimens using Micro RNA isolation kits (Stratagene, La Jolla, CA) were amplified using antisense RNA (aRNA) as described (12). The quality and degradation of the isolated RNA were estimated after electrophoresis using an Agilent 2001 bioanalyzer. The references used for each microarray analysis were aRNA samples prepared from the normal liver tissue of one patient. Test RNA samples fluorescently labeled with Cy5 and reference RNA labeled with Cy3 were used for microarray hybridization as described (12). Each hybridization proceeded at least twice. The typical CNSDC lesion in two stage I PBC patients, lymphoid cell aggregates in two stage III PBC patients, and in zone 1 hepatic lesion in two patients with chronic hepatitis C (CH-C) (F_1A_1) were selectively excised by laser capture microdissection (LCM) using a CRI-337 (Cell Robotics, Inc., Albuquerque, NM) as described (15). Total RNA was isolated from the dissected samples using RNAqueous™-Micro (Ambion, Austin, TX), and 5 ng of isolated RNA were evaluated by electrophoresis in an Agilent 2001 bioanalyzer with a micropico kit. Total RNA was amplified twice as described above. Amplified RNA was similarly used for microarray analysis. As the reference, similarly prepared RNA from a normal liver was used. The optimum conditions of LCM and reproducibility of data were assessed repeatedly. The mRNA expression of each genes determined by the signal intensities using first amplified and second amplified aRNA had a significant high correlation ($p = 0.99$) and the differences in their expression of all genes were within twofold.

In addition, we excised lymphoid cell infiltrates and hepatocytes from eight patients with chronic hepatitis B or C separately and determined which genes were predominantly expressed in these two legions. Some of these data were used for the analysis of gene expression.

Image Analysis and Data Processing

Quantitative assessment of the signals on the slides was done by scanning on a ScanArray 5000 (General Scanning, Watertown, MA), followed by image analysis using ImaGene

3.0 software (Bio Discovery, Los Angeles, CA). The signal intensity of each spot was corrected by subtracting adjacent background signals. To normalize the data, we averaged the intensities of all spots obtained with Cy3 and Cy5 in each of the 16 rectangles, and adjusted the intensity of each corrected DNA spot by the average intensity ratio of Cy5:Cy3 (=1.0). This global normalization of intensity provided a smaller variance of the Cy5:Cy3 ratio and almost the same results as normalization using the housekeeping genes. Because signal values of approximately 500–600 were obtained for luciferase genes, which have no homology with any human gene sequence, all values below 600 were set as background values. The lowess (or intensity-dependent) normalization was also performed, because dye bias may be different for low-intensity spots relative to high-intensity spots. Similar results were obtained as using global normalization and M-A plot were distributed equally. Hierarchical clustering of gene expression of the patients was performed by BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html). The filtered data were log-transferred, -normalized, -centered, and -applied to the average linkage clustering with centered correlation. BRB-ArrayTools contains a class comparison tool based on univariate F tests to find genes differentially expressed between predefined clinical groups. The permutation distribution of the F statistic, based on 2,000 random permutations, was also used to confirm statistical significance. A p value of less than 0.05 and more than 1.8-fold difference in gene expression was considered significant.

Quantitative Real-Time Detection (RTD)-PCR

We performed quantitative real time detection PCR (RTD)-PCR using TaqMan Universal Master Mix (PE Applied Biosystems, CA). Primer pairs and probes for FK506-BP, interferon gamma inducible protein-10 (IP-10), hepatocyte growth factor activator (HGFAC), Charcot-Leyden crystal (CLC), CD1C and, GAPDH were obtained from TaqMan assay reagents library (Applied Biosystems, CA).

Statistical Analysis

All clinical data are expressed as mean ± SEM. A p value of less than 0.05 was considered significant. Fisher's exact test was used to test the significance of hierarchical clustering in the dendrogram using various clinical parameters including the etiology of liver disease, gender, age and blood test results etc. A p value of less than 0.01 was considered significant.

RESULTS

Differentially Expressed Genes in Stage I PBC Hepatic Lesions

The analysis of gene expression in early-stage PBC may reveal the etiology and pathogenesis of this disease. To examine

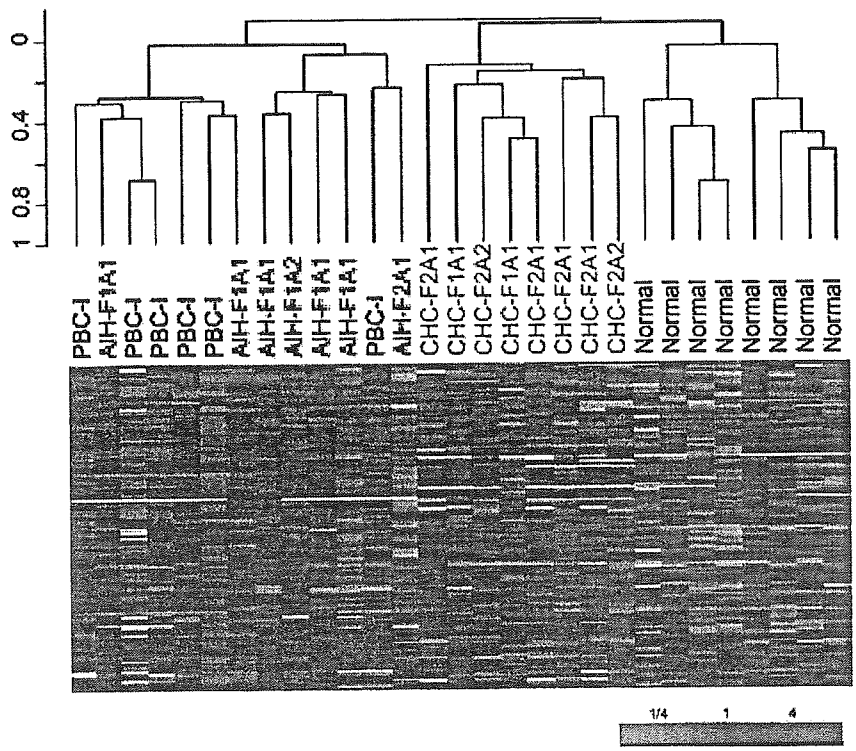

Figure 1. A cluster of 85 genes upregulated in stage I PBC. Hierarchical clustering analysis of 1,080 genes in patients with stage I PBC (blue), AIH (red), CH-C(green), and normal (black) were performed. Dendrogram shows grouping of patients based on similarities in gene expression profiles. The patients were clustered into normal, CH-C, AIH, and PBC, respectively, with a few exceptions.

Table 3. Upregulated Genes in Early-Stage PBC Hepatic Lesion

Gene	Fold	*p* Value	Ly/Hep	Ref Seq ID	Function
Immune response					
Monokine induced by gamma interferon	3.6	0.003	Ly	NM_002416	Chemokine activity
TNF receptor superfamily, member 7	2.4	0.000	Ly	NM_001242	Positive regulation of interferon-gamma biosynthesis
Interleukin 7	2.1	0.000	Ly	NM_000880	Important for B and T cell development
TNF receptor superfamily, member 17	2.0	0.000	Ly/Hep	NM_001192	Promotes B-cell survival
Epstein-Barr virus induced gene 3 (EBI3)	1.8	0.000	Ly/Hep	NM_005755	Hematopoietin receptor family related to the p40 of IL 12
CD20 antigen	1.8	0.001	Ly	NM_021950	Regulation of B-cell activation and proliferation
CD1C antigen, c polypeptide	1.8	0.000	Ly	NM_001765	T-cell surface receptor
FK506-binding protein 4 (59kD)	1.8	0.000	Ly/Hep	NM_002014	Immunoregulatory gene expression in B and T lymphocytes
Transcriptional factors and cell signaling					
Transcription factor AREB6	2.7	0.000	Ly	nse	Inhibits interleukin-2 (il-2) gene expression
Zink finger protein PLIZF	2.7	0.000	Ly/Hep	NM_006006	Expressed within the hematopoietic system
c-myb cDNA clone lambda-LMC8	2.7	0.003	Ly/Hep	NM_005375	Differentiation of hematopoietic progenitor cells
PKC	2.2	0.001	Ly/Hep	NM_002737	Protein kinase C activity
CB1 cannabinoid receptor (CNR1) gene	2.1	0.000	Ly	NM_016083	G-protein signaling
CGI-137 protein	1.8	0.002	Hep	NM_003187	Transcription coactivator activity
POU domain, class 2, transcription factor 1	1.8	0.001	Ly	NM_002697	Transcription factor
Orphan hormone nuclear receptor	1.8	0.007	Ly/Hep	NM_005122	Transcription factor
Bruton agammaglobulinemia tyrosine kinase	1.8	0.038	Ly	NM_000061	B-cell ontogeny
Cell proliferation					
HGF activator	2.1	0.001	Hep	NM_001528	Activates hepatocyte growth factor (hgf)
Mitogen-activated protein kinase 4	2.0	0.011	Ly/Hep	NM_002747	Cell cycle
E2F transcription factor 3	1.8	0.001	Ly/Hep	NM_001949	Transcription factor
Serine/threonine-protein kinase PCTAIRE-1	1.8	0.006	Ly/Hep	NM_033018	Regulation of cell cycle
Cell division cycle 27	1.8	0.014	Ly/Hep	NM_001256	Cell cycle
Cell adhesion and cell-cell signaling					
Endothelin 3	3.4	0.000	Ly	NM_000114	Cell-cell signaling Receptor binding
Cadherin 11 (OB-cadherin, osteoblast)	2.3	0.002	Ly/Hep	NM_033664	Cell adhesion
Cadherin 6, K-cadherin (fetal kidney)	2.3	0.001	Ly/Hep	NM_004932	Cell adhesion
Cadherin 3, P-cadherin (placental)	1.8	0.002	Ly/Hep	NM_001793	Cell adhesion
Cadherin 13, H-cadherin (heart)	1.8	0.037	Ly/Hep	NM_001257	Cell adhesion
Cadherin-associated protein-related (cap-r)	1.8	0.009	Ly/Hep	NM_004389	Cell adhesion
Zyxin	1.8	0.000	Ly/Hep	NM_003461	Cell adhesion
Metabolism and others					
DNA repair protein XRCC9	2.1	0.004	Ly/Hep	NM_004629	Damaged DNA binding DNA repair
p37NB	2.0	0.001	Ly	NM_005824	leucine rich repeat
Cytochrome P450, subfamily IVB	1.8	0.000	Ly/Hep	NM_000779	Electron transport
Glycine decarboxylase	1.8	0.041	Hep	NM_000170	Glycine catabolism
Charot-Leyden crystal protein	1.8	0.002	Ly/Hep	NM_001828	Eosinophil lysophospholipase
Morphogenesis					
Nuclear factor (erythroid-derived 2)-like 1	2.3	0.000	Hep	NM_003204	Morphogenesis
Homeobox protein HOX-D3	2.0	0.004	Ly/Hep	NM_006898	Morphogenesis
Homeobox protein HOX-A4	1.8	0.000	Ly/Hep	NM_002141	Morphogenesis
Brain-derived neurotrophic factor	1.8	0.007	Ly/Hep	NM_001709	Neurogenesis

gene expression in stage I PBC hepatic lesions, six patients with stage I PBC, seven patients with AIH, eight patients with CH-C, and eight patients with normal livers were analyzed. All of these patients were selected because they did not have advanced liver disease (Table 2). Although the male predisposition of patients with AIH was rather high, they were met the criteria at the diagnosis (score >15). Nonsupervised hierarchical clustering analysis using all 1,080 genes revealed

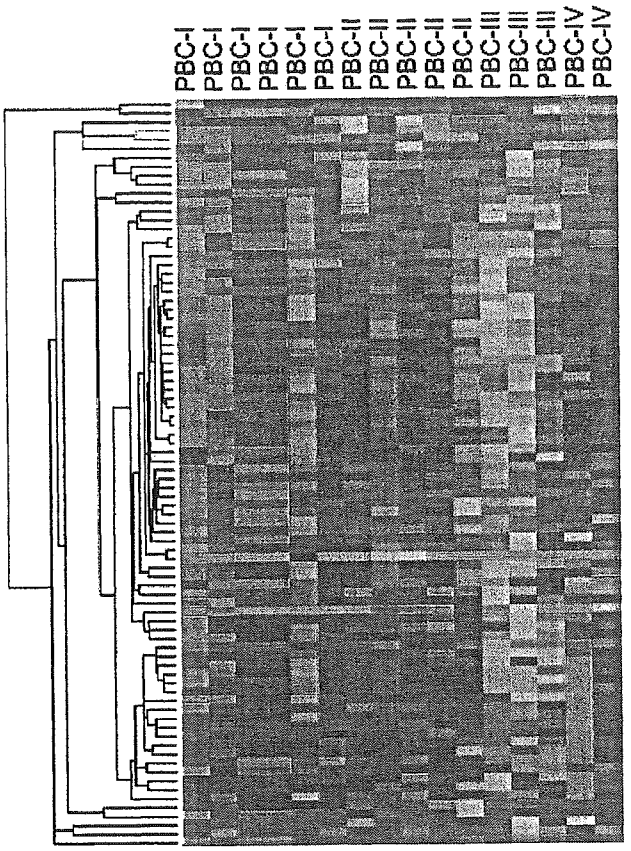

Figure 2. One-way hierarchical clustering analysis based upon 85 upregulated genes in stage I PBC.

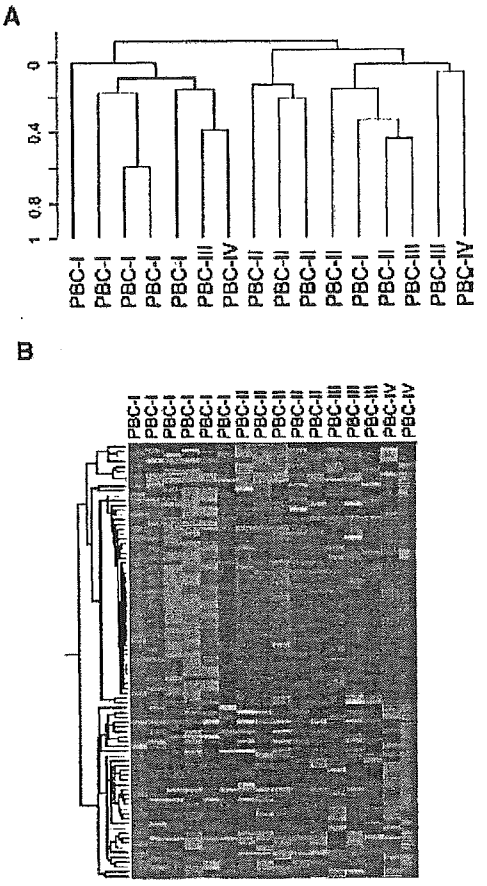

Figure 3. (*A*) Hierarchical clustering analysis of 1,080 genes in patients with all PBC patients was performed. PBC patients were nearly all clustered into two groups; one mainly consisted of patients with stage I PBC, and the other consisted of patients with more advanced PBC (II–IV) (*B*) The class comparison tool was applied to define the significant genes that distinguish stage I PBC, stage II PBC, and stage III–IV PBC. Ninety-seven genes met the criteria of p value of less than 0.05 and one-way hierarchical clustering of these genes is shown.

that patients were clustered into normal, CH-C, AIH, and PBC, respectively, with a few exceptions. Genes with low variability and minimum fold change were excluded (filter criteria was low variability less than 15% of expression data values and minimum fold change of 1.5 in either direction from the gene's median value). Hierarchical clustering analysis using 510 filtered genes revealed exact same clustering of patients as using all 1,080 genes (data not shown). This implied that there were characteristic gene expression patterns depending on the etiology of each disease. Other clinical parameters except the etiology of liver diastase contributed to form this clustering although classification of PBC and AIH was not statistically significant. Out of 1,080 genes analyzed, we found a cluster of 85 genes that were upregulated in PBC patients (Fig. 1), a cluster of 80 genes that were upregulated in CH-C patients, and a cluster of 61 genes that were upregulated in AIH patients. Thirty-eight of the genes in the PBC cluster met criteria of a p value less than 0.05 and more than a 1.8-fold difference compared with others groups (Table 3). Chemokines, cytokines and their receptors such as monokine-induced gamma interferon (Mig), interleukin (IL)-7, and TNF receptor super family were upregulated in stage I PBC, although the affected regions in liver were limited to the portal area. These genes are involved in the development of T and B lymphocytes. In accordance with this, several lymphocyte specific transcriptional factors and morphogenesis-related genes were also upregulated. As mainly lymphocyte predominant genes were upregulated in stage I PBC liver, this might reflect the changes in gene expression in CNSDC lesions.

Differentially Expressed Genes in Late-Stage PBC Hepatic Lesions

A cluster of 85 genes upregulated in stage I PBC was re-evaluated in samples with a more advanced stage of PBC. We performed one-way hierarchical clustering analysis to reveal the changes in gene expression during disease progression from stage I–IV. Surprisingly, many stage I PBC specific genes were downregulated with disease progression (Fig. 2).

Table 4. Upregulated Genes in Late-Stage PBC Lesion

Gene	Fold	p-Value	Ly/Hep	Ref Seq ID	Function
Innume response					
Interleukin 8	2.0	0.005	Hep	NM_000584	Chemotactic factor
Monocyte/macrophage Ig-related receptor (MIR)-7	1.9	0.010	Ly	NM_006669	Inhibitory receptor for major histocompatibility complex
S100 calcium-binding protein A9 (calgranulin B)	1.9	0.013	Ly/Hep	NM_002965	Inflammatory response
Monocyte chemotactic protein 1	1.8	0.004	Ly	NM_002982	Chemotactic factor
S100 calcium-binding protein A8 (calgranulin A)	1.8	0.016	Ly/Hep	NM_002964	Inflammatory response
Cell death					
FADD	2.0	0.012	Ly/Hep	NM_003824	Apoptotic adaptor molecule that recruits caspase-8
Fas	1.8	0.027	Ly/Hep	NM_000043	Apoptosis
Cytoplasmic dynein light chain (hdlc 1)	1.8	0.008	Ly/Hep	NM_003746	Dynein-related intracellular transport and motility
p53	1.8	0.021	Ly/Hep	NM_000546	Cell cycle
Cell cycle and proliferation					
GRB2 (growth factor receptor-bound protein 2)	2.8	0.005	Ly/Hep	NM_002086	Epidermal growth factor receptor signaling pathway
CAS	2.1	0.010	Ly	NM_001316	Intracellular protein transport
Teratocarcinoma-derived growth factor 1	2.0	0.048	Ly	NM_003212	Tumor growth
Cyclin D1	1.8	0.046	Hep	NM_053056	Regulation of cell cycle
Cyclin D2	1.8	0.038	Ly/Hep	NM_001759	Regulation of cell cycle
Retinoic acid receptor beta-2 (RAR-beta-2)	1.9	0.014	Ly/Hep	NM_000965	Cell growth and maintenance
Alpha-tubulin	1.8	0.014	Ly	NM_006082	Constituent of microtubules
Signal transduction					
Regulator of G-protein signalling 1	2.9	0.010	Ly	NM_002922	Regulation of B-cell activation and proliferation
Zinc finger protein 336	2.6	0.005	Ly/Hep	NM_022482	Transcription factor
GTP-binding protein (RAB6)	1.8	0.000	Ly/Hep	NM_002869	Small GTPase mediated signal transduction
76 kDa tyrosine phosphoprotein SLP-76	1.8	0.046	Ly	NM_005565	Involved in T cell antigen receptor mediated signaling
Ras homolog gene family, member E	1.8	0.014	Ly/Hep	NM 005168	Small GTPase mediated signal transduction
Cell adhesion					
Neogenin	1.9	0.018	Ly/Hep	NM 002499	Cell adhesion and development cell motility
Collagen, type VI, alpha 3	1.8	0.013	Ly/Hep	NM_057164	Extracellular matrix
Cadherin 5, VE-cadherin (vascular epithelium)	1.8	0.043	Ly	NM_001795	Cohesion and organization of endothelial cell
Keratin 18	1.8	0.009	Ly/Hep	NM_000224	Intermediate filament family
Development					
Dopamine receptor D2	1.8	0.002	Ly/Hep	NM_000795	Neurogenesis
Sno oncogene snoN protein ski-related	1.8	0.024	Ly/Hep	NM_005414	Cell differentiation
Stress response and other					
Glutathione S-transferase pi	2.0	0.012	Ly/Hep	NM_000852	Xenobiotic metabolism and susceptibility to cancer
Inducible nitric oxide synthase	1.9	0.009	Ly/Hep	NM_000625	Nitric oxide biosynthesis
Heme oxygenase-2	1.9	0.018	Ly/Hep	NM_002134	Heme oxygenase (decyclizing) activity

Hierarchical clustering analysis using all 1,080 genes clustered PBC patients into nearly two groups: one mainly consisted of patients with stage I PBC and the other consisted of patients with more advanced PBC (II–IV) (Fig. 3A). These data strongly suggest that stage I PBC is different from more advanced-stage (II–IV) PBC in terms of molecular events. To examine which genes were upregulated in late-stage PBC, a class comparison tool was applied to define the significant genes which distinguish stage I, stage II, and late-stage PBC (stages III and IV). One-way hierarchical clustering using differentially expressed genes (94, $p < 0.05$) are shown in Figure 3B. Among upregulated genes in late-stage (III and IV) PBC, 28 genes showed more than a 1.8-fold difference and were listed in Table 4. Apoptosis (fas, FADD, etc.), cell cycle, and stress response-related genes (cyclin D1, glutathion S-transferase pi, and inducible nitric oxidase syntheytase, etc.) were upregulated. Chemokines such as IL-8, macrophage inflammatory chemotactic protein-1 (MCP-1), and macrophage inflammatory protein 3-beta (MIP3B), mediators of the inflammatory response were upregulated in

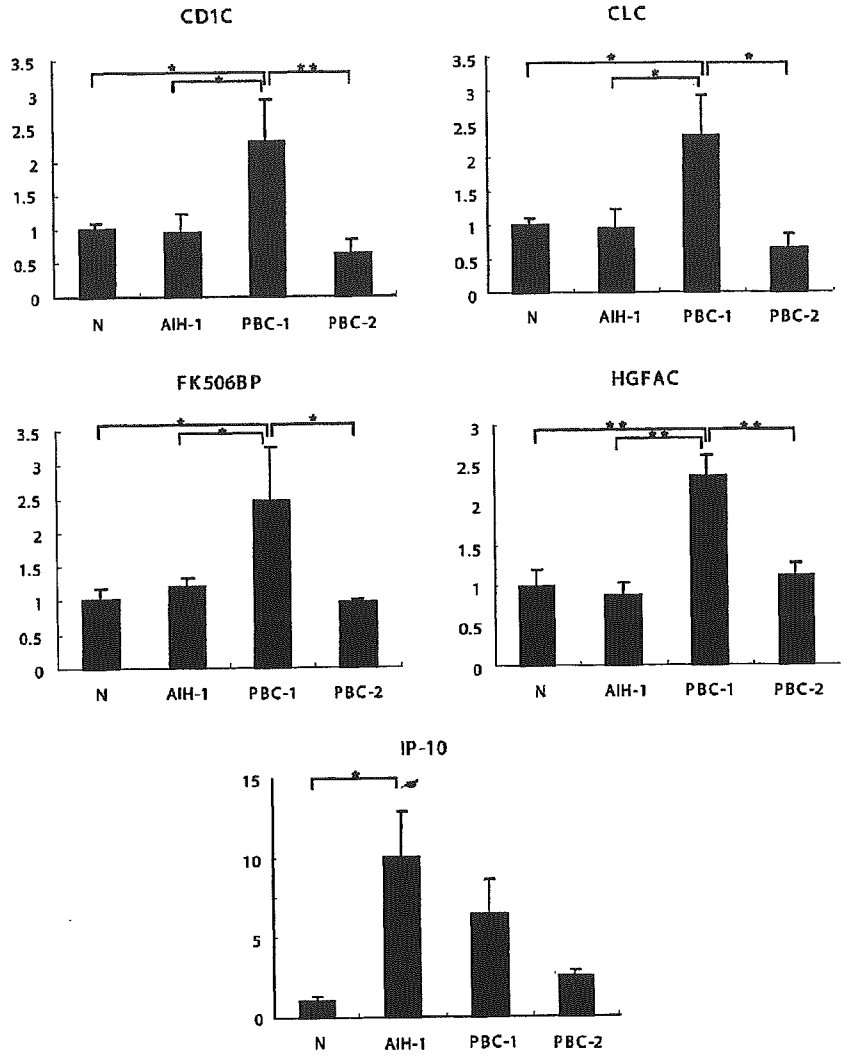

Figure 4. RTD-PCR for CD1C, FK504-BP, HGFAC, CLC and IP-10 expressed in AIH, PBC, and NL (PBC-1, stage = 1, n = 6; PBC-2, stage >1, n = 10).

advanced PBC. Type IV collagen and VE-cadherin were upregulated with increased fibrosis and angiogenesis. Thus, different functional genes were upregulated in late-stage PBC.

Evaluation of Gene Expression by RTD-PCR

Expression of some of the representative genes was confirmed by RTD-PCR (Fig. 4). CD1C antigen, FK506 BP, HGF activator, Charcot-Leyden crystal protein (CLC), and IP-10 were quantified by RTD-PCR and were found to be present at different stages of PBC. These genes were upregulated only in stage I PBC. Although the expression of IP-10 in AIH was as high as in stage I PBC, it also decreased in late-stage PBC (not significant).

Differential Gene Expression of Lymphoid Cell Aggregation in CNSDC Lesions in Stage I and Stage III PBC Hepatic Lesions

Since gene expression profiles in stage I PBC possibly reflected lymphoid cell aggregation and the formation of CNSDC lesions, we selectively analyzed CNSDC lesions in stage I and stage III PBC by LCM. Liver biopsy specimens obtained from four patients were analyzed. As shown in Figure 5A, the representative CNSDC lesion in stage I PBC was precisely dissected by LCM. On the other hand, the typical CNSDC lesion was not preserved, and scarring was present in stage III PBC with the dissection of the lymphoid cell aggregation in the portal area (Fig. 5B). As controls, lymphoid cell aggregation in the portal area in early-stage CH-C (F_1A_1) were used (Fig. 5C). Hierarchical clustering analysis using all 1,080 genes showed that four PBC samples

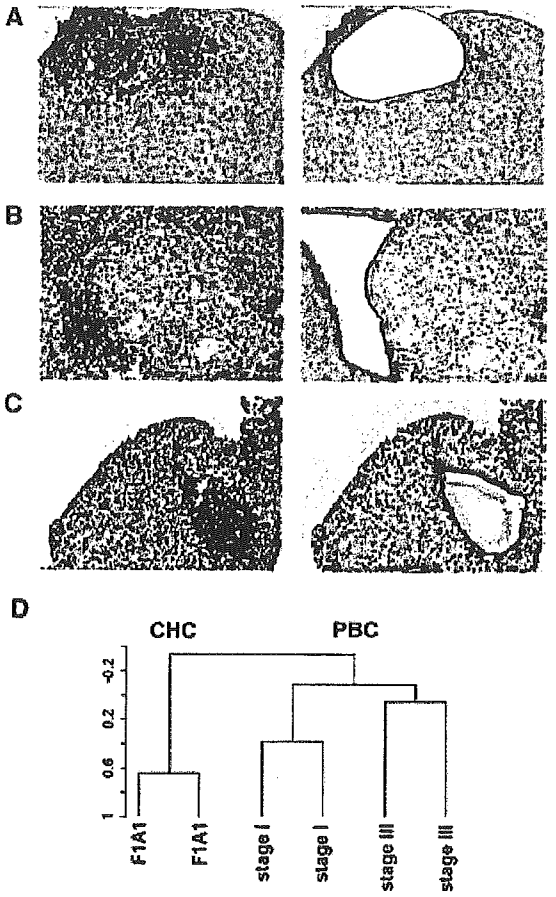

Figure 5. (*A*) The representative CNSDC lesion in stage I PBC was precisely dissected by LCM. (*B*) The typical CNSDC lesion was not preserved and scarring was present in stage III PBC with the dissection of the lymphoid cell aggregation in the portal area. (*C*) The lymphoid cell aggregation in portal area in early stage of CH-C (F1A1) was dissected and used for control. (*D*) The hierarchical clustering analysis using all 1,080 genes showed that four PBC samples were grouped together and were separate from CH-C.

were grouped together, separated from the two CH-C samples and two CNSDC lesions of stage I PBC were grouped together, separated from the two CNSDC lesions of stage III PBC. Gene expression in CNSDC lesions of stage I PBC was compared with that of CH-C lesions (Table 5). IFN-gamma expression was upregulated more than fivefold in stage I CNSDC lesions, suggesting the presence of an active Th1 immune response. IL-7, colony stimulating factor 1 receptor and IL-6 receptor were upregulated. This might imply the development and maturation of T and B cells. Regarding the other cytokines such as IL-2, IL-12, IL-4, and IL-10, no difference could be seen between the two lesions (data not shown). In addition to lymphocytes, myeloid cells such as macrophages and eosinophils were activated, supported by the upregulation of myeloid cell activation antigen c-myb and c-kit proto-oncogene. Coincident with these findings, the transcriptional factor, NF-kappa-B was upregulated, suggesting the development and maturation of a variety of cells in their early stage. Regarding cell adhesion molecules, MHC class I and class II were upregulated. This was compatible with the upregulation of IFN-gamma and active inflammation in CNSDC lesions. The upregulation of other cell adhesion molecules such as keratin 18, collagen IV, and laminin indicate active fibrogenesis in CNSDC lesions. The other characteristic finding in CNSDC lesions was that the immunosuppressive genes such as FK506-binding protein 4 and cyclophilinD were upregulated in stage I PBC. This may partially explain the poor response of PBC to steroid therapy.

Many characteristic genes upregulated in stage I PBC were downregulated and other genes were upregulated with disease progression (Table 6). For example, apoptosis inhibitory genes such as MIHB and vascular-cell adhesion molecules such as VCAM-1 were upregulated. It is interesting that transcriptional factor NF-ATc was upregulated in contrast to the upregulation of NF-kappa-B of stage I PBC. These data implicated that more mature lymphocytes infiltrated in advanced PBC lesions.

DISCUSSION

Although PBC is progressive and a significant proportion of patients eventually develop cirrhosis, little is understood about the molecular mechanisms underlying its features. Recent studies of the expression profiling of hepatic lesions in PBC revealed important differentially expressed genes. However, these studies only analyzed advanced-stage PBC, and the results might not reflex the initial changes in PBC which are more central to the pathogenesis of PBC (17, 18). In this study, we first focused on early-stage PBC and determined PBC-specific genes by comparing analogous stages of AIH, CH-C, and normal liver. We demonstrated here mainly different genes from those described in previous reports were upregulated in early stage of PBC, although many similar genes as previous reports were also upregulated (16, 17). The number of genes we analyzed in this study was relatively low (1,080 genes); however, this gene set included essential genes to study liver-related disease as we reported in other studies (12–14, 18–20).

Surprisingly, most of the genes specifically expressed in stage I PBC no longer expressed in more advanced-stage PBC, suggesting changes in molecular pathogenesis during the transition from early- to late-stage PBC. We examined these findings by using whole liver samples and CNSDC lesions excised by LCM.

Gene expression analysis using whole liver samples was useful for demonstrating the characteristic gene expression in PBC hepatic lesions. Many lymphocyte specific genes were upregulated in stage I PBC hepatic lesions. The CNSDC lesion-specific expression analysis using LCM provided a useful data to compare the early and late stage of PBC. In stage I PBC, many cytokines and their receptors were

Table 5. Upregulated and Downregulated Genes in CNSDC Lesions in Stage I PBC

Gene name	PBCI/CHC	Ly/Hep	Ref Seq ID	Function
Cytokine related				
Interferon, gamma	4.2	Ly/Hep	NM_000619	Immune response
Colony stimulating factor 1 receptor	5.4	Ly/Hep	NM_005211	Controls the function of macrophages
Interleukin 6 receptor	2.7	Ly/Hep	NM_000565	Multiple myeloma, autoimmune diseases
Interleukin 7	2.6	Ly	NM 000880	Important for B and T cell development
TNF receptor superfamily, member 7	2.4	Ly/Hep	NM_001242	Positive regulation of interferon-gamma biosynthesis
Cell proliferation related				
elF 4A, isoform 1	5.8	Ly/Hep	NM_001416	Translation factor
MAP kinase kinase 3	5.2	Ly/Hep	NM_145109	Cell proliferation
c-myb cDNA clone lambda-LMC8	3.5	Ly/Hep	NM_005375	Differentiation of hematopoietic progenitor cells
Myeloid cell nuclear differentiation antigen	2.8	Ly/Hep	NM_002432	Expressed in granulocyte-monocyte lineage
c-kit proto-oncogene	2.5	Ly/Hep	NM_000222	Mast/stem cell growth factor receptor
Transcription				
NF-kappa-B transcription factor p65 subunit	2.9	Ly/Hep	NM_021975	Transcription factor
Pig7 (PIG7)	2.7	Ly/Hep	NM_004862	Regulation of TNF alpha gene transcription
DNA-binding protein inhibitors ID2	2.5	Ly/Hep	NM_002166	Development
c-myc binding protein	2.5	Hep	NM_145896	Repress the transcriptional activity of c-myc
NM23B	2.5	Ly/Hep	NM_002512	Transcriptional activator of the c-myc gene
Immune modulator				
FK506-binding protein 4 (59kD)	2.6	Ly/Hep	NM_002014	Immunoregulatory gene expression in B and T lymphocytes
Peptidylprolyl isomerase D (cyclophilin D)	2.5	Ly/Hep	NM_005038	Bind the immunosuppressant cyclosporin A
Cell-cell interaction				
MHC class II DR	10.4	Ly	NM_021983	Immune response
Keratin 18	3.4	Ly/Hep	NM_000224	Intermediate filament family
MHC class IB	3.0	Ly/Hep	NM_005514	Immune response
Collagen, type IV, alpha 2	2.9	Ly	NM_001846	Major structural component of basement membranes
Laminin, beta 2	2.6	Ly/Hep	NM_002292	Cell adhesion, differentiation, migration, signaling
Others				
Platelet-activating factor receptor	3.1	Ly	NM_000952	Inflammatory, smooth-muscle contractile
Hematopoietic lineage cell specific protein	2.5	Ly	NM_005335	Early stage of myeloid and erythroid differentiation
Downregulated genes				
3d/Epstein Barr virus receptor 2	−15.3	Ly	NM_001877	Receptor for complement and EBV on B-cells and T-cells
CD69 antigen (p60, early T-cell activation antigen)	−14.6	Ly	NM_001781	lymphocyte proliferation and functions
Pre-B-cell leukemia transcription factor 3	−7.9	Ly/Hep	NM_006195	Transcriptional activator
IAP homolog B (MIHB)	−7.8	Ly/Hep	NM_001166	Apoptotic suppressor
Platelet-derived growth factor receptor, alpha	−7.5	Ly	NM_006206	Mitogens for cells of mesenchymal origin
Integrin, alpha 4	−7.0	Ly	NM_000885	Very late antigen 4 on T cell for VCAM-1
Vascular cell adhesion molecule 1 (VCAM 1)	−6.7	Ly	NM_080682	Leukocyte-endothelial cell adhesion
Interleukin-1 receptor, type II	−6.0	Ly	NM_004633	Immune response
Dr-nm23	−5.0	Ly	NM_002513	Inhibits granulocyte differentiation and induces apoptosis
Thymine-DNA glycosylase	−4.7	Ly	NM_003211	DNA repair
ERC-55 mRNA	−4.5	Ly/Hep	NM_002902	Calcium-binding protein

Table 6. Changes of Genes Expression in CNSDC Lesions with Disease Progression

Gene Name	PBCIII/PBCI	Ly/Hep	Ref Seq ID	Function
IAP homolog B (MIHB)	5.4	Ly/Hep	NM_001166	Apoptotic suppressor
Stromal cell-derived factor 1	3.9	Ly/Hep	NM_000609	Activate leukocytes
DNA polymerase alpha-subunit	3.7	Ly/Hep	NM_016937	DNA replication
Platelet-derived growth factor receptor, alpha	3.4	Ly	NM_006206	Mitogens for cells of mesenchymal origin
Nerve growth factor receptor	3.2	Ly/Hep	NM_002507	Mediate cell survival neural cells
Mal, T-cell differentiation protein	3.2	Ly	NM_022438	Intermediate and late stages of T-cell differentiation
ERC-55 mRNA	3.0	Ly/Hep	NM_002902	Calcium-binding protein
NF-ATc	3.0	Ly	NM_006162	Inducible expression of cytokine genes in T cells
Vascular cell adhesion molecule 1 (VCAM 1)	3.0	Ly	NM_080682	Leukocyte-endothelial cell adhesion
CD69 antigen (p60, early T-cell activation antigen)	2.9	Ly	NM_001781	Lymphocyte proliferation and functions
Dr-nm23	2.7	Ly	NM_002513	Inhibits granulocyte differentiation and induces apoptosis.
Cytokine and immune response				
Interferon, gamma	−5.1	Ly/Hep	NM_000619	Immune response
Interleukin 7	−2.6	Ly	NM_000880	B and T cell development
Interleukin 6 receptor	−2.2	Ly/Hep	NM_000565	Multiple myeloma, autoimmune diseases
TNF receptor superfamily, member 7	−2.1	Ly/Hep	NM_001242	Positive regulation of interferon-gamma biosynthesis
TNF receptor superfamily, member 17	−2.1	Ly/Hep	NM_001192	Promotes B-cell survival
Cell proliferation related				
c-myb cDNA clone lambda-LMC8	−2.4	Ly/Hep	NM_005375	Differentiation of hematopoietic progenitor cells
Vascular endothelial growth factor B	−2.2	Ly/Hep	NM_003376	Angiogenesis, endothelial cell proliferation
Transcription				
POU domain, class 2, associating factor 1	−4.1	Ly	NM 002697	Transcription factor
NF-kappa-B transcription factor p65 subunit	−3.4	Ly/Hep	NM_021975	Transcription factor
DNA-binding protein inhibitors ID2	−3.2	Ly/Hep	NM_002166	Development
Spi-B transcription factor (Spi-1/PU.1 related)	−3.1	Ly	NM_003121	Transcription factor
TFIIB	−2.2	Ly/Hep	NM_001514	Transcription factor
Pig7 (PIG7)	−2.2	Ly/Hep	NM_004862	Regulation of TNF alpha gene transcription
Cell-cell interaction				
MHC class II DR	−5.1	Ly	NM_021983	Immune response
Collagen, type XI, alpha 2	−2.6	Ly/Hep	NM_080679	Cell adhesion
CD19 antigen	−2.6	Ly	NM_001770	Growth regulation of B-cells
Others				
Hematopoietic lineage cell specific protein	−2.5	Ly	NM_005335	Early stage of myeloid and erythroid differentiation
Adrenergic, beta-2-, receptor, surface	−2.4	Ly	NM_000024	Catecholamine-induced activation of adenylate cyclase
Protein disulfide isomerase-related protein (PDIR)	−2.4	Ly/Hep	NM_006810	Protein folding and stress response
E2D2	−2.1	Ly/Hep	NM_003339	Ubiquitin-conjugating enzyme

upregulated, reflecting active inflammation in the CNSDC lesions. In particular, IFN-gamma and its inducible genes such as Mig, MHC class I, class II, and TNF receptor super family 7 were upregulated. These findings imply a Th1 dominant immune response through CD4+ helper T cells, as is commonly found in inflammatory regions that are complicated with viral infection and autoimmune hepatitis. In addition, many genes related to B cell development were upregulated, supporting the significance of the Th2 dominant immune responses in CNSDC lesions. Specifically, the IL-7, IL-6 receptor, CD20, and bruton agammaglobulinemia tyrosine kinase play important roles in the development and maturation of B

cells. Besides lymphocyte infiltration, other cell types such as NK cells, macrophages, and eosinophils were found to infiltrate lesions, supported by the upregulation of CD1, colony stimulating factor-1, myeloid cell nuclear differentiation antigen, c-kit proto-oncogene, and Charcot-Leyden crystal. Adhesion molecules such as keratin 18, collagen IV, laminin, and cadherin were all upregulated, reflecting that fibrogenesis was highly activated from early stages of disease. Finally, it is interesting that the immune-suppressive genes such as FK506-binding protein 4 and cyclophilinD were upregulated in stage I PBC. This may partially explain the poor response of PBC to steroid therapy. These gene expression profiles sharply contrast with those of CH-C lesions.

Expression of some of the representative genes was confirmed by RTD-PCR (Fig. 4). CD1C antigen, FK506 BP, HGF activator, Charcot-Leyden crystal protein (CLC), and IP-10 were quantified by RTD-PCR. These genes were upregulated only in stage I PBC. CD1C molecule is a group 1 member of the CD1 family of major histocompatibility complex (MHC)-like glycoproteins. A CD1C-restricted, mycobacteria-specific T-cell line could be detected in peripheral blood T lymphocytes of patients infected with *Mycobacterium tuberculosis*. The cross-reactivity to mycobacteria is thought to be related to the etiopathogenesis of PBC (21,22). The xenobiotics might be important for the pathogenesis of PBC (23). The hypothesis is also supported by the upregulation of CYP and GST in this study.

It was surprising that the expression level of upregulated genes in stage I PBC diminished with disease progression and other gene clusters were upregulated. These changes in gene expression were mainly due to changes in the expression of infiltrating lymphocytes and hepatocytes. In advanced-stage PBC, genes related to apoptosis (fas, FADD, etc.), cell cycle, and stress response-related genes (cyclin D1, glutathion S-transferase pi, and inducible nitric oxidase syntheytase, etc.) were upregulated, reflecting hepatocellular injury and regeneration of hepatocytes. More importantly, gene expression of infiltrating lymphoid cells dramatically changed. Many of the upregulated genes in CNSDC lesions of stage I PBC were downregulated in stage III PBC (Table 6), while other types of genes were upregulated. Lymphocytes infiltrating in advanced PBC might be mature and more resistant to apoptosis, as interpreted by their gene expression. On the other hand, they may be induced by the various nonspecific antigens in liver in which the target bile duct was already lost. Chemokines such as IL-8, MCP-1, and MIP3B were upregulated with disease progression. These chemokines potentially accelerate the severity of the disease.

In conclusion, we clarified the characteristic gene expression profiles of PBC hepatic lesions by using whole liver biopsy samples and CNSDC lesions excised by LCM. These findings should have significance on the understanding of the pathogenesis of PBC with unknown etiology.

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Differential gene alteration among hepatoma cell lines demonstrated by cDNA microarray-based comparative genomic hybridization

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Abstract

We assayed chromosomal abnormalities in hepatoma cell lines using the microarray-based comparative genomic hybridization (array-CGH) method and investigated the relationship between genomic copy number alterations and expression profiles in these hepatoma cell lines. We modified a cDNA array-CGH assay to compare genomic DNAs from seven hepatoma cell lines, as well as DNA from two non-hepatoma cell lines and from normal cells. The mRNA expression of each sample was assayed in parallel by cDNA microarray. We identified small amplified or deleted chromosomal regions, as well as alterations in DNA copy number not previously described. We predominantly found alterations of apoptosis-related genes in Hep3B and HepG2, cell adhesion and receptor molecules in HLE, and cytokine-related genes in PLC/PRF/5. About 40% of the genes showing amplification or loss showed altered levels of mRNA ($p < 0.05$). Hierarchical clustering analysis showed that the expression of these genes allows differentiation between α-fetoprotein (AFP)-producing and AFP-negative cell lines. cDNA array-CGH is a sensitive method that can be used to detect alterations in genomic copy number in tumor cells. Differences in DNA copy alterations between AFP-producing and AFP-negative cells may lead to differential gene expression and may be related to the phenotype of these cells.

Keywords: Microarray-based comparative genomic hybridization; Hepatocellular carcinoma; α-Fetoprotein

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide [1]. Among the factors implicated in the etiology of HCC are infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), dietary aflatoxin, alcohol consumption, and exposure to chemical carcinogens [2–4]. Alterations in genomic DNA copy number are key genetic events in the development and progression of human cancers, including HCC [5–7].

Comparative genomic hybridization (CGH) is a highly specific molecular cytogenetic approach that allows positional identification of gains and losses of DNA sequences throughout the entire genome [8–12]. CGH is based on the use of competitive fluorescence in situ hybridization (FISH) on normal chromosome spreads of differently labeled total genomic DNA from appropriate control and tumor tissue [13]. CGH, however, is unable to detect DNA copy number changes within narrow regions of chromosomes, and alterations of <1 Mb are difficult to detect [14].

Microarray-based CGH (array-CGH) was developed to detect genome-wide alterations in tumor samples by Pinkel et al. using cDNA microarray slides [14–16]. This technique has enabled rapid surveys of known copy number alterations in tumor samples, but resolution can be hampered when only small regions of the genome are amplified [17,18]. Using cDNA clones instead of BAC or PAC clones as probes, however, would make it possible to directly detect amplification and deletion

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of copy numbers of individual genes. Moreover, using cDNAs arrayed on slides would enable parallel measurements of mRNA levels, which may reveal the degree to which variation in gene copy number contributes to variation in gene expression in tumor cells [19,20]. We previously analyzed the alterations of mRNA expression in hepatoma cell lines using an in-house cDNA microarray [21,22], and we showed that α-fetoprotein (AFP)-producing and AFP-negative hepatoma cell lines had different gene expression profiles [23].

In this study, we assayed chromosomal abnormalities in hepatoma cell lines using the cDNA array-CGH method and investigated the relationship between genomic copy number alterations and mRNA expression profiles in these hepatoma cell lines.

Materials and methods

Cell lines and cells. We used three positive control cell lines: the HL-60 promyelocytic leukemia cell line, the IMR-32 neuroblastoma cell line, and the RCF-26 hepatoma cell line, which contain amplified copy numbers of the c-myc, N-myc, and luciferase genes [24–26]. Five AFP-producing hepatoma cell lines: Huh7, Hep3B, HepG2, PLC/PRF/5, and Huh6; two AFP-negative hepatoma cell lines: SK-Hep1 and HLE; two non-hepatoma cell lines: HeLa and KMBC (bile duct cancer cell derived) were used for gene expression profiling and analysis of genomic copy number alterations. As a reference sample in these microarray experiments, we used peripheral blood mononuclear cells (PBMCs) from healthy volunteers and a SV40-T antigen-immortalized normal human hepatocyte cell line (THLE-5b) [27].

cDNA clones and sequence verification. The cDNA microarrays used in this study were made in collaboration with Hitachi Software Engineering (Yokohama, Japan), using an SPBIO2000 robotic arraying machine. The cDNA clones used for making microarrays were selected from UniGene cluster (http://www.ncbi.nlm.nih.gov/UniGene/), and most of them were obtained from the IMAGE Consortium libraries (http://image.llnl.gov/) through Research Genetics (Huntsville, AL). Each microarray contained a total of 1080 cDNA clones, consisting of 930 unique sequence-verified clones and four housekeeping genes. The 930 clones included 141 apoptosis-related genes, 99 cell cycle-related genes, 154 cell–cell interaction-related genes, 198 cytokine and growth factor genes, 123 oncogenes, 81 transcription factor genes, 26 DNA repair-related genes, 93 stress response-related genes, and 87 hematology-related genes. Polymerase chain reaction (PCR) products from the clones were prepared and printed onto glass slides as previously described [28,29].

Copy number analyses by cDNA microarrays. CGH experiments on cDNA microarrays were performed using a modification of a previously described procedure [30]. Nuclei were isolated from cells and dissolved in "hypotonic buffer," consisting of 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dichlorodiphenyltrichloroethane-1,1,1–trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Following digestion in proteinase K in the presence of SDS, genomic DNA was isolated by phenol–chloroform extraction. From each sample, 20 μg of genomic DNA was sonicated for 10 min and digested for 32 h with *Dpn*II (New England Biolabs, Beverly, MA), followed by phenol–chloroform extraction. Each digested hepatoma cell line or positive control DNA was labeled with Cy5-dUTP (Amersham-Pharmacia Biotech, Uppsala, Sweden), and each normal DNA was labeled with Cy3-dUTP (Amersham-Pharmacia) using a Bioprime Labeling Kit (Invitrogen, Carlsbad, CA). Briefly, digested sample DNA was mixed with 15 μg random octamers in a total volume of 41 μl reaction buffer, heated at 100 °C for 5 min, and chilled on ice. Five microliters of 10× dNTPs (i.e., 1.2 mM each of dATP, dGTP, and dCTP, and 0.6 mM dTTP), 3 μl Cy5-dUTP or Cy3-dUTP, and 1 μl Klenow fragment were added to the DNA and then incubated at 37 °C for 2 h. Reactions were stopped by adding 5 μl of 0.5 M EDTA, pH 8.0, and labeled probes were purified on a Microcon 30 column (Millipore, Bedford, MA). Thirty micrograms of human Cot-1 DNA (Invitrogen), 100 μg yeast tRNA (Sigma–Aldrich, St. Louis, MO), and 20 μg poly(A) (Sigma–Aldrich) were added as blocking reagents and then the samples were concentrated to 12 μl. 2.55 μl of 20× SSC and 0.45 μl of 10% SDS were added, and each 15 μl sample was heated at 100 °C for 90 s and then used as a hybridization probe for the DNA-spotted slides. The slides were covered with glass coverslips (22 mm × 22 mm), fixed in a Hybridization Cassette (TeleChem, Sunnyvale, CA), and hybridization was performed at 70 °C for 12 h. The slides were washed in 2× SSC, 0.03% SDS at 70 °C for 5 min, 1× SSC at 70 °C for 5 min, and 0.2× SSC at room temperature for 5 min.

Expression analyses by cDNA microarrays. Total RNA was isolated using a ToTally RNA Kit (Ambion, Austin, TX), and mRNA was isolated from total RNA samples using a MicroPoly(A)Pure Kit (Ambion), according to the manufacturer's instructions. RNA prepared from THLE-5b cells was used as a reference for all cDNA microarray analyses. Fluorescently labeled cDNA probes were made from 2 μg aliquots of mRNA by reverse transcription using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). Labeling and hybridization were performed as previously described [23].

Imaging and analyses. Fluorescence intensities generated by the Cy5 and Cy3 probes immobilized on the microarray slides were measured by a laser confocal microscope equipped with a scanning system (ScanArray 5000, GSI Lumonics, Billerica, MA) with appropriate excitation and emission filters. The fluorescence images for Cy5 and Cy3 were scanned separately and stored for image analysis using ImaGene Ver. 3.0 Software (BioDiscovery, Marina del Rey, CA). The signal intensity of each spot was corrected by subtracting background signals in the immediate vicinity, and each Cy5/Cy3 ratio was calculated using global normalization. Clones with a copy number ratio >1.80 were considered to be amplified and those with a ratio <0.55 were considered to be deleted. Over- and underexpression of mRNAs used the same ratios.

Southern hybridization. Genomic DNAs from each cell line and PBMCs were digested overnight with the appropriate restriction enzyme, electrophoresed in 0.8% agarose gels, transferred onto Hybond-N^+ nylon membranes (Amersham-Pharmacia), and hybridized with a ^{32}P-labeled probe previously shown to be amplified or deleted in our array-CGH experiments, according to the standard method [31,32].

Statistical analysis. BRB-Array Tools Ver. 3.1.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html) were used to compare gene amplification in AFP-producing and AFP-negative hepatoma cell lines. Genes showing significant differences were determined by univariate significance test, with a threshold of $p < 0.05$, using a randomized variance model. Hierarchical clustering was performed using centered genes and correlations were determined using average linkage analysis.

Results

Establishment of a sensitive CGH analysis of cDNA arrays

To evaluate the reliability of our CGH method using cDNA microarray slides, we first assayed our positive control cell lines, IMR-32, HL60, and RCF-26, which are known to have amplified copies of c-myc (HL60), N-myc (IMR-32), or luciferase (RCF-26) [24–26]. Using

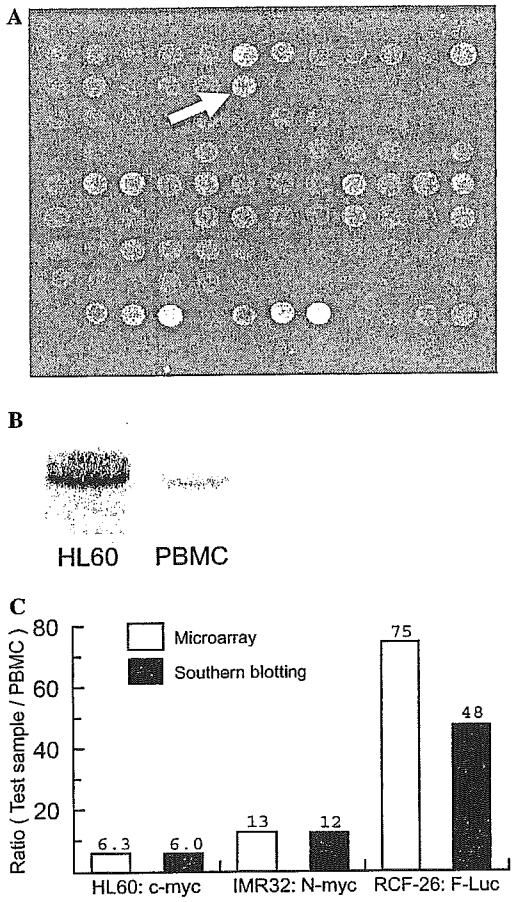

Fig. 1. Evaluation of the reliability of the cDNA microarray-based CGH method by comparison with Southern blotting. (A) Portion of typical array patterns of in-house microarrays (red, HL60; green, PBMC). The spot located by the white arrow indicates the c-myc gene. (B) Southern blot analysis of HL60 and PBMC DNA hybridized with c-myc probes. (C) DNA copy number of c-myc in HL60 cells, N-myc in IMR-32 cells, and the luciferase gene in RCF-26 cells determined by cDNA microarray and by Southern blotting. *Abbreviation:* F-Luc, firefly luciferase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

our method, we found that each of these genes was significantly amplified in their respective cell lines and that the estimated copy number determined by our cDNA array-CGH was well correlated with that from Southern blotting analysis (Fig. 1). Our cDNA array-CGH method also found that the DNA copy number of the p53 gene in HL60 cells was about half that in PBMCs (data not shown), in agreement with previously reported results [33].

Amplified and/or deleted genes detected by cDNA array-CGH

A typical scatter plot of intensities generated by Cy5 or Cy3 immobilized at the target sequence on the microarray slides is shown in Fig. 2. Using a fold-change of 1.8 or higher as the filtering criteria, we selected those genes with a >95% probability of being changed differentially. The seven hepatoma cell lines tested differed with respect to the number and identity of genes amplified or deleted (Table 1). As expected, we found that the HBV copy number was increased in the Hep3B and PLC/PRF/5 cell lines [1,34]. Although few genes were commonly amplified or deleted among the seven hepatoma cell lines, there were common biological characteristics among amplified or deleted genes in the individual cell lines. In the Hep3B and HepG2 cell lines, apoptosis-related gene copy numbers [e.g., glutathione-*S*-transferase T1 (GST T1), fas-associated via death domain (FADD), defender against apoptotic cell death (DAD1), and mammalian inhibitors of apoptosis homolog B (MIHB)] changed, whereas in the HLE cell line, cell adhesion and receptor molecule encoding gene copy numbers (e.g., integrin, glutamate receptor, and endothelin receptor) changed, and, in the PLC/PRF/5 cell line, the cytokine-related gene copy numbers (e.g., small inducible cytokine A2, interferon-α, and interferon regulatory factor 2) changed. Differences observed in amplified and deleted genes in these cell lines may reflect differences in their oncogenetic pathways and tumor phenotypes.

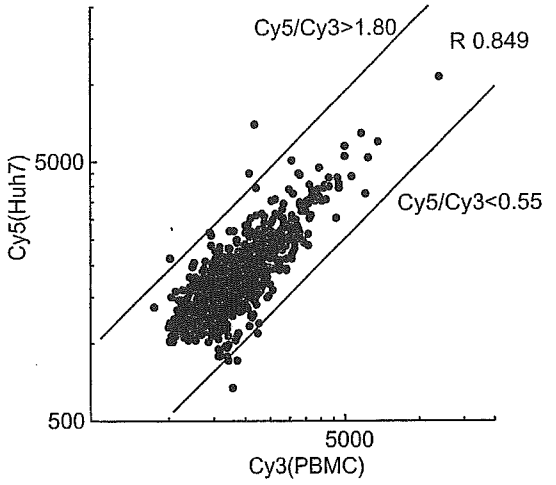

Fig. 2. Log_{10} (intensity of Cy5) and log_{10} (intensity of Cy3) of Huh7 cells are graphed on a scatter plot, and their linear relationship was examined statistically.

Southern blotting data

The changes in DNA copy number determined by our cDNA array-CGH were reevaluated by Southern blotting analysis (Fig. 3). In agreement with array-CGH results, we observed amplification of the HBV genome in the Hep3B and PLC/PRF/5 cell lines, and amplification of cyclin-dependent kinase 3 in Huh7 cells

Table 1
Genes amplified or deleted in seven hepatoma cell lines

Cell line	Gene name	Cy5/Cy3 (array-CGH)	Cy5/Cy3 (expression profiles)	Function	GenBank	Chromosome location
Huh7						
>1.80	RNA-binding motif protein 4 (RBM4)	3.15 ± 0.70	1.60	RNA-binding	NM_002896	11q13
	Cyclin-dependent kinase 3	2.19 ± 0.23	7.75	Cell cycle	NM_001258	17q22-qter
	Alpha platelet-derived growth factor receptor precursor	1.83 ± 0.40	1.77	Cell receptor	NM_006206	4q11-q13
<0.55	Inhibin, alpha	0.49 ± 0.05	1.28	Growth factor	NM_002191	2q33-q36
	Granzyme A	0.54 ± 0.11	0.49	Immune response	NM_006144	5q11-q12
Hep3B						
>1.80	Glutathione-*S*-transferase T1	8.91 ± 8.74	3.32	Apoptosis	NM_000853	22q11.23
	HBV-P	6.84 ± 7.16		Virus genome		
	HBV-full	3.60 ± 1.61		Virus genome		
	Homo sapiens CGI-137 protein	2.40 ± 1.66	1.46	Transcription factor	NM_003187	5q11.2-q13.1
	Fas-associated via death domain (FADD)	2.23 ± 0.39	1.54	Apoptosis	NM_003824	11q13.3
	H. sapiens PAC clone DJ0855D21	2.02 ± 0.90	1.48	PAC clone	AC004908	Unknown
	BCL-2 homologous antagonist/killer (BAK) protein	2.02 ± 1.37	1.81	Apoptosis	NM_001188	6p21.3
	Immunoglobulin mu-binding protein 2	1.91 ± 0.37	2.2	DNA-binding	NM_002180	11q13.2-q13.4
	Discs, large (*Drosophila*) homolog 1	1.88 ± 1.32	2.03	Guanylate kinase	NM_004087	3q29
	RNA-binding motif protein 4 (RBM4)	1.83 ± 0.04	4.02	RNA-binding	NM_006144	11q13
<0.55	Burkitt lymphoma receptor 1, GTP-binding protein	0.53 ± 0.18	2.27	Cell receptor	NM_032966	11q23.3
HepG2						
>1.80	Glutathione-*S*-transferase T1	2.70 ± 0.57	2.16	Apoptosis	NM_000853	22q11.23
	Defender against cell death (DAD1)	2.31 ± 0.27	1.46	Apoptosis inhibitor	NM_001344	14q11-q12
	Oxidase (cytochrome *c*) assembly 1-like	2.26 ± 0.19	2.66	Electron transport	NM_005015	14q11.2
	Transcriptional regulator ISGF3 gamma subunit	1.99 ± 0.53	11.1	Transcription factor	NM_006084	14q11.2
	Mammalian inhibitor-of-apoptosis homolog B (MIHB)	1.92 ± 0.85	0.59	Apoptosis inhibitor	NM_001166	11q22
	Guanine nucleotide-binding protein (G protein), alpha stimulating activity polypeptide 1	1.85 ± 0.31	1.85	Transcription factor	NM_080425	20q13.2-q13.3
<0.55	Nothing					
SKHep1						
>1.80	*H. sapiens* PAC clone DJ0855D21	2.10 ± 0.27	0.4	PAC clone	AC004908	Unknown
	Decorin	1.83 ± 0.17	1.28	Cell–cell interaction	NM_133503	12q13.2
<0.55	Inhibin, alpha	0.44 ± 0.02	0.34	Growth factor	NM_002191	2q33-q36
	H. sapiens insulin-like growth factor II receptor (IGF2R)	0.53 ± 0.01	0.3	Cell receptor	NM_000876	6q26
HLE						
>1.80	Interleukin enhancer-binding factor 1	2.90 ± 0.21	NA	Cytokine	NM_004514	17q25
	Integrin, beta 4	2.70 ± 0.45	NA	Cell–cell interaction	NM_000213	17q11-qter
	RB130 retinoblastoma-like 2	2.33 ± 0.47	NA	Protein kinase	NM_004203	16p13.11
	Glutamate receptor, ionotropic, AMPA 1	1.93 ± 0.26	0.8	Cell receptor	NM_000827	5q31.1
	E2D2	1.85 ± 0.38	1.95	Protein ligase	NM_003339	5q31.3
<0.55	Human vitamin K-dependent protein Z	0.38 ± 0.11	0.52	Protein-binding	NM_003891	13q34
	Endothelin receptor type B	0.40 ± 0.05	NA	Cell receptor	NM_000115	13q22
PLC/PRF/5						
>1.80	HBV-P	19.3 ± 4.62	NA	Virus genome		
	HBV-full	9.04 ± 5.36	NA	Virus genome		
	H. sapiens PAC clone DJ0855D21	2.81 ± 0.32	1.19	PAC clone	AC004908	Unknown
	Small inducible cytokine A2 (monocyte chemotactic protein 1, homologous to mouse Sig-je)	1.81 ± 0.02	4.05	Cytokine	NM_002982	17q11.2-q21.1
<0.55	Interferon-alpha	0.40 ± 0.10	1.44	Cytokine	NM_000605	9p22
	Interferon regulatory factor 2	0.45 ± 0.05	1.22	Cytokine	NM_002199	4q34.1-q35.1
	H. sapiens transcriptional coactivator p52	0.55 ± 0.29	1.13	Transcription factor	NM_033222	9p22.2
Huh6						
>1.80	Nothing					
<0.55	Nothing					

Notes. Clones showing a copy number ratio >1.80 were considered to be amplified and those with a copy number ratio <0.55 were considered to be deleted. The array-CGH data are expressed as means ± SD. *Abbreviation:* NA, data not available.