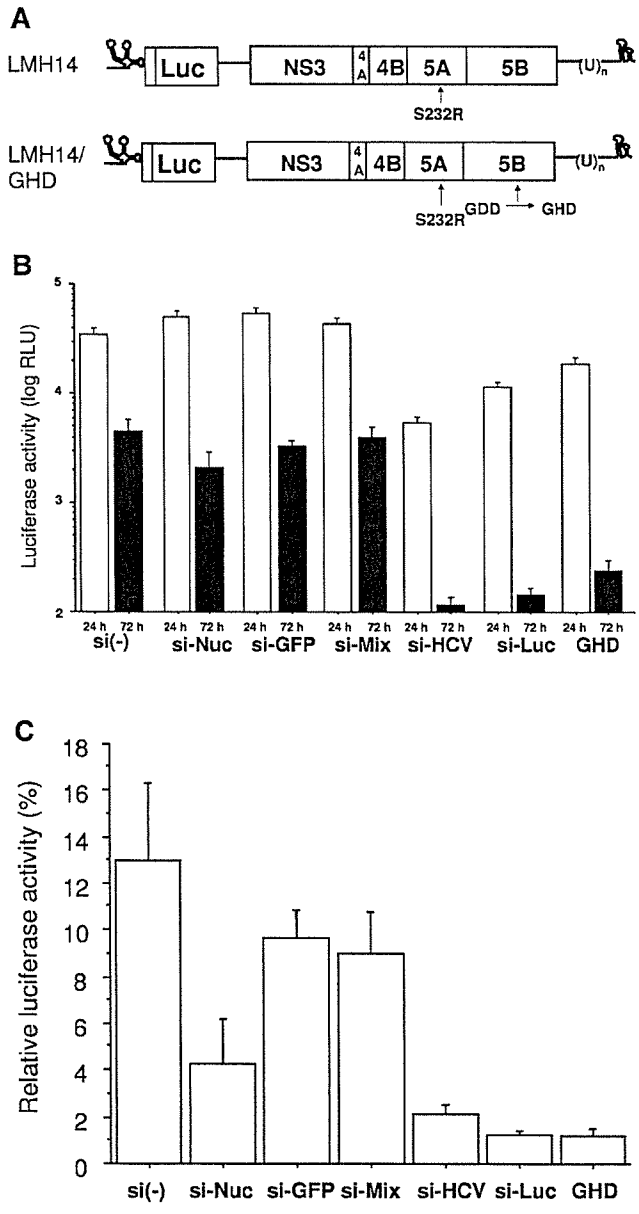

FIG. 5. Effect of suppression of endogenous nucleolin on HCV replication in the LMH14 replicon. (A) Schematic representation of the luciferase replicon. In the LMH14 replicon, the neomycin resistance gene was replaced by a luciferase gene, and S232 of NS5A was replaced by R. In the LMH14/GHD replicon, the NS5B GDD motif in LMH14 was changed to GHD and used as a negative control. (B) Cells were transfected with in vitro-transcribed LHH14 or LMH14/GHD RNA along with 2 μM of si-Mix, si-GFP, si-Nuc, si-Luc, si-HCV, or no siRNA [si(−)] using the DMRIE-C reagent, and luciferase activity (relative light units [RLU]) was measured 24 and 72 h after transfection. Shown are the activities at 24 and 72 h. Error bars indicate the standard deviations of the results from at least three independent experiments. (C) Activity at 24 h was used as an indication of each transfection. Shown are the ratios of activity (percent) at 72 h relative to that at 24 h. Error bars indicate the standard deviations of the results from at least three independent experiments.

To rule out the cytotoxic effects of the suppression of endogenous nucleolin, we transfected pGL3 control, with or without each siRNA, and measured luciferase activity 48 and 72 h after transfection. We found that cotransfection of each siRNA did not inhibit luciferase activity at both 48 and 72 h (Fig. 6), indicating that both suppression of nucleolin and transfection of siRNA did not have detrimental effects on transfected cells.

DISCUSSION

HCV replication has been found to take place in a distinctly altered membrane structure, or membranous web, of the endoplasmic reticulum (11). When HCV NS proteins are coexpressed in stable cell lines harboring replicons, they colocalize to these membrane structures, indicating that they might form a complex (16, 39, 47). These nonstructural proteins, together with host factors, form the viral replicase, the complex in which viral replication is thought to take place. The in vitro level of the RdRp activity of NS5B is low (12), indicating that cofactors, whether viral and/or host proteins and/or the appropriate cellular environment, are necessary for optimal activity of HCV RdRp. HCV NS5B has been reported to interact with NS3, NS4A, NS4B, NS5A, and NS5B itself (9, 48, 57, 65). Using an HCV subgenomic replicon, we previously reported the critical role of the interaction between NS5A and NS5B and the oligomerization of NS5B itself in HCV replication (36, 56). NS3 and NS4B have been shown to be positive and negative regulators, respectively, of NS5B in the replication complex (46).

In addition to interacting with HCV nonstructural proteins, NS5B has been reported to interact with many host proteins, including a SNARE-like protein (62); eIF4AII, an RNA-dependent ATPase/helicase; a component of the translation initiation complex (30), protein kinase C-related kinase 2, which specifically phosphorylates NS5B (27); and p68, a human RNA helicase I (15). The suppression of protein kinase C-related kinase 2 has been reported to reduce the phosphorylation of NS5B and to inhibit HCV RNA replication (27), and the suppression of p68 has been reported to inhibit the synthesis of negative-strand HCV RNA from the positive strand (15).

Several host proteins have been shown to interact with RdRp of other RNA viruses. For example, in poliovirus, an RdRp and an RdRp precursor interact with human Sam68 (38) and heterogeneous nuclear ribonucleoprotein C1/C2 (5), respectively, and modulate RdRp activity directly or indirectly. Bromo mosaic virus RdRp and tobacco mosaic virus RdRp interact with eukaryotic initiation factor 3 and eukaryotic initiation factor 3-related factor, altering RdRp activity (45, 50).

Here and in a previous report, we identified and characterized the interaction between nucleolin and HCV NS5B (20). Nucleolin was originally identified as a common phosphoprotein of growing eukaryotic cells, although its function is not completely understood. Nucleolin is a multifunctional protein that shuttles between the nucleus and cytoplasm. In addition, it is expressed on the surface of various cells, acting as a receptor for various ligands, including lipoproteins (55), cytokines, growth factors (6, 52, 60), the extracellular matrix (10, 18, 25), bacteria (58), and viruses (4, 8, 21, 41–44).

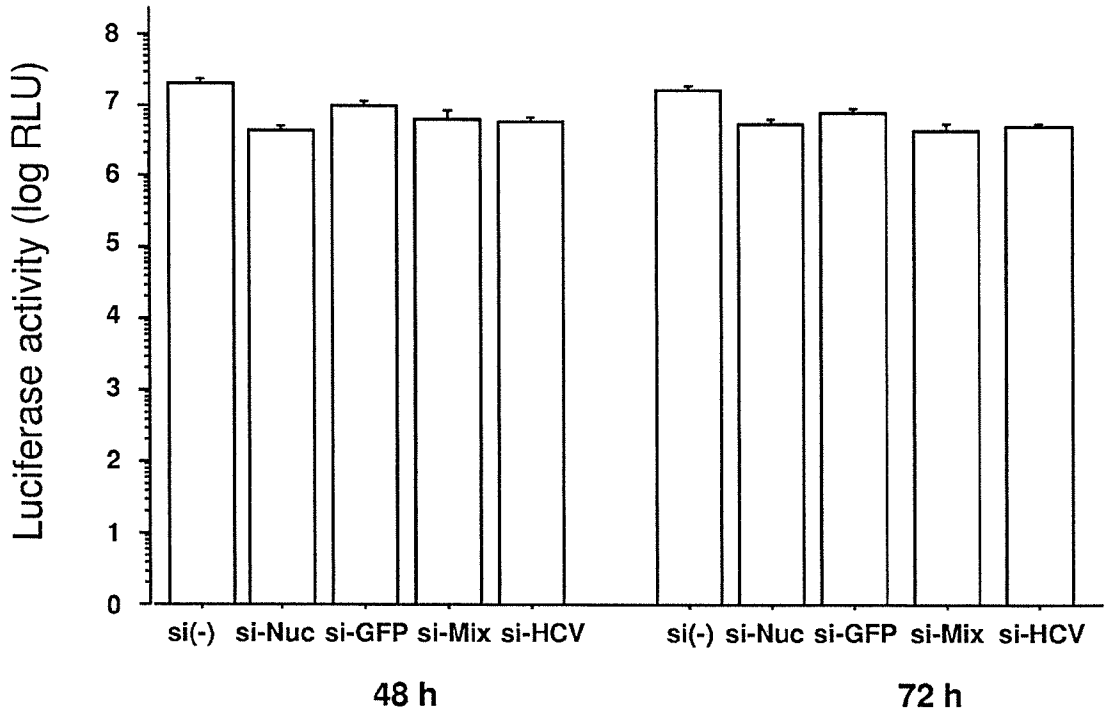

FIG. 6. Effect of suppression of endogenous nucleolin on cell proliferation. The plasmid pGL3 control, encoding the luciferase gene under the control of the CMV promoter/enhancer, was cotransfected with 2 μM of si-Mix, si-GFP, si-Nuc, si-HCV, or no siRNA [si(−)] using DMRIE-C reagent, and luciferase activity was measured 48 and 72 h after transfection. The error bars indicate the standard deviations of the results from at least three independent experiments.

We found that recombinant C-terminal nucleolin proteins can bind NS5B and inhibit its RdRp activity in a dose-dependent manner (20), suggesting that nucleolin may affect HCV replication by interacting with NS5B. The direct interaction of nucleolin with HCV NS5B in vivo and in vitro was shown to require two critical stretches of NS5B. Here, we showed that within one of these regions, aa 208 to 214, the W208 residue was critical for both binding of nucleolin and HCV replication. Transient down-regulation of endogenous nucleolin by siRNA considerably inhibited HCV replication in Huh7 cells. These results strongly indicate that nucleolin has an important role in HCV replication through its direct interaction with NS5B.

Our finding of an important positive role for nucleolin in HCV replication is apparently inconsistent with previous findings of an inhibitory role for nucleolin. It was previously reported that purified C-terminal nucleolin proteins inhibited the RdRp activity of NS5B in vitro. The latter result, however, may have been due to the use of recombinant truncated nucleolin proteins, because recombinant full-length nucleolin was not available (70). Taken together, however, these results indicate that N-terminal nucleolin may be important for the positive function of nucleolin in HCV replication, although the NS5B-binding region is within the RGG domain and RNA-binding domain 4 is at the C terminus.

Transfection of the mutant replicon containing NS5B W208A, which could not bind nucleolin, led to almost no HCV replication. By contrast, the suppression of nucleolin by siRNA moderately inhibited HCV replication, a result also observed with the transient assay using luciferase reporter replicon and G418-resistant colony formation. While HCV replication was completely inhibited by MA/W208A, replication was only partially inhibited by si-Nuc, indicating that si-Nuc can transiently suppress, but cannot eliminate, expression of endogenous nucleolin. Recently, nucleolin was reported to inhibit cell cycle progression after heat shock and genotoxic stress by increasing complex formation with human replication protein A (26). When pGL3 control or pCI-Neo was cotransfected with si-Nuc, the luciferase activity or the number of G418-resistant colonies was not reduced, strongly suggesting that the moderate inhibition of nucleolin expression did not have severe cytotoxic effects on siRNA-transfected cells. More efficient suppression of nucleolin may result in more severe inhibition of HCV RNA replication. It is therefore important to determine whether nucleolin is dispensable in mammalian cells as it is in *Saccharomyces pombe* (17) and *Saccharomyces cerevisiae* (31), since nucleolin may constitute a putative therapeutic target to inhibit HCV replication.

Using a clustered alanine substitution mutant library (CM) of NS5B, we previously showed that two stretches of NS5B amino acids, aa 208 to 214 and 500 to 506, were critical for nucleolin binding. According to the crystal models of NS5B, the former stretch is in the palm and the latter stretch is in the bottom of the thumb domain. We focused on identifying residues in aa 208 to 214 that are essential for nucleolin binding and HCV replication, as the CM mutant of aa 500 to 506 was defective in RdRp activity in vitro and HCV replication in vivo (36, 48, 49). We found that the W208 residue was critical for

both nucleolin binding and HCV replication. This residue is exposed to solvent at the edge of the palm and is not close to the catalytic pocket.

Nucleolin may stabilize monomeric NS5B, making it ready for oligomerization to NS5B, or it may facilitate the formation of a complex between NS5B and template RNA. In both cases, a substoichiometric amount of nucleolin may be required transiently at a step prior to the catalytic RdRp reaction of NS5B. Efforts to determine the contribution of amino acid residues 500 to 508 to nucleolin binding and HCV replication in vivo are ongoing and may reveal further correlations. We found that another mutant replicon, MA/K211A, reduced the number of G418-resistant colonies compared with the wild type and the other mutants. Because K211A of NS5Bt is close to the pocket of catalytic activity and did not affect binding to nucleolin, K211 may contribute to the structural integrity of the pocket or the heat-stable property of RdRp as reported previously (36).

Efficient HCV replication and infection in tissue-cultured cells by using full-length HCV RNA replicons have been reported previously (32, 63, 72). HCV replication occurs in differentiated subcellular fractions and involves dynamic complexes of structural proteins, nonstructural proteins, and HCV RNA demarcated by membrane structures. It is therefore of great interest to determine whether nucleolin is involved in such HCV-replicating intermediates in compartmented subcellular structures.

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Virological effects and safety of combined double filtration plasmapheresis (DFPP) and interferon therapy in patients with chronic hepatitis C: A preliminary study

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Abstract

Purpose: In patients with chronic genotype 1b hepatitis C and a high viral load, the viral load was reduced by double filtration plasmapheresis (DFPP), followed by combined interferon and ribavirin therapy. The safety and virological effects of this treatment method were preliminarily investigated.
Methods: In nine patients with chronic hepatitis C, DFPP was performed three times on days 1, 2, and 4, and the administration of interferon and ribavirin was initiated immediately after DFPP on day 1.
Result: The HCV RNA was undetectable in all patients after the plasma was passed through a plasma fractionator (second filter) in the DFPP circuit. After 2 weeks, the HCV RNA tended to decrease in the DFPP group more than in the control group (-2.45 ± 1.12 versus -1.57 ± 0.95, $P = 0.073$). However, this decrease was not attributable to a sustained virological response (SVR) (22.2% versus 18.2%, $P = 0.822$). Most of the adverse events were caused by the interferon and ribavirin combination therapy.
Conclusion: DFPP can be safely performed concomitantly with interferon and ribavirin combination therapy in chronic hepatitis C patients. The combination may contribute to an early virological response. The effect of DFPP on the SVR and its significance remain to be clarified.

Keywords: Chronic hepatitis C; Interferon therapy; Double filtration plasmapheresis

1. Introduction

Hepatitis C virus (HCV) infection induces acute hepatitis, and approximately 70–80% of these cases progress to chronic hepatitis. The course of the disease is stable in approximately 30% of chronic hepatitis cases; however, the remaining 70% of cases progress to liver cirrhosis after approximately 30–40 years. Further, the cases that progress to liver cirrhosis develop hepatocellular carcinoma at an annual rate of approximately 8% [1].

From the viewpoint of viral eradication, interferon therapy is the only radical therapy for chronic hepatitis C. Once complete viral elimination is achieved by interferon therapy, liver fibrosis improves, and the risk of liver carcinogenesis is reduced [1].

In interferon therapy for chronic hepatitis C, administration of $(3–6) \times 10^6$ IU interferon alone, three times a week for 24 and 48 weeks, has been reported to produce a sustained virological response (SVR) in approximately 6% and 16% of patients, respectively [2]. Treatment with interferon in combination with ribavirin for 24 and 48 weeks has been reported to produce an SVR in 21% [3] and 41% [2,4,5] of patients, respectively.

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A combination of pegylated interferon (PEG-IFN) – a recently marketed long-acting interferon for once-a-week administration – and ribavirin induced an SVR in 47% of patients with chronic genotype 1b HCV infection and high viral loads in whom previous interferon therapy failed to produce a response [6,7].

However, even the combination therapy with PEG-IFN and ribavirin for 48 weeks, which is currently the most promising therapy, does not improve viremia in the other patients for whom there is no appropriate therapy for the eradication of HCV viremia other than time-course observation and the administration of liver-protective drugs. An NS3 protease inhibitor has been developed as a novel antiviral agent, and clinical studies have been performed using this agent; however, the clinical application of this drug will take time because of the occurrence of certain adverse events [8]. The development of an NS5 polymerase inhibitor and an internal ribosome entry site (IRES)-targeting agent is in progress [9–11].

The presence or absence of concomitant ribavirin therapy, the duration of therapy, the virus genotype, the viral load before administration, the grade of liver fibrosis, and the gender and age of the patient are all factors that influence the therapeutic effect of interferon therapy. An early virological response (EVR) is defined as a decline of more than 2 $\log_{10}$ units in the viral load 8 or 12 weeks after the initiation of treatment; it has also been reported to be one of the important factors for an SVR [12,13].

There have been several studies in which the HCV level was investigated in HCV-positive patients during extracorporeal circulation therapies, such as plasmapheresis and hemodialysis. In these studies, the HCV RNA level decreased transiently by approximately 50–90% immediately after plasmapheresis or hemodialysis; however, it returned to either the pre-treatment level or to a higher level within approximately 4–6 h [14–17].

In double filtration plasmapheresis (DFPP), which is a plasmapheresis therapy, the patient's whole blood is separated into plasma and blood cell components by using a plasma separator (first filter). The separated plasma components are further separated into high and low molecular weight components by using a plasma fractionator (second filter); the high molecular weight components including immunoglobulins are removed, and the low molecular weight components including albumin are returned to the body. Although this technique using two filters is relatively more complicated than the normal simple plasma exchange, its advantage is that supplemental plasma transfusion is not necessary.

The diameter of the HCV particle is approximately 55–65 nm [18]. In theory, these viruses are unable to pass through the second filter that has a pore size smaller than the diameter of the viral particle; they are therefore eliminated from the plasma.

In this study, we attempted to reduce the viral loads of patients with chronic genotype 1b hepatitis C and a high viral load by using DFPP. We focused on HCV RNA levels both before plasmapheresis therapy and before interferon and ribavirin combination therapy, and then investigated the early virological effects and also the safety of the treatment.

2. Experimental/materials and methods

2.1. Patients

The study involved nine patients (four males and five females, mean age: 51.7 ± 11.3 years) with chronic genotype 1b hepatitis C that was histologically diagnosed at our department between December 2002 and July 2004. In these patients, the HCV RNA level determined by reverse transcriptase-polymerase chain reaction (RT-PCR) was not less than 100 KIU/ml.

The inclusion criteria were as follows. (1) Minimum age: 20 years; maximum age: 70 years. (2) Blood test values before therapy: hemoglobin, 12 g/dl or higher; platelet count, 100,000 mm^{-3} or higher; white blood cell count, 3000 mm^{-3} or higher; neutrophil count, 1500 mm^{-3} or higher.

The exclusion criteria were as follows. (1) Pregnancy or possible pregnancy, and lactation; (2) depression; (3) serious complications, particularly uncontrollable hypertension and impaired function of the bone marrow, kidneys, or the lungs; (4) autoimmune diseases or suspicion of the same; (5) diabetes or suspicion of the same; (6) allergic predispositions; (7) history of hypersensitivity to interferon or nucleic acid analogues; (8) history of hypersensitivity to biological products such as vaccines; (9) suspicion of alcoholic liver injury, autoimmune hepatitis, or drug-induced liver injury; (10) multiple infections with hepatitis B virus within 48 weeks before the initiation of therapy or suspicion of the same; (11) previous hepatic encephalopathy, rupture of the esophageal varix, orascites; (12) complications of hepatic cirrhosis or hepatocellular carcinoma on examination within 4 weeks before the initiation of therapy or ongoing treatment for the same; (13) treatment with drugs having antiviral actions, immunoregulatory actions, or bone marrow-inhibiting actions such as interferon, Ara-A, zidopudine, glucocorticoid, interleukin 2, or Shosaikoto within 12 weeks of the initiation of therapy or administration of injections containing glycyrrhizin as the main ingredient, theophylline, antipyrine, or warfarin within 4 weeks before therapy.

2.2. Study design

This study was conducted in accordance with the Good Clinical Practice guidelines, conforming to the Helsinki Declaration. It was approved by the Ethics Committee of Kanazawa University Clinical Study Center, and written informed consent was obtained from the patients before their participation in the study.

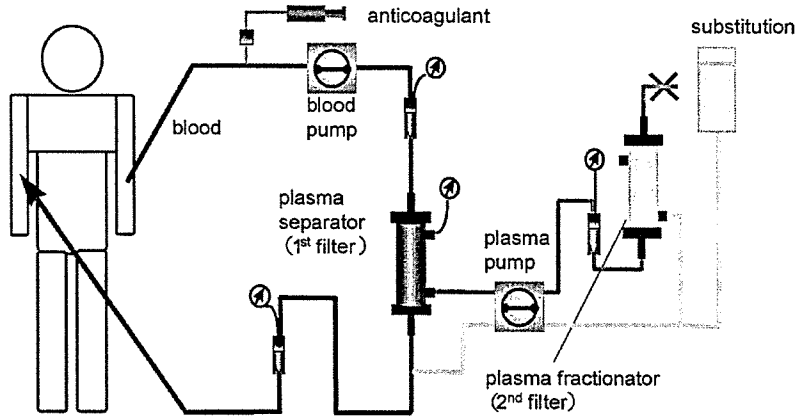

Fig. 1. Mechanism of double filtration plasmapheresis (DFPP). The blood is separated into plasma and blood corpuscles by the plasma separator (first filter), and then filtered using a plasma fractionator (second filter) which separates the plasma into low- and high-molecular weight components.

2.2.1. Treatment schedule

DFPP was performed on day 1 of the therapy to decrease the viral load, and the administration of interferon and ribavirin was initiated 1 h after the completion of DFPP. DFPP was performed three times, on days 1, 2, and 4, and a blood test was performed before each treatment to determine the efficacy of the treatment.

2.2.2. Double filtration plasmapheresis

In order to access the blood during DFPP, a Soft-Cell double-lumen catheter (GamCath catheter N®; Gambro, Stockholm, Sweden) was inserted and indwelled in the right femoral vein for 5 days.

For DFPP, a Plasmaflo KM8800 (Kuraray Medical Inc., Tokyo, Japan) was used as the dialysis apparatus. The plasma separator and plasma fractionator (first and second filters, respectively) used were Plasmaflo OP-08W® and Cascadeflo EC-50W®, respectively (Asahi Kasei Medical Co. Ltd., Tokyo, Japan) (Fig. 1). With regard to the frequency of treatment, in a previous study it was observed that the level of fibrinogen decreased to less than 100 mg/dl when DFPP was continuously performed twice. Thus, DFPP was discontinued on day 3 to prevent fibrinogen-associated complications, such as a tendency to bleed, and the applicability of DFPP on day 4 was determined based on laboratory test results.

In order to process 50 ml/kg of blood in a single round of DFPP, DFPP was performed for approximately 3 h at a blood flow rate of approximately 80 ml/min. The potent protease inhibitor, Nafamostat mesilate (Naotamin®; Asahi Kasei Pharma Co. Ltd., Tokyo, Japan), was used as an anticoagulant because heparin is considered to influence RT-PCR when this procedure is used for HCV RNA measurement. For fluid replacement, we used either 50 or 100 ml of 25% albumin (Kenketsu Albumin-Wf®; Mitsubishi Pharma Co., Tokyo, Japan) that was diluted with 200 ml of saline.

Blood tests were performed before each DFPP to ensure that the plasmapheresis could be performed without the occurrence of any adverse events; DFPP was not performed if the platelet count was 50,000 mm^{-3} or less, or if the fibrinogen level was 100 mg/dl or less. The DFPP was resumed after the recovery of these test values was confirmed.

2.2.3. Interferon therapy

For the interferon therapy, interferon (IFN) α-2b (Intron A®; Schering-Plough KK, Kenilworth, NJ) and ribavirin (Rebetol®; Schering-Plough KK) were concomitantly administered. IFN α-2b was administered intramuscularly 1 h after the completion of DFPP on day 1; the oral administration of ribavirin was initiated after the completion of DFPP on day 1. IFN α-2b was administered six times a week for 2 weeks at a daily dose of 6×10^6 IU, followed by three times a week for 22 weeks or three times a week intermittently for 46 weeks. The ribavirin dose was determined based on the body weight measured at the time of patient registration. The dose used was 600 and 800 mg for body weights of less than 60 and 60 kg or higher, respectively. The daily dose was divided into two doses and administered orally for 24 weeks.

2.2.4. Evaluation

The HCV RNA was measured during and after therapy by using an RT-PCR assay (Amplicor HCV RNA Monitor®; BML, Tokyo, Japan: measurement sensitivity, 0.5 KIU or higher); the sample was diluted when the HCV RNA level was higher than the upper quantification limit (850 KIU/ml). When the HCV RNA was less than the lower quantification limit, a qualitative method was used (Amplicor HCV RNA®, BML; measurement sensitivity, 50 IU).

The HCV RNA was measured after 2, 4, 8, and 12 weeks of therapy, either before DFPP or every morning on days 1–6. It was measured after 24 and 48 weeks of therapy as well as at 24 weeks after the completion of the interferon therapy. Whenever possible, the HCV RNA was also measured immediately after DFPP completion. The HCV RNA in the plasma before and after the second filtration in the DFPP circulation was also measured 1 h after the initiation of the DFPP and immediately before the completion of the DFPP. A negative

viral detection at 24 weeks after the interferon administration was defined as an SVR.

As a control group for a comparison of the decrease in HCV RNA during the 2-week therapy period, we used the HCV RNA data of 11 patients with chronic genotype 1b hepatitis C and a high viral load who underwent IFN and ribavirin combination therapy without DFPP at our department during the same period.

In all patients, a liver biopsy was performed immediately before therapy, and fibrosis and inflammation were evaluated based on the New Inuyama classification. The inflammatory activity in the portal vein and the periportal area as well as the degrees of intralobular inflammation and hepatocyte degeneration were graded from A0 to A3 (0: none, 1: mild, 2: moderate, and 3: severe) based on the "degree of inflammatory activity". Fibrosis was also graded from F0 to F4 (0: none, 1: mild without septa, 2: moderate with few septa, 3: numerous septa without cirrhosis, and 4: cirrhosis) [19].

For the blood tests, we performed white blood cell, red blood cell, platelet, and differential leukocyte (neutrophils, eosinophils, basophils, lymphocytes, and monocytes) counts as well as the zinc sulfate turbidity test (ZTT). Further, the percentage of hemoglobin, hematocrit, and reticulocytes as well as the levels of fibrinogen, total protein, albumin, γ-globulin, and total cholesterol were all measured.

Adverse events were evaluated in accordance with the WHO guidelines, and these were classified into mild, moderate, severe, and life-threatening events.

The significance of the differences was analyzed using the χ^2-test, Fisher's exact test, a *t*-test, and logistic regression analysis.

3. Results

3.1. Patients' backgrounds

The patients' backgrounds are listed in Table 1. In the DFPP group, there were four male and five female patients with a mean age of 51.7 ± 11.3 years. The HCV RNA before therapy was at least 100 KIU/ml and less than 500 KIU/ml in three patients and 500 KIU/ml or higher in six patients, indicating that many of the patients had a high viral load. Three patients had previously undergone IFN therapy, which was virologically ineffective. For the remaining six patients, this was their first experience of IFN therapy. With regard to liver histology, the fibrosis was graded as F2 or lower in all patients, and in all patients there was no evidence of advanced chronic hepatitis. Compared with the control group, gender, age, serum HCV RNA, previous IFN treatment, liver histology, and blood biochemical data for the treatment group were statistically not significantly different.

3.2. Virological changes after DFPP

To confirm the elimination of the virus by DFPP, HCV RNA was measured before and after the second filtration. One hour after the initiation of DFPP, once the DFPP exchange blood flow had stabilized, the HCV RNA in the blood after it had passed through the second filter was quantitatively undetectable in all nine patients. This confirmed the elimination of the virus by the second filter (Fig. 2). Further, at the completion of DFPP – approximately 3 h after its initiation – the HCV RNA was undetectable in all nine patients (data not shown). These findings confirmed that DFPP is capable of

Table 1
Baseline characteristics of chronic genotype 1b hepatitis C patients with a high viral load, treated with a combination of DFPP with interferon (IFN) and ribavirin therapy (DFPP group) and IFN and ribavirin therapy without DFPP (control group)

	DFPP + IFN-R	IFN-R	*P*-value
Gender (M/F)	4/5	7/4	0.684
Age	51.7 ± 11.3	50.6 ± 10.6	0.856
Serum HCV RNA (KIU/ml)			
Mean (minimum–maximum)	2162 (224–12000)	818 (340–1700)	0.254
100–500/500≦	3/6	3/8	
Previous IFN treatment			
Naïve/retreatment	6/3	5/6	0.343
Liver histology			
Stage (F0/F1/F2/F3/F4)	1/1/7/0/0	0/5/6/0/0	0.167
Grade (A0/A1/A2/A3)	0/5/3/1	1/7/3/0	0.541
ALT (IU/ml)	88.4 ± 47.7	89.5 ± 34.5	0.957
Hemoglobin (g/dl)	14.0 ± 1.7	14.2 ± 1.2	0.728
Platelet count ($\times 10^4\ \mu l^{-1}$)	16.9 ± 5.2	15.7 ± 3.4	0.551
Fibrinogen (mg/dl)	231.0 ± 38.9	222.7 ± 92.3	0.804
ZTT (IU)	12.4 ± 4.9	14.2 ± 6.4	0.496
Total protein (mg/dl)	6.9 ± 0.4	6.9 ± 0.4	>0.999
Albumin (g/dl)	4.2 ± 0.3	4.1 ± 0.4	0.373
γ-Globulin (g/dl)	1.3 ± 0.2	1.4 ± 0.3	0.492
Total cholesterol (mg/dl)	178.6 ± 28.3	172.0 ± 45.2	0.714

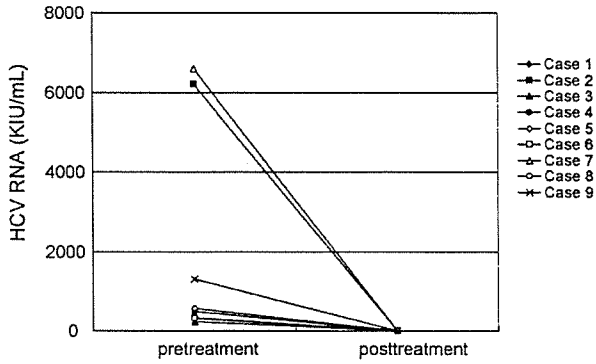

Fig. 2. Change in plasma HCV RNA pre- and post-plasma fractionator (second filter). In all nine cases, HCV RNA was not detected in the plasma of the post-plasma fractionator filtrate 1 h after starting the DFPP.

eliminating the HCV particles and that the elimination efficiency does not decrease with time.

3.3. Early virological response

In order to investigate the EVR, the HCV RNA was measured 2 weeks after the initiation of therapy. It was quantitatively undetectable in four of the nine patients (44.4%). In the control group treated with IFN and ribavirin combination therapy without DFPP, the HCV RNA was quantitatively undetectable in two patients (18.2%). The number of patients with undetectable HCV RNA 2 weeks after the initiation of therapy was higher in the DFPP group, but the difference was not significant ($P = 0.201$).

The EVR is defined as a viral load decline of 2 $\log_{10}$ units or more from the baseline level at an early stage in the therapy, i.e., 2 weeks after the initiation of the therapy in the case of this study. An EVR was achieved in 6 of the 9 patients in the DFPP group (66.7%) and in 4 of the 11 patients in the control group (36.4%), indicating that the HCV RNA tended to decrease earlier during concomitant DFPP and interferon therapy; however, the difference was not statistically significant ($P = 0.178$, Fig. 3A).

The change in HCV viral load also tended to decline more in the DFPP group than in the control group (-2.45 ± 1.12 versus -1.57 ± 0.95; $P = 0.073$, Fig. 3B).

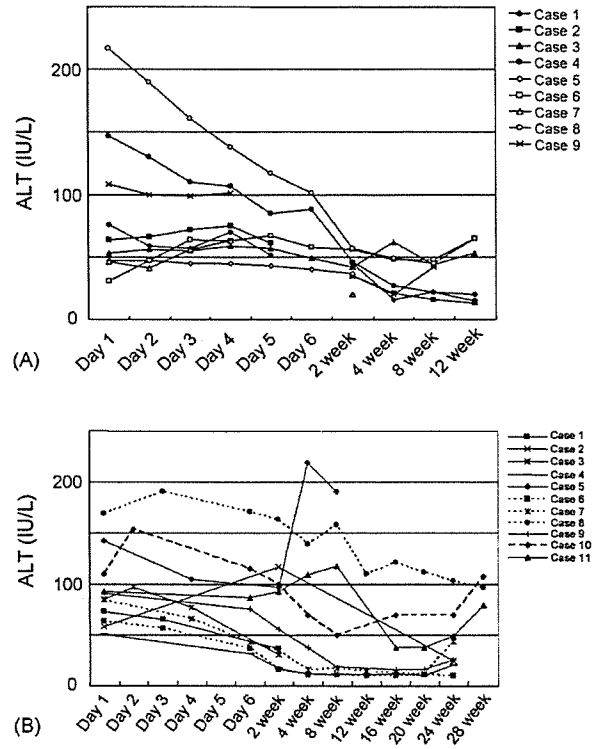

Fig. 4. Change in the serum ALT level during treatment. The ALT level was normalized in eight cases in the second week of treatment.

3.4. Biochemical response

The time-course changes in alanine aminotransferase (ALT) are shown in Fig. 4. The ALT level was normalized in seven of the nine patients (77.8%) by 4 weeks of the therapy. In the control group, it was normalized in 7 of the 11 patients (63.6%). The difference was not statistically significant ($P = 0.845$).

3.5. Sustained virological response

An SVR was observed in two of the nine patients (22.2%) treated with concomitant DFPP and interferon therapy (an intent-to-treat approach). Of these, one patient received IFN

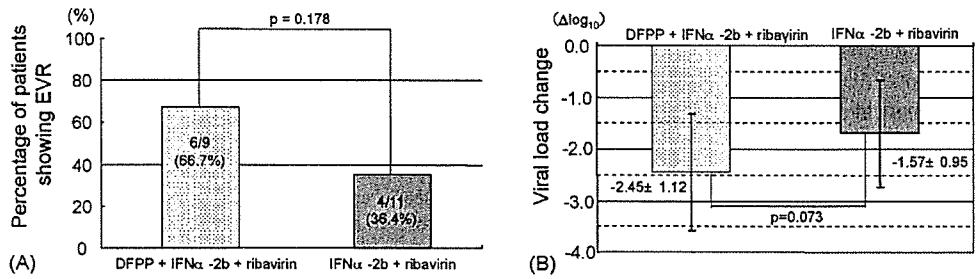

Fig. 3. Change of HCV RNA load 2 weeks after treatment. (A) An EVR is defined as a viral load decline of 2 $\log_{10}$ units or more from the baseline level after 2 weeks of treatment. The numbers in each column indicate the ratio of EVR cases/all treatment cases. (B) Viral load change after 2 weeks. Viral load change was calculated by the formula; $\log_{10}$ (HCV RNA load after 2 weeks/HCV RNA load at pre-treatment).

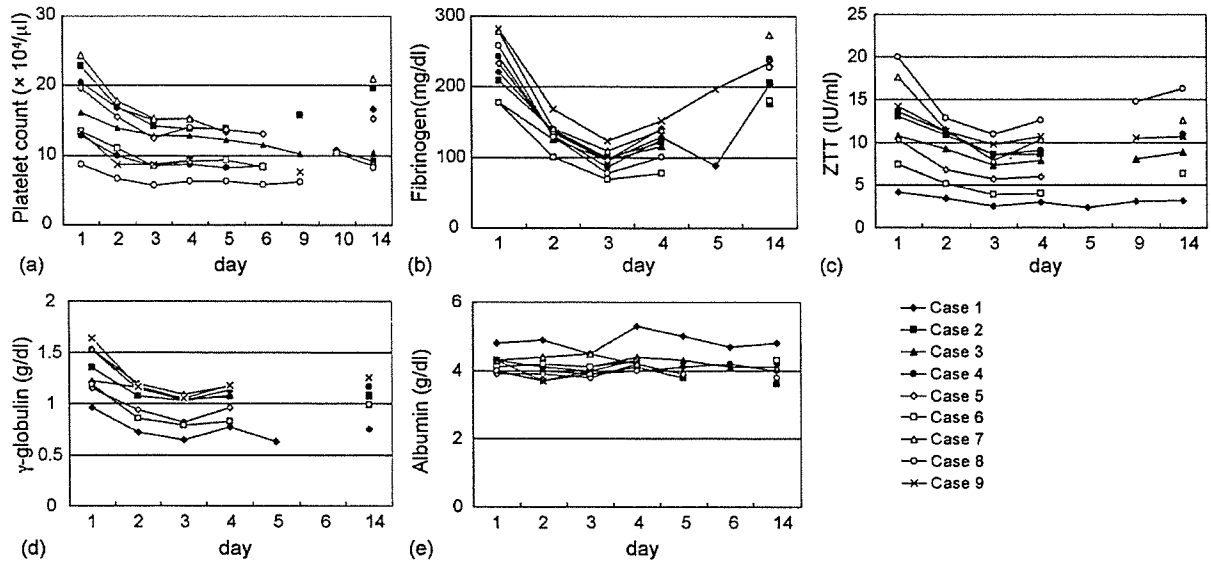

Fig. 5. Changes in the laboratory findings during treatment: (a) platelet count, (b) fibrinogen, (c) ZTT, (d) γ-globulin, and (e) albumin.

and ribavirin combination therapy for 24 weeks, and the other received IFN and ribavirin combination therapy for 24 weeks, followed by additional IFN monotherapy for 24 weeks. These patients were two of the four patients in whom the HCV RNA was quantitatively undetectable after 2 weeks of therapy. Treatment of the other two patients was discontinued before 24 weeks due to adverse events (skin eruption, anorexia, and general malaise). In the six patients for whom complete treatment was possible, the SVR rate was 33.3% (2/6) (a per-protocol approach).

In the control group, the SVR rate was 18. About 2% (2/11) as determined using an intent-to-treat approach and 20.0% (2/10) as determined using a per-protocol approach. The difference in the SVR rate between the two groups was not statistically significant ($P = 0.822$ and 0.551, respectively).

The factors associated with the SVR in our patients were analyzed using uni- and multi-variate analysis. Using univariate analysis, the EVR was found to be the only factor associated with SVR ($P = 0.025$). Associations with other factors, such as DFPP, age, sex, pre-treatment history, and fibrosis, were not detected in our series ($P = 0.822$, 0.170, 0.822, 0.822, and 0.052, respectively). Using multivariate analysis, the association of all of these factors was found to be statistically insignificant.

In the DFPP group, the only factor showing a relationship with the SVR was the viral negativity at 2 weeks ($P = 0.014$).

3.6. Safety and adverse events

In eight of the patients, DFPP was performed three times on days 1, 2, and 4. It was not performed on day 4 in one patient because this patient's fibrinogen level was lower than 100 mg/dl before DFPP; hence, DFPP was performed only twice in this patient.

The changes in laboratory test values as a result of DFPP treatment were also investigated. After DFPP on day 1, the platelet count decreased on average by 22.5%. The decrease persisted until the completion of DFPP and then slowly recovered by 2 weeks after the initiation of the therapy. The fibrinogen level decreased by 30–50% after DFPP on day 1. It decreased to its lowest level on day 3, slowly recovered after the completion of DFPP, and then returned to a level similar to the pre-DFPP level by 2 weeks after the initiation of the therapy. The ZTT values and the γ-globulin and total cholesterol levels also showed similar changes. No change was observed in the serum albumin level (Fig. 5).

The adverse events that occurred during the IFN and DFPP combination therapy are listed in Table 2. Most of these were influenza-like symptoms and digestive symptoms that were associated with the IFN and ribavirin combination therapy. The adverse events attributable to concomitant DFPP and IFN therapy were mild hypotension in two patients and mild

Table 2
Rates of discontinuation of treatment, dose reduction, and occurrence of adverse events during treatment

Adverse events	*n* (%)
Influenza-like symptoms	8 (89)
Skin eruption	1 (11)
Gastrointestinal symptoms	6 (67)
Insomnia	2 (22)
Hypotension	2 (22)
Vagal reflux	1 (11)
Depression	5 (56)
Anemia	7 (78)
Neutropenia	1 (11)
Thrombocytopenia	1 (11)
Dose reduction	4 (44)
Discontinuation of treatment	3 (33)

transient vagal reflex in one patient. These events occurred during DFPP, and fluid drip injection resulted in rapid recovery from these symptoms. A blood test revealed decreases in the hemoglobin as well as in the neutrophil and platelet counts; however, these were observed during IFN therapy, suggesting that they were unrelated to the DFPP. The ribavirin dose was reduced in three patients due to anorexia, anemia, and skin eruption. The IFN dose was reduced in one patient due to neutropenia. It was difficult to continue the IFN and ribavirin combination therapy in three patients due to skin eruption, anorexia, and systemic malaise; therefore, in these patients the therapy was discontinued.

4. Discussion

Currently, the most promising therapy for chronic hepatitis C is a 48-week PEG-IFN and ribavirin combination therapy. Using this approach, the SVR is increased in approximately 50% of patients [20]. However, in the other half, HCV viremia persists and, although ALT is stabilized, hepatitis activity may again increase several years later. Alternatively, chronic hepatitis persists without the normalization of ALT and may progress to liver cirrhosis resulting in hepatocellular carcinoma. To prevent these events from occurring, it is necessary to develop other novel ideas or novel drugs for therapy. With regard to ideas for therapy, long-term IFN therapy extended over 48 weeks, the use of intravenously administrable IFN-β, and concomitant IFN and high-dose ribavirin therapy with simultaneous monitoring of the blood ribavirin concentration, have been investigated [21]. With regard to novel drugs, amantadine, IL-12, and thymosin-α1 are concomitantly used with IFN therapy or with IFN and ribavirin combination therapy [22–24]. With regard to novel antiviral agents, the development of an antisense complex targeting the viral IRES, a serine protease inhibitor targeting NS3 protease, and a polymerase inhibitor targeting NS5B have been investigated [8–11].

Previous reports have described the various factors that affect the SVR produced by IFN therapy for chronic hepatitis. The patient-related factors include gender, age, the presence or absence of concomitant ribavirin treatment, and the stage of liver fibrosis; the viral factors include the viral genotype, HCV RNA level before therapy, and the number of mutations in the NS5A interferon sensitivity-determining region (ISDR) of genotype 1b [25]. Recently, an EVR has been recognized as an important factor for achieving an SVR using IFN therapy. An EVR is defined as virus elimination at an early stage after the initiation of treatment. An EVR in 2 weeks with IFN monotherapy and 12 weeks with IFNα-2b and ribavirin combination therapy has been shown to be frequent in SVR cases, and the probability of achieving an SVR is high when the virus is eliminated from the circulation within these early stages [12,13].

In this study, we focused on the HCV RNA level before therapy and attempted to reduce the viral load of chronic genotype 1b hepatitis C patients, with a high viral load of 100 KIU/ml or higher, by performing plasmapheresis therapy, DFPP, before the initiation of IFN therapy. Thus far, this is the first attempt to study the efficacy of a combination of IFN therapy and plasmapheresis with chronic hepatitis C patients.

In previous studies, Manzin et al. measured the HCV RNA in chronic hepatitis C patients with cryoglobulinemia before and after plasma exchange; they found that the HCV RNA was reduced by 45.3–93.3% after treatment but returned to its previous level after 4–6 h [14]. Similarly, Ramratnam et al. observed a decrease in the viral load after plasmapheresis in HIV-1- and HCV-positive patients [26]. It has also been reported that heparin-induced extracorporeal low-density lipoprotein (LDL) precipitation (HELP) apheresis decreased the HCV RNA in chronic hepatitis C patients with the complication of hypercholesterolemia [16,17,27]. The decrease in the HCV RNA was transient according to these reports. By fitting a mathematical model to the changes in viral load during IFN therapy, HCV production in the liver has been estimated to be 10^{12} particles per day [28]. Although the virus is eliminated from the blood, virus production continues in the liver unless it is inhibited by IFN; hence, the HCV RNA level may return to the pre-therapy level after a few hours. Therefore, we planned to administer IFN immediately after the first plasmapheresis.

We selected DFPP as a plasmapheresis technique because there was no necessity to exchange plasma and supplement albumin during the apheresis.

Since DFPP is based on the principle of size separation, the membrane with a mean pore size of 30 nm that was used as the second filter in this study can theoretically be expected to eliminate HCV particles with a diameter of 55–65 nm. After the DFPP second filtration, the HCV RNA was quantitatively undetectable after performing DFPP for 1 and 3 h; this implies that the filter could eliminate HCV particles and that the efficiency did not change with time.

Other low molecular weight substances, including albumin (MW 66 kDa) and ribavirin (MW 244 kDa), passed through the second membrane and were returned to patient's blood. Hence, low molecular weight substances were not eliminated by this DFPP system.

In seven patients, in whom the serum HCV RNA level could be measured before and after DFPP on day 1, the mean rate at which the HCV RNA level decreased was 48% (18–78%); the HCV RNA level did not increase again. This exclusion may have been due to the inhibition of virus production by the IFN therapy that was initiated immediately after DFPP.

The EVR after 2 weeks of therapy tended to be higher in the DFPP group than in the control group. The change in viral load after 2 weeks also tended to decrease more in the DFPP group than in the control group. However, both these differences were statistically insignificant. The virus negativity in the DFPP group after 4 and 8 weeks was 66.7% and 62.5%, respectively. In the control group, the HCV RNA levels were not measured periodically and therefore no comparison was

possible. However, in a double-blind, controlled study in Japan on IFNα-2b and ribavirin combination therapy for chronic genotype 1b hepatitis C patients with a high viral load, Iino et al. found that the HCV RNA negativity after 4 and 8 weeks after the initiation of therapy was 18.8% and 38.2%, respectively. This suggested that IFN therapy with a concomitant reduction of the viral load using DFPP may induce an early conversion to the HCV RNA-negative state.

In our study, an SVR was observed in 2 of the 9 patients (22.2%) in the DFPP group and in 2 of the 11 patients (18.2%) in the control group. These rates are comparable to the result in which an SVR was observed in 19.0% of chronic genotype 1b HCV patients after 24 weeks after the administration of an interferon and ribavirin combination therapy [30]. An SVR observed in DFPP group was not higher than that in control group although an EVR on 2 weeks in DFPP was relatively higher than that in control group. One of the possible reasons for this observation is that, in two of the six patients exhibiting an EVR, treatment was discontinued because of the adverse events. EVR was demonstrated to be the only factor related to SVR by univariate analysis in our study; this may have been because the number of patients was insufficient to conduct an accurate analysis of these factors.

With regard to the safety of the IFN and ribavirin combination therapy with concomitant DFPP, only mild hypotension and transient vagal reflex were observed after the initiation of DFPP, and these were rapidly resolved. There were no other adverse events attributable to concomitant DFPP, and the treatment was performed safely. Since DFPP is frequently used in the treatment of severe disease conditions, such as malignant rheumatoid arthritis, thrombotic thrombopenic purpura, and multiple sclerosis, there may be no problems with the application of this procedure to chronic hepatitis patients. During the treatment, a catheter was inserted in the right femoral vein for DFPP; however, no infection or accident occurred as a consequence of its indwelling. The other adverse events were attributable to the ribavirin and IFN therapy.

With regard to results of the blood test, the platelet count and fibrinogen level slowly decreased from the initiation of therapy until the completion of DFPP; however, these gradually recovered after the completion of DFPP. On day 3, the fibrinogen level was lower than 100 mg/dl in all but two patients. To ensure the safety of the patients, and to prevent complications such as hemorrhage, DFPP conducted three times on days 1, 2, and 4 during the first week of therapy may be appropriate for patients with chronic hepatitis C.

In view of the fact that we found no significant difference in the EVR and SVR between the DFPP and control groups, and because the number of patients was very small, it was not possible in this preliminary study to draw conclusions regarding the applicability of the DFPP used in conjunction with ribavirin and IFN therapy. It could be argued that a simple physical reduction in the viral load, induced by concomitant DFPP or other apheresis, is really significant for the ribavirin and IFN therapy for chronic hepatitis C patients with a high viral load. Based on our results, it may be possible to use DFPP to facilitate early viral elimination. However, the early viral elimination by DFPP was not related to an SVR. Although five of the eight patients (62.5%, therapy was discontinued in one patient after 7 weeks) became virus-negative after 8 weeks, an SVR was observed in only two patients; thus, the relationship between the early virus negativity obtained by DFPP and the SVR remains unclear. Since the early conversion to the virus-negative state is a result of the reactivity of the host and virus to ribavirin and IFN, it may be possible that a simple physical reduction in the amount of the virus is not significant. However, DFPP eliminates not only the virus but also macromolecules including immunoglobulins. The elimination of some humoral factors or complement components by the dialysis membrane might be involved. The state of HCV in the blood, such as the immunoglobulin-bound state, the lipoprotein-associated state, and the non-bound (free) state, may also be important. In two preliminary cases, the state of HCV during the treatment was investigated using differential flotation centrifugation. In differential floatation centrifugation, the hyperbaric fraction, including the immunoglobulin-bound HCV particles, settles to the bottom, whereas the hypobaric fraction, including free-state HCV particles, rises to the top. In the two examined cases, the bottom: top ratio was reduced after the first DFPP treatment; from 214 to 62 in case 1 and from 108 to 46 in case 2. Since a decline in the bottom: top ratio indicates an increased amount of free-state HCV particles compared to the amount of immunoglobulin-bound HCV particles, these results suggest that the occurrence of the free-state HCV increased after DFPP. This increase in the amount of free-state HCV may possibly involve an SVR; this supposition is based on a previous study that demonstrated that a high proportion of free-state HCV was related to an SVR [29].

In conclusion, in order to achieve a reduction in the level of HCV RNA before IFN therapy and to facilitate an early conversion to a virus-negative state, plasmapheresis therapy (DFPP) was performed on chronic genotype 1b hepatitis C patients with a high viral load. The therapy was performed without the occurrence of severe adverse events, and the results suggested the possibility that concomitant DFPP increased the rate of early conversion to a virus-negative state. Since only nine patients were treated and IFN administration was discontinued in three patients, the relationship between DFPP and an EVR or SVR is unclear. If an EVR is achieved by a combination of DFPP and interferon therapy, and this is related to an SVR, the combination therapy with DFPP will not only be of benefit to chronic hepatitis C patients with a high viral load but may also shorten the period of interferon therapy. Furthermore, the cost of interferon therapy will also be reduced, although the cost of performing DFPP three times will remain high at approximately 2500 dollars. Because our study was limited by the small number of patients, further investigations using a study design that clarifies the relationship between DFPP and an EVR or SVR are necessary. It is also important to further investigate the

antiviral effect of the PEG-IFN and ribavirin combination therapy with concomitant DFPP, and the effects of DFPP other than virus elimination. In order to clarify this relationship, a multi-center clinical trial is being undertaken in Japan, and the data from this study is currently being collected.

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Different Signaling Pathways in the Livers of Patients With Chronic Hepatitis B or Chronic Hepatitis C

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The clinical manifestations of chronic hepatitis B (CH-B) and chronic hepatitis C (CH-C) are different. We previously reported differences in the gene expression profiles of liver tissue infected with CH-B or CH-C; however, the signaling pathways underlying each condition have yet to be clarified. Using a newly constructed cDNA microarray consisting of 9614 clones selected from 256,550 tags of hepatic serial analysis of gene expression (SAGE) libraries, we compared the gene expression profiles of liver tissue from 24 CH-B patients with those of 23 CH-C patients. Laser capture microdissection was used to isolate hepatocytes from liver lobules and infiltrating lymphoid cells from the portal area, from 16 patients, for gene expression analysis. Furthermore, the comprehensive gene network was analyzed using SAGE libraries of CH-B and CH-C. Supervised and nonsupervised learning methods revealed that gene expression was correlated more with the infecting virus than any other clinical parameters such as histological stage and disease activity. Pro-apoptotic and DNA repair responses were predominant in CH-B with p53 and 14-3-3 interacting genes having an important role. In contrast, inflammatory and anti-apoptotic phenotypes were predominant in CH-C. These differences would evoke different oncogenic factors in CH-B and CH-C. In conclusion, we describe the different signaling pathways induced in the livers of patients with CH-B or CH-C. The results might be useful in guiding therapeutic strategies to prevent the development of hepatocellular carcinoma in cases of CH-B and CH-C. ***Supplementary material for this article can be found on the HEPATOLOGY website (http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html).*** **(HEPATOLOGY 2006;44:1122-1138.)**

The human liver infected with hepatitis B virus (HBV) and hepatitis C virus (HCV) develops chronic hepatitis, cirrhosis, and in some instances, hepatocellular carcinoma (HCC).[1-3] The virological features of these 2 viruses are completely different. HBV is a DNA virus that integrates into the host genome.[4,5] HBV proteins, which have been reported to have transcriptional transactivator activity, may be related to the occurrence of HCC.[6-9] By contrast, HCV is a positive stranded RNA virus that replicates in the cytoplasm.[2] There are some reports that HCV proteins localize to the nucleus or interact with nuclear proteins.[10,11] Nevertheless, both viruses infect the liver and cause chronic hepatitis, which is not distinguishable by histological examination or clinical manifestations.[12] In chronic viral hepatitis, increased numbers of immunoregulatory cells infiltrate the liver, but the functional relevance of these cells to the pathogenesis of chronic hepatitis is not known.

We previously reported that the gene expression profiles in the livers of patients with chronic hepatitis B (CH-B) or chronic hepatitis C (CH-C) are different, and revealed some characteristic features of each disease.[13] However, the independent expression profiles of infiltrated lymphocytes and hepatocytes have yet to be clarified, as do the detailed signaling pathways underlying these 2 conditions.

In this study, we investigated the signaling pathways underlying CH-B and CH-C using cDNA microarray and serial analysis of gene expression (SAGE) techniques. Using laser capture microdissection (LCM), we selectively isolated hepatocytes from liver lobules and infiltrat-

Abbreviations: CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; SAGE, serial analysis of gene expression; HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma; GO, gene ontology; LCM, laser capture microdissection; ALT, alanine aminotransferase; aRNA, antisense RNA; CTL, cytotoxic lymphocyte; Cy, cyanine; EGFR, epidermal growth factor receptor; cDNA, complementary DNA; IFN, interferon; NF-κB, nuclear factor-κB; NK cells, natural killer cells.

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ing lymphoid cells from the portal area, from biopsy specimens, and analyzed their gene expression profiles.

Patients and Methods

Patients. The subjects were 27 patients with CH-B and 26 with CH-C at the Graduate School of Medicine, Kanazawa University Hospital, Japan, between 1999 and 2003 (Table 1). Informed consent was obtained from all patients and ethics approval for the study was obtained from the ethics committee for human genome/gene analysis research at Kanazawa University Graduate School of Medicine. Liver biopsy samples were taken from 24 CH-B patients and 23 CH-C patients, and were divided into 3 portions: one was immersed in formalin for histological assessment, another was immediately frozen in liquid nitrogen for further RNA isolation, and the final portion was frozen in OCT compound for LCM analysis and stored at −80°C until use. Tissue samples from the remaining 6 patients with HCC were surgically obtained from the noncancerous parts of the liver and immediately frozen in liquid nitrogen for SAGE analysis. For normal liver, surgically obtained tissue samples of 6 patients who showed no clinical signs of hepatitis were used, as described.[13]

The grading and staging of chronic hepatitis were histologically assessed according to the method described by Desmet et al.[14] (Table 1). There were no significant differences in the degree of histological activity or staging, nor in the sex or age of patients with CH-B or CH-C (Table 1).

Treatment of Cultured Cells With Interferon-α. Huh-7 cells were treated with recombinant interferon-α (IFN-α) (Schering-Plough Corp., Osaka, Japan) at a concentration of 1000 IU/mL for 6 hours, and were harvested for analysis of induced gene expression by cDNA microarray.

Preparation of cDNA Microarray Slides. In addition to the in-house cDNA microarray slides consisting of 1080 cDNA clones as described,[13,15-19] we made a new cDNA microarray slide for a detailed analysis of the signaling pathways involved in metabolism and enzyme function in liver disease. Besides cDNA microarray analysis, a total of 256,550 tags were obtained from hepatic SAGE libraries (derived from normal liver, CH-C, CH-C related HCC, CH-B, and CH-B related HCC), including 52,149 unique tags. Among these, 16,916 tags with more than 2 hits were selected to avoid the effect of sequencing errors in the libraries. From these candidate genes, 9614 nonredundant clones were obtained from Incyte Genomics (Incyte Corp., Beverly, MA), Clontech (Nippon Becton Dickinson, Tokyo, Japan), and Invitrogen (Invitrogen Japan K.K., Tokyo, Japan). Each clone was sequence validated and PCR amplified by Dragon Genomics (Takara Bio, Otsu, Japan), and the cDNA microarray slides (Liver chip 10k) were constructed using SPBIO 2000 (Hitachi Software, Fukuoka, Japan) as previously described.[13,15-19]

Laser Capture Microdissection. Hepatocytes in liver lobules and infiltrated lymphoid cells in the portal area were isolated by LCM using a CRI-337 LCM system (Cell Robotics, Albuquerque, NM)[18] (Fig. 1). Frozen liver biopsy specimens in OCT compound were sliced into sections 8 μm thick, immediately fixed in methanol for 5 minutes, and kept on dry ice. Tissue samples were quickly stained with toluidine blue and dissected. Around 500 lymphoid cells and a similar number of hepatocytes were excised from 3 slides and immersed in a denaturing solution. Dissection was completed within 5 minutes for each slide.

RNA Isolation and Antisense RNA Amplification. Total RNA was isolated from liver biopsy samples using an RNA extraction kit (Micro RNA Extraction Kit, Stratagene, La Jolla, CA). Aliquots of total RNA (5 μg) were subjected to amplification with antisense RNA (aRNA) using a Message Amp aRNA kit (Ambion, Austin, TX) as recommended by the manufacturer. About 25 μg of aRNA was amplified from 5 μg of total RNA, assuming that 500-fold amplification of mRNA was obtained. Total RNA from LCM samples was isolated with a carrier nucleic acid (20 ng poly C) using RNAqueous-Micro (Ambion). The quality and degradation of the isolated RNA were estimated after electrophoresis using an Agilent 2001 bioanalyzer (Agilent Technologies, Palo Alto, CA) (Fig. 1B). RNA isolation typically yielded 20-40 ng total RNA from 500 cells. Half of the obtained RNA was amplified twice as described above to yield 20-40 μg aRNA. Antisense RNA (20 μg) was used for further labeling procedures. The optimum conditions of LCM and reproducibility of data were assessed repeatedly.

Hybridization on cDNA Microarray Slides and Image Analysis. As a reference for each microarray analysis, aRNA samples prepared from the normal liver tissue from 1 of the patients were used. Test RNA samples fluorescently labeled with cyanine 5 (Cy5) and reference RNA labeled with Cy3 were used for microarray hybridization as described.[13,15-19] Quantitative assessment of the signals on the slides was carried out by scanning on a ScanArray 5000 (General Scanning, Watertown, MA) followed by image analysis using GenePix Pro 4.1 (Axon Instruments, Union City, CA) as described.

Processing of cDNA Microarray Data. Hierarchical clustering of gene expression was performed by BRB-Ar-

Table 1. Characteristics of Patients, as Used for Analyses of Whole Liver Biopsy, LCM, and SAGE Samples

Patient No.	Virus	Age	Sex	ALT	A	F	Viral load (LEG/mL, KIU/mL)	HCV serotype	HBeAg	LCM Hep	LCM Ly
Whole liver biopsy samples											
1	HBV	34	F	45	1	1	8.2	na.	+		
2	HBV	64	F	119	1	1	>8.7	na.	+		
3	HBV	49	M	21	1	1	<3.7	na.	−		
4	HBV	29	M	194	2	1	7.5	na.	+		
5	HBV	47	M	10	1	2	<3.7	na.	−		
6	HBV	53	F	43	1	2	7.3	na.	+		
7	HBV	24	M	42	2	2	7.1	na.	+		
8	HBV	18	M	400	2	2	8.0	na.	+		
9	HBV	20	M	188	2	2	6.2	na.	+		
10	HBV	59	M	68	2	3	4.4	na.	+		
11	HBV	36	F	29	2	3	4.2	na.	−		
12	HBV	60	M	33	2	3	7.4	na.	+		
13	HBV	60	F	28	2	3	<3.7	na.	−		
14	HBV	35	F	145	3	3	7.6	na.	+		
15	HBV	64	M	48	1	4	7.1	na.	−		
16	HBV	55	M	30	1	4	7.4	na.	−		
17	HBV	34	F	45	2	4	8.5	na.	+		
18	HBV	54	M	159	2	4	5.5	na.	+		
19	HBV	60	M	121	3	4	4.6	na.	+		
20	HCV	24	M	34	1	1	>850	I	−		
21	HCV	68	F	43	1	1	720	II	−		
22	HCV	64	F	117	1	2	590	na.	−		
23	HCV	69	M	6	1	2	300	II	−		
24	HCV	42	M	59	1	2	410	II	−		
25	HCV	73	M	19	1	2	140	I	−		
26	HCV	43	M	98	2	2	60	I	−		
27	HCV	70	M	56	2	2	600	I	−		
28	HCV	70	F	26	2	3	350	I	−		
29	HCV	65	M	21	2	3	290	I	−		
30	HCV	47	M	225	2	3	120	I	−		
31	HCV	58	M	200	2	3	410	I	−		
32	HCV	57	F	116	2	3	490	I	−		
33	HCV	63	F	39	2	4	290	I	−		
34	HCV	76	M	54	2	4	660	I	−		
35	HCV	67	M	67	2	4	240	I	−		
36	HCV	46	M	111	2	4	>850	I	−		
37	HCV	63	M	64	2	4	60	na.	−		
LCM samples											
38(2)	HBV	64	F	119	1	1	>8.7	na.	+	+	+
39	HBV	31	F	114	1	1	8.5	na.	+	+	+
40	HBV	68	F	41	2	2	5.5	na.	+		+
41	HBV	29	M	140	2	2	>8.7	na.	+		+
42	HBV	40	M	80	2	2	<3.7	na.	−		+
43	HBV	45	M	83	2	3	6.1	na.	+		+
44(10)	HBV	59	M	68	2	3	4.4	na.	+	+	+
45(14)	HBV	35	F	145	3	3	7.6	na.	+	+	+
46(21)	HCV	68	F	43	1	1	720	II	−	+	+
47	HCV	47	M	33	1	1	50	I	−	+	+
48	HCV	67	M	80	2	2	114	II	−		+
49	HCV	73	M	71	2	2	>850	II	−		+
50	HCV	67	M	70	2	2	>851	I	−		+
51	HCV	59	F	43	2	3	>852	I	−		+
52(31)	HCV	58	M	200	2	3	410	I	−	+	+
53(32)	HCV	57	F	116	2	3	490	I	−	+	+
SAGE samples											
54	HBV	55	M	34	1	1	5.9	na.	−		
55	HBV	70	F	31	2	2	7.7	na.	−		
56	HBV	72	M	22	1	4	6.3	na.	+		
57	HCV	71	M	128	1	4	440	I	−		
58	HCV	69	F	84	2	4	212	I	−		
59	HCV	49	M	150	2	4	60	I	−		

Abbreviations: na., not applicable; LCM, laser capture microdissection; ALT, alanine aminotransferase; SAGE, serial analysis of gene expression; A, activity; Hep., hepatocyte obtained by LCM; Ly., lymphocyte obtained by LCM; F, fibrosis; No., if the sample was obtained from the same patient, the new sample number is shown with the old one; HCV RNA was assayed by Amplicor Monitor Test (KIU/mL); HBV DNA was assayed by transcription-mediated amplification (LEG/mL).

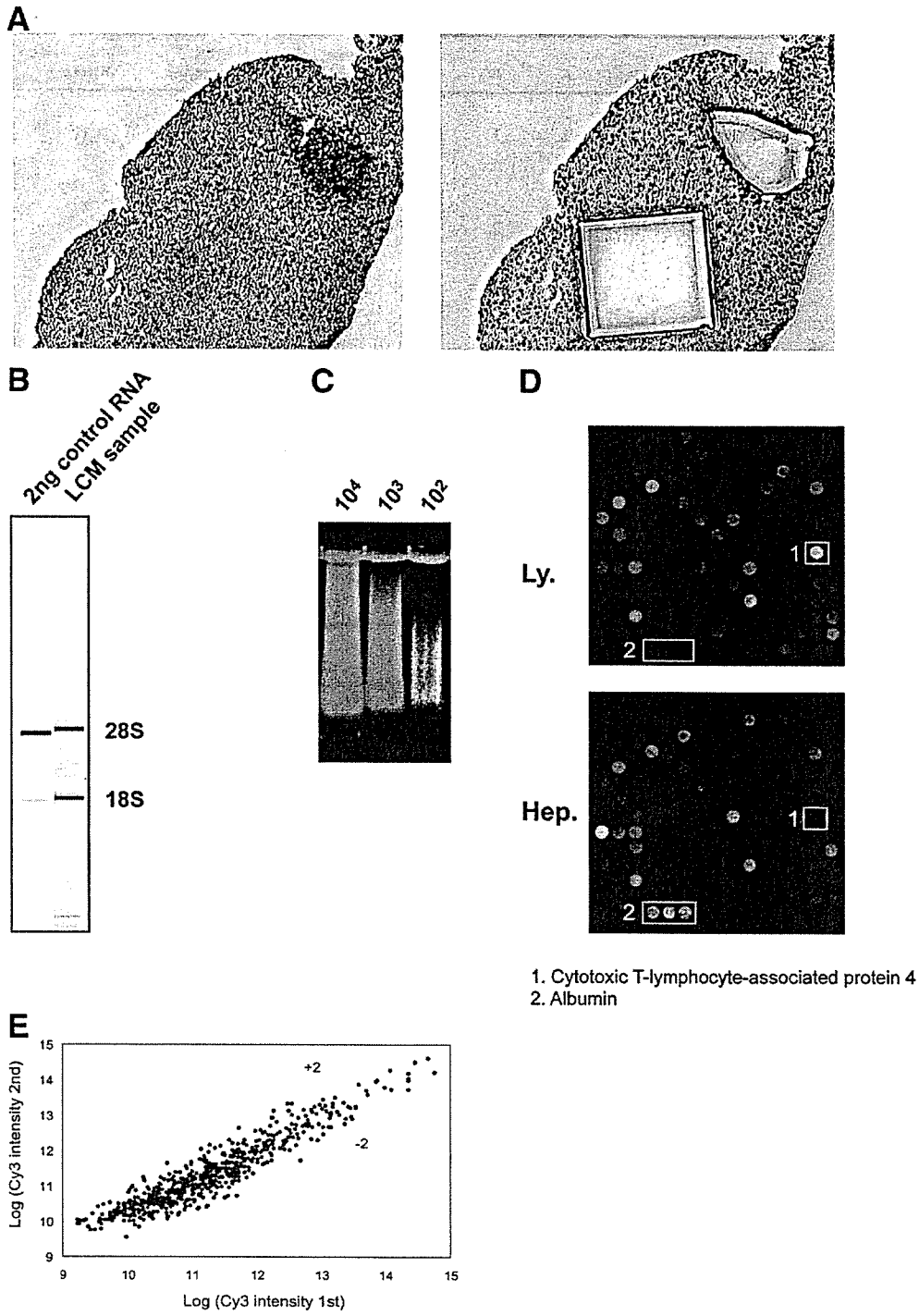

Fig. 1. Optimization of LCM and cDNA microarray analysis. (A) Toluidine blue staining of liver biopsy specimens before (left) and after (right) LCM. (B) Electrophoresis of isolated RNA using an Agilent 2001 bioanalyzer. (C) Two round-amplified aRNA from 10^2-10^4 excised hepatocytes. (D) Typical hybridization result from LCM samples. (E) Correlation of signal intensity between first and second amplified genes. Two values were significantly correlated ($P < .001$, $r^2 = .97$) within 2-fold differences.

rayTools (http://linus.nci.nih.gov/BRB-ArrayTools.htm). The filtered data were log-transferred, normalized, centered, and applied to the average linkage clustering with centered correlation. A class prediction was performed by compound covariate predictor incorporating genes that were differentially expressed at the $P = .002$ significance

Table 2. Supervised Learning Methods to Differentiate CHB and CHC

Classifier Category	Clinical Groups	Total Number of Cases	Number of Cases Misclassified	Classifier *P* Values	Number of Genes in the Classifiers ($P < .002$)
HBV versus HCV	HBV	19	1	<0.001	160
	HCV	18	3		
Histological stage	F1F2	17	10	0.402	55
	F3F4	20	7		
Histological activity	A0A1	13	6	0.173	106
	A2A3	24	6		
Age	≥50	22	9	0.298	39
	<50	15	6		
ALT at biopsy	≥80	14	7	0.200	21
	<80	23	6		

level as assessed by the random variance t test (BRB-ArrayTools). The univariate t test values for comparing the classes were used as the weights. The cross-validated misclassification rate was computed and at least 2,000 permutations were performed for a valid permutation P value. The Fisher and Kolmogorov-Smirnov tests were performed for gene ontology (GO) comparison ($P < .005$) (BRB-ArrayTools).

Pathway Analysis of Expression Data. The pathway analysis of the differentially expressed genes was performed using MetaCore software suite (GeneGo, St. Joseph, MI). Possible networks were created according to the list of the differentially expressed genes using the MetaCore database, a unique, curated database of human protein-protein and protein-DNA interactions; transcription factors; and signaling, metabolic, and bioactive molecules. The P value was calculated as:

$$p\text{-Value} = \frac{R!n!(N-R)!(N-n)!}{N!} \sum_{i=\max(r,R+n-N)}^{\min(n,R)} \frac{1}{i!(R-i)!(n-i)!(N-R-n+i)!}$$

where N is total number of nodes in the MetaCore database, R the number of network objects corresponding to the genes list, n the total number of nodes in each small network generated from the genes list, and r the number of nodes with data in each small network generated from the genes list. Moreover, direct interactions among the differentially expressed genes were examined. Each connection represents a direct, experimentally confirmed, physical interaction.

SAGE. Total RNA isolated from each of 3 patients with CH-B or CH-C was mixed to 200 μg in total, and polyadenylated RNA was extracted using a FastTrac mRNA Purification Kit (Invitrogen). The SAGE protocol was as described.[20,21] SAGE libraries were sequenced at random using an ABI Prism 377 DNA Sequencer and BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA). Sequenced files were analyzed with the SAGE version 1.00 software.

Quantitative Real-time Detection PCR. We performed quantitative real-time detection PCR (RTD-PCR) using TaqMan Universal Master Mix (PE Applied Biosystems). Primer pairs and probes for MxA, IP10, IFI15, OAS2, GZMA, TP53, PDECGF, IFNG, DIABLO, FGFB, BGA2, CASP9, PEX5, ANGPT1, VEGF, and β-actin were obtained from TaqMan assay reagents library. Results were expressed as means ± SEM. Significance was tested by 1-way ANOVA with Bonferroni's methods and differences were considered statistically significant at $P < .05$.

Results

Optimization of LCM and cDNA Microarray Analysis. Before analysis of region-specific gene expression, the sensitivity and reliability of linear aRNA amplification was examined. The quality and degradation of the isolated RNA were estimated after electrophoresis using an Agilent 2001 bioanalyzer (Fig. 1B). We successfully amplified aRNA from 10^2-10^4 excised hepatocytes with 2 rounds of amplification (Fig. 1C). The estimated amount of isolated RNA from around 150 excised hepatocytes (Fig. 1A) was 5-10 ng, and 10-20 μg of aRNA was obtained by 2 rounds of amplification, assuming that a 25×10^4-fold amplification (500-fold by single amplification) was carried out. A typical hybridization result is shown in Fig. 1D. Cytotoxic T lymphocyte-associated protein 4

Fig. 2. (A) Hierarchical clustering analysis of gene expression in hepatocytes and liver-infiltrating lymphocytes. Hep, hepatocyte; Ly, lymphocyte; B, hepatitis B; C, hepatitis C. (B) Hierarchical clustering analysis of 1,360 filtered genes (we excluded genes with an expression level within 1.5-fold of median value in more than 80% of samples) demonstrated more clear clusters of CH-B and CH-C.

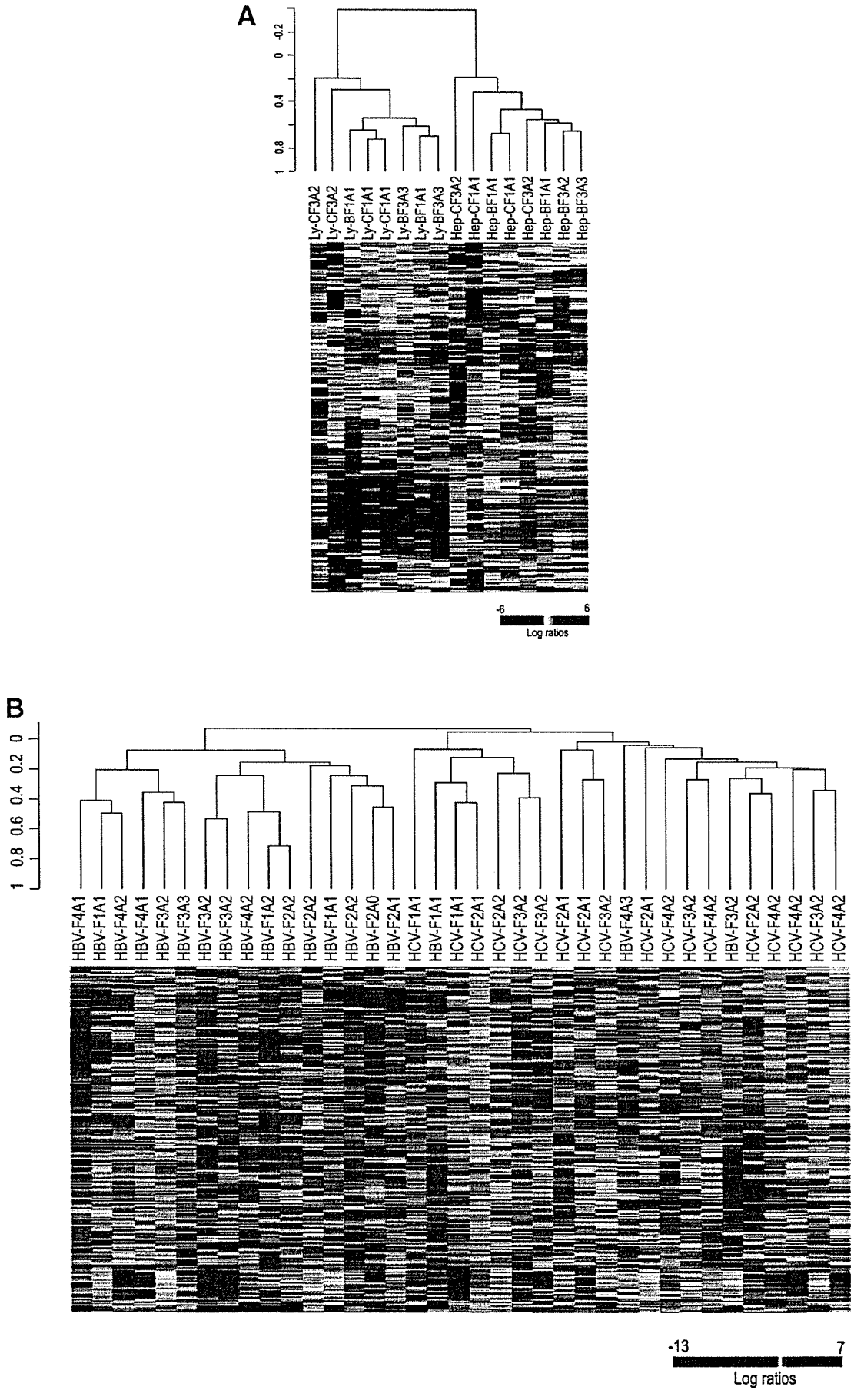
A
1 0.8 0.4 0 -0.2
Ly-CF3A2
Ly-CF3A2
Ly-BF1A1
Ly-CF1A1
Ly-CF1A1
Ly-BF3A3
Ly-BF1A1
Ly-BF3A3
Hep-CF3A2
Hep-CF1A1
Hep-BF1A1
Hep-CF1A1
Hep-CF3A2
Hep-BF1A1
Hep-BF3A2
Hep-BF3A3
-6
6
Log ratios
B
1 0.8 0.6 0.4 0.2 0
HBV-F4A1
HBV-F1A1
HBV-F4A2
HBV-F4A1
HBV-F3A2
HBV-F3A3
HBV-F3A2
HBV-F3A2
HBV-F4A2
HBV-F1A2
HBV-F2A2
HBV-F2A2
HBV-F1A1
HBV-F2A2
HBV-F2A0
HBV-F2A1
HCV-F1A1
HBV-F1A1
HCV-F1A1
HCV-F2A1
HCV-F2A2
HCV-F3A2
HCV-F3A2
HCV-F2A1
HCV-F2A1
HCV-F3A2
HBV-F4A3
HCV-F2A1
HCV-F4A2
HCV-F3A2
HCV-F4A2
HBV-F3A2
HCV-F2A2
HCV-F4A2
HCV-F4A2
HCV-F3A2
HCV-F4A2
-13
7
Log ratios

Fig. 2

was predominantly expressed in liver-infiltrating lymphocytes, whereas albumin was predominantly expressed in hepatocytes (Fig. 1D). To determine whether multiple amplifications affected the original gene expression, the signal intensities of first- and second-round amplified genes were compared. There was a significant correlation between the 2 values ($P < .001$, $r^2 = .97$), within a 2-fold difference (Fig. 1E), suggesting that the linear amplification procedure maintained the original level of gene expression.

Identification of Genes Differentially Expressed in Hepatocytes and Liver-Infiltrating Lymphocytes. Pairwise *t* test comparisons were applied and differentially expressed genes were identified in lymphocytes and hepatocytes in 4 patients with CH-B and 4 patients with CH-C (Supplementary Table 1-1). In hepatocytes, liver-specific proteins and enzymes such as fibrinogen, afamin, and cytochrome P450 were all expressed. In lymphocytes, cytokines, chemokines, and lymphocyte surface markers such as interleukin-7 receptor, chemokine (C-X-C motif) receptor 4, CD83 antigen, and CD69 antigen were all expressed (Supplementary Table 1-2). Hierarchical clustering analysis of gene expression in hepatocytes and liver-infiltrating lymphocytes demonstrated clear differences in gene expression (Fig. 2). Representative differentially expressed genes in lymphocytes and hepatocytes in CH-B and CH-C are summarized in Supplementary Tables 2-1, 2-2, 3-1, and 3-2.

Supervised and Nonsupervised Learning Methods to Classify Gene Expression Profiling According to Different Clinical Parameters. To examine which clinical parameters contributed to the changes in gene expression, supervised and nonsupervised learning methods were applied to classify gene expression profiles. The gene expression profiles of whole liver biopsy specimens, obtained from 19 patients with CH-B and 18 with CH-C, were analyzed. Hierarchical clustering analysis; a nonsupervised learning method, using 9641 nonfiltered genes, clearly demonstrated 2 clusters in CH-B and CH-C with a few exceptions (data not shown). Hierarchical clustering analysis with 1360 filtered genes (we excluded genes with an expression level within 1.5-fold of the median value in more than 80% of samples) demonstrated clearer clusters in CH-B and CH-C (Fig. 2B). Supervised learning methods based on the compound covariate predictor revealed that, among various clinical parameters including etiology (HBV or HCV), histological stage (F_1F_2 or F_3F_4), activity (A_0A_1 or A_2A_3), age ($\geq$50 or <50 years), and alanine aminotransferase (ALT) level at biopsy ($\geq$80 or <80 IU/mL), only etiology significantly classified these patients (Table 2). Thus, HBV or HCV infection determines gene expression to a greater degree than any other clinical parameters, such as histological stage and disease activity.

Differentially Expressed Genes in CH-B and CH-C Hepatic Lesions. The 160 genes were differentially expressed in CH-B and CH-C by class prediction analysis ($P < .005$); representative genes (greater than 3-fold difference in *t* value) are listed in Tables 3 and 4. Based on the expression profiles of hepatocytes and lymphocytes isolated using LCM, genes expressed in both hepatocytes and lymphocytes are described as Hep/Ly (Tables 3 and 4). Genes expressed at a significantly greater level in hepatocytes than lymphocytes were described as Hep. Genes expressed at a significantly greater level in lymphocytes than hepatocytes were described as Ly. In CH-B, genes involved in cell cycle arrest and induction of apoptosis were preferentially expressed. Several hepatocyte-specific and apoptosis-inducing genes such as Diablo homolog (cytochrome c/apaf-1/caspase-9 pathway activator) and BCL2-associated athanogene 2 (inhibitor of heat shock protein 70) were upregulated (Table 3, Fig. 7). In CH-C, cell cycle accelerating, immune-related, and antigen-presenting genes were preferentially upregulated. Many type 1 IFN-induced genes such as IFN-α-inducible protein 27 and IFN-α-inducible protein (clone IFI-15K) were upregulated in CH-C. The induction of these genes was confirmed by examining gene expression in Huh-7 cells treated with recombinant IFN-α (Tables 3 and 4, Fig. 7).

The frequent pathway processes observed in CH-B and CH-C using MetaCore are shown in Table 5. Induction of genes related to apoptosis (caspase activation via cytochrome C), transcription, and fibrosis (intermediate filament-based process and TGF-β receptor signaling pathway) were upregulated in CH-B, whereas genes related to immune reaction (defense response, antigen presentation, Golgi vesicle transport, and ubiquitin cycle), lipid metabolism (regulation of cholesterol absorption), and epidermal growth factor receptor (EGFR) signaling were upregulated in CH-C. This suggests that there are different signaling pathways in CH-B and CH-C.

Go Comparison of Expressed Genes in CH-B and CH-C Hepatic Lesions. The analysis of differentially expressed genes could underestimate the presence of mean full signaling pathways that were coordinately upregulated or downregulated, with subtle differences at an individual gene level. The biological significance of these coordinately regulated signaling pathways has recently been demonstrated.[22] Therefore, we applied the GO comparison tool to expressed genes in CH-B and CH-C hepatic lesions. The comparison tool provided a list of GO categories that were coordinately regulated between CH-B and CH-C.