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Impact of Diabetes on Recurrence of Hepatocellular Carcinoma after Surgical Treatment in Patients With Viral Hepatitis

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OBJECTIVES: Consensus has been reached that diabetes is a risk factor for development of HCC, but the impact on postoperative recurrence is still controversial. To clarify this point, we analyzed the relationship of postoperative recurrence rate of HCC and coexistence of diabetes in the patients with viral hepatitis.

METHODS: A total of 90 patients who had undergone curative resection for HCC were analyzed. They were divided into two groups with and without diabetes, and the recurrence-free survival rates after surgical treatment and the factors contributing to recurrence were examined.

RESULTS: Kaplan-Meier survival analysis showed the recurrence-free survival rates in the diabetic group were significantly lower than those in the nondiabetic group ($P = 0.005$) and overall survival rates in the diabetic group were significantly lower than those in the nondiabetic group ($P = 0.005$). These results were emphasized in the analysis of patients infected with hepatitis C virus. Univariate and multivariate analyses showed diabetes was a significant factor contributing to HCC recurrence after treatment. Furthermore, multivariate analysis in HCC patients with diabetes showed Child-Pugh classification B ($P = 0.001$) and insulin therapy ($P = 0.049$) were significant factors contributing to HCC recurrence after treatment.

CONCLUSIONS: The results of the present study suggest that diabetes is a risk factor for the recurrence of HCV-related HCC and decreases the overall survival rates after surgical treatment. HCV-related HCC patients with diabetes should be closely followed for postoperative recurrence.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent malignant neoplasm in the world (1), and its prevalence is particularly high in Asia and Africa. The recent increase in its prevalence has attracted the attention of researchers (2, 3). Surgical therapy provides complete cure for HCC, but the indication is limited to a relatively small number of patients. Recent remarkable advances in diagnostic imaging techniques and systematic hepatectomy have been improving the prognosis of patients with HCC, but these techniques have not provided satisfactory results (4, 5), because of a high post-treatment recurrence rate characterizing HCC. Previous studies have noted that factors contributing to recurrence include gender, alcohol consumption, hepatitis C virus (HCV) infection, hepatic reserve, liver fibrosis degree, tumor size, tumor differentiation degree, vascular factor, and alpha-fetoprotein (AFP) level (6–9).

On the other hand, recent studies have reported that coexistence of diabetes is a risk factor for the progression of liver fibrosis and the development of HCC in chronic hepatitis C (10, 11). These reports suggest that coexistence of diabetes is also involved in the high postoperative recurrence rate of HCC. However, it is controversial whether diabetes is an independent risk factor for the post-treatment recurrence of HCC. Ikeda *et al.* (12) reported that diabetes was a risk factor for the recurrence of HCC after surgical treatment, but Poon *et al.* (13) and Toyoda *et al.* (14) reported that this was not the case. The discrepancy among these reports is probably due in part to the difference in the etiology of liver disease in the patients studied.

In this study, to clarify the controversial point about diabetes and HCC recurrence, we examined the impact of diabetes on the postoperative recurrence of HCC in 90 patients who had undergone curative resection for HCC. In addition, we classified the HCC patients into groups of hepatitis B

virus (HBV)- and HCV-related HCC patients, and performed a close analysis of the impact of diabetes on the postoperative recurrence of HCC in each group.

PATIENTS AND METHODS

Patients

A total of 150 patients were diagnosed with primary HCC and underwent surgical treatment in Kanazawa University Hospital between June 1987 and May 2004. Of these patients, 90 were analyzed who had HBV or HCV infection and underwent curative resection.

HCCs were detected by imaging modalities such as ultrasound scan, dynamic CT scan, MR imaging, and abdominal arteriography. The diagnosis of HCC was made by typical hypervascular tumor staining on angiography in addition to using typical findings, which showed hyperattenuation areas in the early phase and hypoattenuation in the late phase on dynamic CT (15).

All resected tumors were examined pathologically for the degree of differentiation of HCC, vascular invasion, and persistence of tumor in the surgical stump. Pathological degree of differentiation of HCC was assessed according to the general rules for the clinical and pathologic study of primary liver cancer (16).

Treatment and Follow-Up

In selecting surgery as a treatment option for HCC, we considered the following criteria: (a) good general condition of the patient whose Karnofsky performance status was over 80, (b) primary HCC, (c) Child-Pugh classification A or B, (d) the number of HCC was solitary and no CT, MRI, or angiographic evidence of vascular invasion or distant metastasis. Curative resection was defined as complete excision of the tumor with tumor-free surgical margins and no local recurrence at the surgical margin within 6 months after surgery.

Patients were followed postoperatively on an outpatient basis by abdominal ultrasound, dynamic CT, or MRI at 3-month intervals for at least 60 months. Recurrence was diagnosed by dynamic CT or MRI, and the date of recurrence was defined as the date of examination when the recurrence of HCC was noted. In patients with recurrent HCC, the recurrence-free period was defined as the time between the date of surgery and the date of recurrence. We confirmed the date of the patient's last visit to our hospital and checked the status of HCC using each patient's medical record.

Laboratory and Virologic Testing

Blood samples were tested for hepatitis B surface antigen (HBs-Ag) and hepatitis C virus antibody (HCV-Ab) by commercial immunoassays (Fuji Rebio, Tokyo, Japan). Serum AFP level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan). Diabetes was diagnosed according to the American Diabetes Association criteria for type II diabetes (17) and the severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet *et al.* (18).

Statistical Analysis

Between-group differences were assessed by univariate analysis with Student's *t*-test for numerical data and the $\chi 2$ test with Yates' correction (or Fisher's exact test where appropriate) for nominal data. Overall survival and recurrence-free survival was examined using the method of Kaplan-Meier, and differences were assessed by the log-rank test. Impact factors for the recurrence of HCC after hepatic resection were analyzed by univariate and multivariate analysis using Cox proportional hazards model. Seventeen variables were analyzed, consisting of age, gender, etiology, body mass index (BMI), prevalence of alcohol abuse, diabetes, hemoglobin A1c (HbA1c), liver fibrosis degree, Child-Pugh classification, platelet count, alanine aminotranseferase (ALT), T-bil, Alb, AFP, tumor size, tumor differentiation degree, and the presence of vascular invasion. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of Baseline Characteristics

Of the 90 patients (75 men and 15 women, with a mean age of 61.0 yr) who were followed and analyzed, 30 were diagnosed as having coexistence of diabetes, and 60 had no diabetes. The characteristics of the patients in both groups

Table 1. Characteristics of Patients

Characteristic	Patients With Diabetes (N = 30)	Patients Without Diabetes (N = 60)	*P* Value
Median age (yr)	62.0	60.6	0.453
Gender (male/female)	24/6	51/9	0.560
Etiology (HBV/HCV/ HBV + HCV)	8/22/0	17/40/3	0.438
Body mass index (kg/m^2)	23.5	22.73	0.316
Alcohol abuse (+/−)	13/17	27/33	0.881
HbA1c (%)	6.4	4.8	<0.001
HOMA-IR	4.1	3.3	0.399
Platelet count ($\times 10^4/\mu$L)	12.2	13.5	0.284
ALT (IU/L)	69.8	56.4	0.318
Total bilirubin (mg/dL)	0.9	0.8	0.510
Albumin (g/dL)	4.1	4.2	0.341
AFP (ng/mL)	417	395	0.931
Fibrosis (F1/F2/F3/F4)	0/4/4/22	4/5/5/46	0.732
Inflammatory grading (A1/A2/A3)	11/17/2	24/33/3	0.385
Child-Pugh grade (A/B/C)	24/6/0	53/7/0	0.294
Tumor size (mm)	34.3	29.8	0.359
Diff. degree (wel/mod/por)*	11/12/7	20/19/21	0.264
Vascular invasion (+/−)	11/19	20/40	0.757
Date of operation (1987–1995/1995–2000/ 2001–2004)	8/14/8	13/30/17	0.538

*Histological degree of HCC: wel = well differentiated; mod = moderately differentiated; por = poorly differentiated.

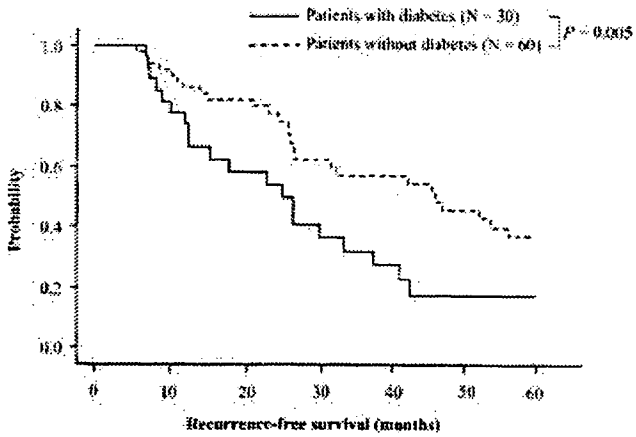

Figure 1. Kaplan-Meier curves for recurrence-free survival in the groups of patients with and without diabetes.

are shown in Table 1. No significant differences were noted between the two groups in age, gender, HBV or HCV infection rate, BMI, or prevalence of alcohol abuse. HbA1c was significantly higher, at 6.4%, in the diabetic group than in the nondiabetic group with an HbA1c of 4.8% ($P < 0.001$). There were no significant differences in platelet count, ALT, T-bil, Alb, AFP, or Child-Pugh classification between the two groups. In addition, no significant differences were observed in the liver fibrosis degree, or in the size, degree of differentiation, and presence of microscopic vascular invasion of resected HCC. Homeostasis model assessment-insulin resistance (HOMA-IR) of the patients with diabetes, which was high compared with that of Japanese healthy subjects (19), was higher than that of the patients without diabetes, although it was not statistically significant.

Impact of Diabetes on Recurrence After Surgical Treatment of HCC

Next, the diabetic and nondiabetic groups were compared for the rate of HCC recurrence after surgical treatment. HCC recurred after surgical treatment in 49 patients, consisting of 22 diabetic patients (73.3%) and 27 nondiabetic patients (45.0%). The mean time to recurrence was 32.8 months (range 8–60 months) and the median time to recurrence was 29.4 months.

Figure 1 shows the Kaplan-Meier curves for recurrence-free survival of the patients with and without diabetes. The recurrence-free survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 77.8%, 55.6%, 36.0%, 16.7%, and 16.7%, respectively, in the diabetic patient group, and 89.5%, 80.4%, 56.8%, 45.0%, and 36.6%, respectively, in the nondiabetic patient group; all rates except 1 yr were significantly lower in the diabetic patient group ($P = 0.155$, $P = 0.010$, $P = 0.009$, $P = 0.002$, and $P = 0.005$).

To further examine the degree of contribution of diabetes to the postoperative recurrence of HCC, we performed univariate and multivariate analysis. Univariate analysis identified the following variables as factors significantly contributing to HCC recurrence after surgical treatment: presence of diabetes ($P = 0.003$), high HbA1c level ($P < 0.001$), and Child-Pugh classification B against A ($P < 0.001$) (Table 2). When we conducted multivariate analysis, we chose variables that had already pointed out a risk factor for HCC recurrence and the P value was lower than 0.1 in univariate analysis. As a result, multivariate analysis of these variables showed that the presence of diabetes (risk 2.9, 95% CI 1.5–5.4, $P < 0.001$) and Child-Pugh classification B against A (risk 3.6, 95% CI 1.7–7.7, $P = 0.001$) were significant factors contributing to HCC recurrence after surgical treatment (Table 3).

Table 2. Univariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment

Variable	Hazard Ratio	95% CI	P Value
Age (yr)	1.0	1.0–1.1	0.258
Gender (male)	0.5	0.3–1.1	0.104
Etiology (HCV)	1.0	0.5–1.8	0.965
Body mass index (>25 kg/m^2)	1.2	0.6–2.3	0.554
Alcohol abuse (+)	1.1	0.7–2.0	0.644
Diabetes (+)	2.4	1.3–4.2	0.003
HbA1c (%)	1.5	1.2–1.9	<0.001
Fibrosis (F4)	1.4	0.7–3.1	0.349
Child-Pugh grade (B)	3.1	1.6–6.0	<0.001
Platelet count ($\times 10^4/\mu$L)	1.0	0.9–1.0	0.465
ALT (IU/L)	1.0	1.0–1.1	0.717
Total bilirubin (mg/dL)	1.4	0.6–3.6	0.451
Albumin (g/dL)	1.4	0.8–2.4	0.225
AFP (>200 ng/mL)	1.0	0.5–1.8	0.906
Tumor size (>50 mm)	1.5	0.7–1.2	0.213
Diff. degree (P)	1.1	0.6–2.1	0.675
Vascular invasion (+)	1.2	0.4–1.7	0.490

Impact of Diabetes on Prognosis After Surgical Treatment of HCC

To examine the impact of diabetes on prognosis of HCC patients, we analyzed the overall survival rates after surgical treatment. Figure 2 shows the Kaplan-Meier curves for overall survival of the patients with and without diabetes after surgical treatment of HCC. The overall survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 100%, 88.9%, 75.0%, 63.6%, and 45.5%, respectively, in the diabetic patient group, and 100%, 98.1%, 88.9%, 85.7%, and 76.3%, respectively, in the nondiabetic patient group; the rates of more than 3 yr were significantly lower in the diabetic patient group ($P = 1.000$, $P = 0.073$, $P = 0.028$, $P = 0.039$, and $P = 0.005$).

Table 3. Multivariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment

Variable	Hazard Ratio	95% CI	P Value
Diabetes	2.9	1.5–5.5	<0.001
Fibrosis (F4)	1.9	0.8–4.5	0.148
Child-Pugh grade (B)	3.6	1.7–7.7	0.001
AFP(>200 ng/mL)	0.7	0.3–1.5	0.390
Diff. degree (P)	0.9	0.5–1.8	0.776
Vascular invasion (+)	2.0	1.0–4.0	0.061

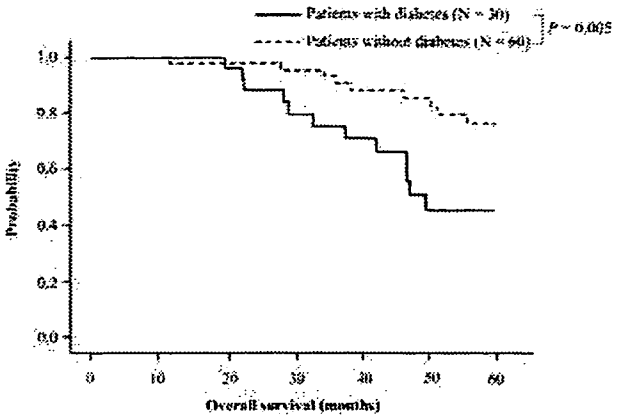

Figure 2. Kaplan-Meier curves for overall survival in the groups of patients with and without diabetes.

These curves indicated that overall survival rates were significantly lower in the diabetic patient group ($P = 0.005$).

Differential Impact of Diabetes on Prognosis After Surgical Treatment Between HBV- and HCV-Infected Patients

Next, we classified the HCC patients into HBV- and HCV-related HCC patients, and examined the impact of diabetes on recurrence-free and overall survival rates after surgical treatment. We divided all patients into 25 HBs-Ag (+), HCV-Ab (−) patients (with HBV-related HCC) and 62 HBs-Ag (−), HCV-Ab (+) patients (with HCV-related HCC), and further divided these two groups of patients into four groups according to the presence or absence of diabetes. In 62 patients who were HCV-Ab positive, 53 patients were also positive for HCVRNA. The other 9 patients were not examined for HCVRNA. The clinical profiles of these four groups of patients are shown in Table 4. Three HBs-Ag (+), HCV-Ab (+) patients, who were not complicated by diabetes, were excluded from the analysis. There were no significant differences between the groups of HBV-related HCC patients with and without diabetes in age, gender, BMI, prevalence of alcohol abuse, platelet count, ALT, total bilirubin, Child-Pugh classification, liver fibrosis degree, tumor size, tumor differentiation degree, or the presence of vascular invasion, except for Alb and AFP. Similarly, there were no significant differences between the groups of HCV-related HCC patients with and without diabetes. The HbA1c levels were higher in the groups of diabetic patients with HBV- or HCV-related HCC.

The Kaplan-Meier curves for recurrence-free survival in the groups of HBV-related HCC patients with and without diabetes are shown in Figure 3. The recurrence-free survival rates 1, 3, and 5 yr after surgical treatment were 85.7%, 57.1%, and 42.9%, respectively, in the diabetic patient group, and 76.5%, 46.7%, and 40.0%, respectively, in the nondiabetic patient group, showing no significant differences between the two groups ($P = 0.596$, $P = 0.670$, and $P = 0.827$). In the analysis of overall survival in the groups of HBV-related HCC patients with and without diabetes by the method of Kaplan-Meier, it indicated that there was no difference between the two groups ($P = 0.505$) (Fig. 4).

Figure 5 shows the Kaplan-Meier curves for recurrence-free survival in the groups of HCV-related HCC patients with and without diabetes. The recurrence-free survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 75.0%, 38.9%, 22.2%, 11.1%, and 11.1%, respectively, in the diabetic patient group, and 94.6%, 83.9%, 62.1%, 35.0%, and 29.2%, respectively, in the nondiabetic patient group, indicating that the recurrence-free survival rates were significantly lower in the diabetic patient group than in the nondiabetic patient group ($P = 0.030$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$).

In the analysis of overall survival in the groups of HCV-related HCC patients with and without diabetes, the overall survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 100%, 88.9%, 76.5%, 64.7%, and 43.8%, respectively, in the diabetic patient group, and 100%, 100%, 96.7%, 92.5%, and

Table 4. Characteristics of Patients With HBV- or HCV-Related HCC

	Patients With HBV-Related HCC			Patients With HCV-Related HCC		
Characteristic	With Diabetes (N = 8)	Without Diabetes (N = 17)	*P* Value	With Diabetes (N = 22)	Without Diabetes (N = 40)	*P* Value
Median age (yr)	61.6	57.3	0.3	62.2	62.9	0.737
Gender (male/female)	7/1	13/4	0.5	17/5	35/5	0.302
Body mass index (kg/m^2)	23.4	23.2	0.9	23.5	22.7	0.348
Alcohol abuse (+/−)	2/6	6/11	0.6	11/11	21/19	0.853
HbA1c (%)	5.9	4.6	0.07	6.6	4.9	<0.001
Fibrosis (F1/F2/F3/F4)	0/0/2/6	3/2/0/12	0.8	0/4/2/16	1/3/5/31	0.680
Child-Pugh grade (A/B)	6/2/0	15/2	0.4	18/4	35/5	0.551
Platelet count (× 10^4/μL)	11.5	13.9	0.3	12.4	13.5	0.520
ALT (IU/L)	31.8	42.3	0.6	84.2	63.5	0.233
Total bilirubin (mg/dL)	0.9	0.9	1.0	0.9	0.8	0.303
Albumin (g/dL)	3.8	4.2	0.02	4.2	4.2	0.983
AFP (ng/mL)	1,056	121	0.001	162	328	0.460
Tumor size (mm)	28.9	31.2	0.8	36.0	29.6	0.295
Diff.degree (W/M/P)	2/2/4	5/7/5	0.3	9/10/3	14/11/15	0.058
Vascular invasion (+/−)	1/7	4/13	0.5	12/10	15/25	0.548

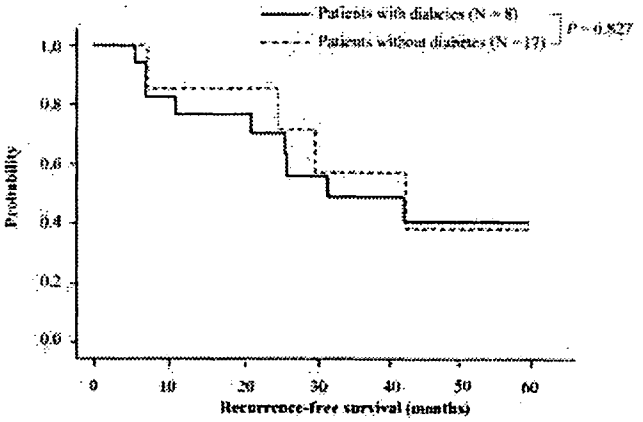

Figure 3. Kaplan-Meier curves for recurrence-free survival in HBV patients with diabetes and HBV patients without diabetes.

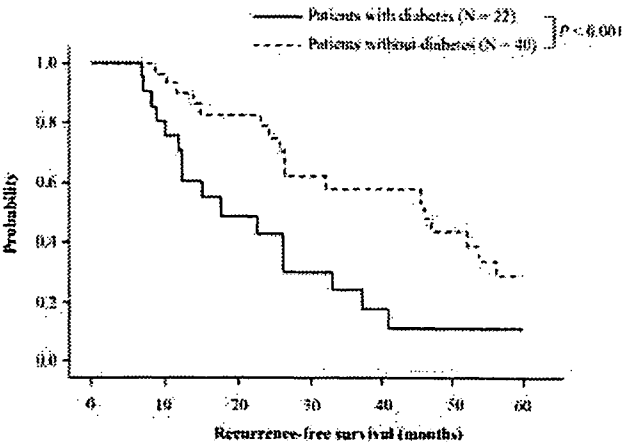

Figure 5. Kaplan-Meier curves for recurrence-free survival in HCV patients with diabetes and HCV patients without diabetes.

82.6%, respectively, in the nondiabetic patient group, indicating that the overall survival rates of more than 3 yr were significantly lower than in the nondiabetic patient group ($P = 1.000$, $P = 1.000$, $P = 0.035$, $P = 0.015$, $P = 0.004$) (Fig. 6).

Factors Associated With Recurrence-Free Survival After Surgical Treatment for HCC in Patients With Diabetes

Finally, we performed univariate and multivariate analyses to determine the variables that might affect the postoperative recurrence of HCC in the 30 HCC patients with diabetes, consisting of 17, 4, and 9 patients receiving insulin therapy, oral hypoglycemic drugs, and no treatment, respectively. Univariate analysis identified Child-Pugh classification B as a factor significantly contributing to the postoperative recurrence of HCC ($P < 0.001$) (Table 5). When we conducted multivariate analysis, we chose variables that had been already pointed out as a risk factor for HCC recurrence and whose P value was lower than 0.1 in univariate analysis. Multivariate analysis identified Child-Pugh classification B (risk 40.0, 95% CI 4.4–362.1, $P = 0.001$) and the presence of insulin therapy (risk 3.9, 95% CI 1.0–15.3, $P = 0.049$) as factors significantly contributing to the postoperative recurrence of HCC (Table 6). In multivariate analysis, both factors showed significant P value. Based on the results, we considered that both factors contribute to recurrence of HCC independently.

DISCUSSION

In the present study, univariate and multivariate analyses identified the presence of diabetes as a factor significantly contributing to the recurrence of HCC after surgical treatment. The results are consistent with the findings of Ikeda *et al.* (12). They analyzed a population of 64 HBV-related HCC patients and a larger population of 144 HCV-related HCC patients, but did not compare the postoperative recurrence rate between the two populations. In our study, 25 and 62 patients with HBV- and HCV-related HCC, respectively, were included, similar to the proportion of such patients in the study by Ikeda *et al.* (12), presumably leading to similar results. On the other hand, none of the variables that have been reported to contribute to the postoperative recurrence of HCC, such as liver fibrosis degree, Alb level, AFP level, tumor differentiation degree,

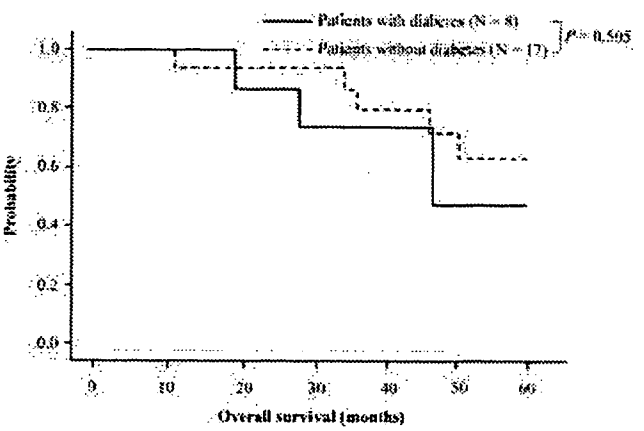

Figure 4. Kaplan-Meier curves for overall survival in HBV patients with diabetes and HBV patients without diabetes.

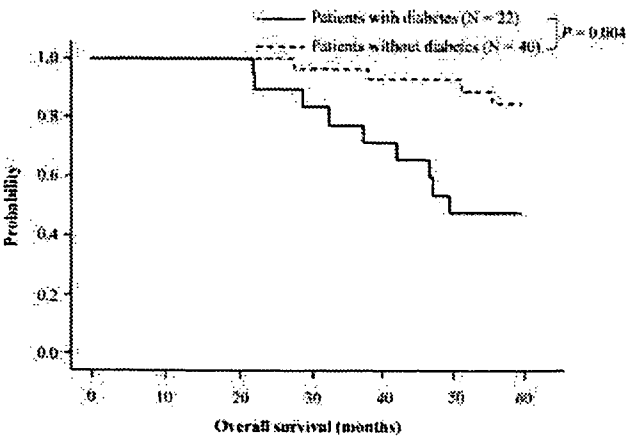

Figure 6. Kaplan-Meier curves for overall survival in HCV patients with diabetes and HCV patients without diabetes.

Table 5. Univariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment in Diabetic Patients

Variable	Hazard Ratio	95% CI	*P* Value
Age (yr)	1.0	1.0–1.1	0.534
Gender (male)	0.8	0.3–2.2	0.681
Etiology (HCV)	0.5	0.2–1.4	0.183
Body mass index (>25 kg/m^2)	1.0	0.4–2.6	0.938
Alcohol abuse (+)	1.0	0.4–2.3	0.972
HbA1c (%)	1.4	1.0–1.9	0.059
Fibrosis (F4)	1.2	0.5–3.3	0.651
Child-Pugh grade (A/B)	11.8	3.2–43.9	<0.001
Platelet count (× 10^4/μL)	1.0	0.9–1.1	0.548
ALT(IU/L)	1.0	1.0–1.0	0.699
Total bilirubin (mg/dL)	0.6	0.2–2.5	0.506
Albumin (g/dL)	1.2	0.5–2.9	0.689
AFP (>200 ng/dL)	0.9	0.4–2.3	0.899
Tumor size (>50 mm)	0.8	0.7–1.2	0.668
Diff degree (W/M/P)	0.9	0.4–2.0	0.723
Vascular invasion	0.9	0.4–2.2	0.843
Insulin therapy	2.5	1.0–6.6	0.058

and the presence of vascular invasion, were identified as significant factors. This is probably because of our criteria for surgical treatment and that patients who recurred within 6 months after surgery were excluded from the study, resulting in the inclusion of only a population with little variation in these variables.

Next, groups of patients with HBV- and HCV-related HCC were separately examined for the impact of diabetes on the recurrence of HCC after surgical treatment. No significant differences in the recurrence-free survival rates determined by the Kaplan-Meier curve were noted between the HBV-related HCC patient groups with and without diabetes, which was similar to the results reported by Poon *et al.* (13), Toyoda *et al.* (14), and Huo *et al.* (20). In contrast, the recurrence-free survival rate was significantly lower in the group of HCV-related HCC patients with diabetes than in the group of HCV-related HCC patients without diabetes. From the above findings, we concluded that the coexistence of diabetes was a factor contributing to the recurrence of HCC after surgical treatment in HCV-related HCC patients, and that the results of analysis of all HCC patients reflected those in the HCV-related HCC patients. In addition, the results of the analysis for the prognosis of HCV-related HCC patients after surgical treatment showed that the overall survival rate was significantly lower in the diabetic patient group than in the nondiabetic group. These results suggest that more frequent recurrence may contribute to shorter survival in HCV-related HCC patients with diabetes.

Table 6. Multivariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment in Diabetic Patients

Variable	Hazard Ratio	95% CI	*P* Value
Insulin therapy (+)	3.9	1.0–15.3	0.049
Fibrosis (F4)	2.2	0.5–9.8	0.306
Child-Pugh grade (B)	40.0	4.4–362.1	0.001
AFP (>200 ng/mL)	2.1	0.5–8.8	0.289
Diff degree (P)	0.6	0.1–2.8	0.542
Vascular invasion (+)	1.7	0.4–7.6	0.513
Etiology (HCV)	2.0	0.3–12.2	0.460
HbA1c (%)	1.1	0.8–1.6	0.629

To our knowledge, only one study has examined the impact of diabetes on the recurrence of HCC after surgical treatment separately in HBV- and HCV-related HCC patients. Contrary to the results of this study, Huo *et al.* (20) have reported that diabetes is not a risk factor for the recurrence of HCV-related HCC. The clinical characteristics of HCC patients, such as the number of tumors, tumor diameter, and background liver histology, differed between their study and ours, and the presence or absence of vascular invasion and hepatic reserve indicated by Child-Pugh classification were unknown in their study, which makes direct comparison difficult, but partially accounts for the different results. Although, to date, no studies have reported that, as shown in this study, there is a possibility that diabetes differently affects the postoperative recurrence of HCC in the groups of patients with HBV- or HCV-related HCC.

This may be because of different mechanisms of carcinogenesis in the two groups (21). It appears that neither HBV nor HCV damages liver cells, but these viruses induce chronic inflammation in the liver, and facilitate mutations in liver cells, leading to their malignant transformation (22, 23). Our previous study using the microarray technique showed that the genes expressed in the liver differed markedly between HBV- and HCV-related liver disease patients (24). This genetic heterogeneity is considered to be associated with different modes of pathogenesis of HBV- and HCV-related HCC (25–28). Previous studies have shown that, in HCV-related HCC, chronic inflammation and oxidative stress are closely associated with hepatocellular death and regeneration (29–33). Highly insulin-resistant diabetics show increased peripheral lipolysis and hepatic accumulation of free fatty acids (34, 35). The β-oxidation of fatty acids in mitochondria is decreased in these patients, and they are under high oxidative stress. We also previously reported that the gene expression profile in the liver of diabetic patients shows increasing fibrogenic, angiogenic, tumorigenic, and stress responsive factors (36). Taken together, these observations suggest that the coexistence of diabetes promotes the progression of liver fibrosis and development of HCC in HCV-related liver disease (37). In contrast, in HBV-related liver disease, integration of the virus genome into the host DNA appears to induce HCC (38–40). Therefore, in such a mechanism of carcinogenesis, the coexistence of diabetes may have little synergistic effect.

Finally, we examined variables that might contribute to the recurrence of HCC after surgical treatment in HCC patients with diabetes. Since insulin therapy is often administered to diabetic patients who have difficulty in controlling blood sugar, or who have advanced liver disease, this factor may be involved in the recurrence of HCC in those under insulin therapy. Therefore, we included HbA1c, liver fibrosis degree,

and Child-Pugh classification together with insulin therapy in multivariate analysis. As a result, multivariate analysis identified Child-Pugh classification B and insulin therapy as significant factors contributing to the postoperative recurrence of HCC. These findings suggest that insulin therapy and Child-Pugh classification B are independent risk factors for postoperative recurrence.

The mechanism by which insulin promotes HCC recurrence is unknown. However, the results of this study are consistent with the report that insulin acts as a tumor growth factor *in vitro* (41). In animal models, insulin has been shown to be a promoter of colonic carcinogenesis (42). Although there has been much debate about the use of insulin and the risk of cancer development, no consensus has been reached (43–45). A recent study has indicated that insulin therapy is a risk factor for the postoperative recurrence of colorectal cancer (46). These findings show the possibility that insulin therapy promotes HCC recurrence after surgical treatment. It should be discussed how to use insulin therapy in HCC patients with diabetes in the future.

There is a limitation to our study, because our study is retrospective and on a not so large population. However, the results of the present study suggest that diabetes is a risk factor for the recurrence of HCV-related HCC and decreases the overall survival rates after surgical treatment. HCV-related HCC patients with diabetes should be closely followed for post-treatment recurrence, and blood sugar control may also be important to reduce the rate of recurrence. However, since the use of insulin to treat diabetes in HCC patients may promote tumor recurrence, treatment methods for blood sugar control require further evaluation.

STUDY HIGHLIGHTS

What Is Current Knowledge

- Diabetes accumulates liver fibrosis for chronic hepatitis C.
- Diabetes is a risk factor for hepatocellular carcinoma (HCC).
- HCC has high recurrence rate after curative surgery.

What Is New Here

- Hepatitis C virus (HCV) related patients with diabetes have a higher possibility of HCC recurrence.
- HCV-related patients with diabetes have poorer prognosis.
- Controlling of blood sugar may reduce HCC recurrence.
- Insulin therapy may accumulate HCC recurrence.

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REFERENCES

1. Bosch FX, Ribes J, Borras J. Epidemiology of primary liver cancer. Semin Liver Dis 1999;19:271–85.
2. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745–50.
3. Heathcote EJ. Prevention of hepatitis C virus-related hepatocellular carcinoma. Gastroenterology 2004;127:S294–302.
4. Yamamoto J, Kosuge T, Takayama T, et al. Recurrence of hepatocellular carcinoma after surgery. Br J Surg 1996;83:1219–22.
5. Poon RT, Fan ST, Lo CM, et al. Intrahepatic recurrence after curative resection of hepatocellular carcinoma: Long-term results of treatment and prognostic factors. Ann Surg 1999;229:216–22.
6. Adachi E, Maeda T, Matsumata T, et al. Risk factors for intrahepatic recurrence in human small hepatocellular carcinoma. Gastroenterology 1995;108:768–75.
7. Ikeda K, Saitoh S, Tsubota A, et al. Risk factors for tumor recurrence and prognosis after curative resection of hepatocellular carcinoma. Cancer 1993;71:19–25.
8. Predictive factors for long term prognosis after partial hepatectomy for patients with hepatocellular carcinoma in Japan. The Liver Cancer Study Group of Japan. Cancer 1994;74:2772–80.
9. Koike Y, Shiratori Y, Sato S, et al. Risk factors for recurring hepatocellular carcinoma differ according to infected hepatitis virus–an analysis of 236 consecutive patients with a single lesion. Hepatology 2000;32:1216–23.
10. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology 2004;126:460–8.
11. Davila JA, Morgan RO, Shaib Y, et al. Diabetes increases the risk of hepatocellular carcinoma in the United States: A population based case control study. Gut 2005;54:533–39.
12. Ikeda Y, Shimada M, Hasegawa H, et al. Prognosis of hepatocellular carcinoma with diabetes mellitus after hepatic resection. Hepatology 1998;27:1567–71.
13. Poon RT, Fan ST, Wong J. Does diabetes mellitus influence the perioperative outcome or long term prognosis after resection of hepatocellular carcinoma? Am J Gastroenterol 2002;97:1480–8.
14. Toyoda H, Kumada T, Nakano S, et al. Impact of diabetes mellitus on the prognosis of patients with hepatocellular carcinoma. Cancer 2001;91:957–63.
15. Araki T, Itai Y, Furui S, et al. Dynamic CT densitometry of hepatic tumors. AJR Am J Roentgenol 1980;135:1037–43.
16. Japan. LCSGo. Classification of primary liver cancer, English 2nd Ed. Tokyo: Kanehara & Co., Ltd., 1997.
17. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997;20:1183–97.
18. Desmet VJ, Gerber M, Hoofnagle JH, et al. Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 1994;19:1513–20.
19. Katsuki A, Sumida Y, Gabazza E, et al. Homeostasis model assessment is a reliable indicator of insulin resistance during follow-up of patients with type 2 diabetes. Diabetes Care 2001;24:362–5.
20. Huo TI, Wu JC, Lui WY, et al. Diabetes mellitus is a recurrence-independent risk factor in patients with hepatitis B virus-related hepatocellular carcinoma undergoing resection. Eur J Gastroenterol Hepatol 2003;15:1203–8.
21. Szabo E, Paska C, Kaposi Novak P, et al. Similarities and differences in hepatitis B and C virus induced hepatocarcinogenesis. Pathol Oncol Res 2004;10:5–11.

22. Brechot C. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: Old and new paradigms. Gastroenterology 2004;127:S56–61.
23. Liang TJ, Heller T. Pathogenesis of hepatitis C-associated hepatocellular carcinoma. Gastroenterology 2004;127:S62–71.
24. Honda M, Kaneko S, Kawai H, et al. Differential gene expression between chronic hepatitis B and C hepatic lesion. Gastroenterology 2001;120:955–66.
25. Edamoto Y, Hara A, Biernat W, et al. Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. Int J Cancer 2003;106:334–41.
26. Iizuka N, Oka M, Yamada-Okabe H, et al. Differential gene expression in distinct virologic types of hepatocellular carcinoma: Association with liver cirrhosis. Oncogene 2003;22:3007–14.
27. Laurent-Puig P, Legoix P, Bluteau O, et al. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. Gastroenterology 2001;120:1763–73.
28. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339–46.
29. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med 2001;345:41–52.
30. Sung VM, Shimodaira S, Doughty AL, et al. Establishment of B-cell lymphoma cell lines persistently infected with hepatitis C virus in vivo and in vitro: The apoptotic effects of virus infection. J Virol 2003;77:2134–46.
31. Guo JT, Zhou H, Liu C, et al. Apoptosis and regeneration of hepatocytes during recovery from transient hepadnavirus infections. J Virol 2000;74:1495–505.
32. Felsher DW, Bishop JM. Reversible tumorigenesis by MYC in hematopoietic lineages. Mol Cell 1999;4:199–207.
33. Shiratori Y, Shiina S, Teratani T, et al. Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. Ann Intern Med 2003;138:299–306.
34. Pessayre D, Berson A, Fromenty B, et al. Mitochondria in steatohepatitis. Semin Liver Dis 2001;21:57–69.
35. Chitturi S, Farrell GC. Etiopathogenesis of nonalcoholic steatohepatitis. Semin Liver Dis 2001;21:27–41.
36. Takamura T, Sakurai M, Ota T, et al. Genes for systemic vascular complications are differentially expressed in the livers of type 2 diabetic patients. Diabetologia 2004;47:638–47.
37. Fong DG, Nehra V, Lindor KD, et al. Metabolic and nutritional considerations in nonalcoholic fatty liver. Hepatology 2000;32:3–10.
38. Brechot C, Pourcel C, Louise A, et al. Presence of integrated hepatitis B virus DNA sequences in cellular DNA of human hepatocellular carcinoma. Nature 1980;286:533–5.
39. Kim CM, Koike K, Saito I, et al. HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 1991;351:317–20.
40. Bressac B, Galvin KM, Liang TJ, et al. Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. Proc Natl Acad Sci U S A 1990;87:1973–7.
41. Giovannucci E. Nutrition, insulin, insulin-like growth factors and cancer. Horm Metab Res 2003;35:694–704.
42. Tran TT, Medline A, Bruce WR. Insulin promotion of colon tumors in rats. Cancer Epidemiol Biomarkers Prev 1996;5:1013–5.
43. Swerdlow AJ, Laing SP, Qiao Z, et al. Cancer incidence and mortality in patients with insulin-treated diabetes: A UK cohort study. Br J Cancer 2005;92:2070–5.
44. Schiel R, Muller UA, Braun A, et al. Risk of malignancies in patients with insulin-treated diabetes mellitus – results of a population-based trial with 10-year follow-up (JEVIN). Eur J Med Res 2005;10:339–44.
45. Chuang TY, Lewis DA, Spandau DF. Decreased incidence of nonmelanoma skin cancer in patients with type 2 diabetes mellitus using insulin: A pilot study. Br J Dermatol 2005;153:552–7.
46. Yang YX, Hennessy S, Lewis JD. Insulin therapy and colorectal cancer risk among type 2 diabetes mellitus patients. Gastroenterology 2004;127:1044–50.

CONFLICT OF INTEREST

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Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon

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Abstract **We developed a reverse genetics system of hepatitis C virus (HCV) genotypes 1a and 2a using infectious clones and human hepatocyte chimeric mice. We inoculated cell culture-produced genotype 2a (JFH-1) HCV intravenously. We also injected genotype 1a CV-H77C clone RNA intrahepatically. Mice inoculated with HCV by both procedures developed measurable and transmissible viremia. Interferon (IFN) alpha treatment resulted in greater reduction of genotype 2a HCV levels than genotype 1a, as seen in clinical practice. Genetically engineered HCV infection system should be useful for analysis of the mechanisms of resistance of HCV to IFN and other drugs.**

Keywords: Human hepatocyte chimeric mouse; Human serum albumin; HCV RNA; Interferon

1. Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1]. HCV causes persistent infection in adults leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. The most effective therapy for viral clearance is a 48-week combination therapy of pegylated interferon (IFN)-alpha and ribavirin. However, the success rate of this combination therapy is only about 50% [4]. Development of new anti-HCV drug had been severely restricted by the absence of a cell culture system that supports the efficient replication of HCV, as well as the lack of a small animal model. A cell culture system has been developed recently using a unique genotype 2a HCV gnome (JFH-1), which does not require adaptive mutations for efficient replication [5–7]. Chimpanzee was the only useful animal for the study of HCV until recently, although the availability of this model is severely restricted [8]. Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into chimeric urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice with engrafted human hepatocytes [9]. This HCV-infected mouse model has been reported to be useful for evaluating anti-HCV drugs such as IFN-alpha and anti-NS3 protease [10]. We have generated a human hepatocyte chimeric mouse where mouse hepatocytes were extensively replaced by human hepatocytes [11], and established a genetically engineered hepatitis B virus (HBV) system [12]. Using this mouse, we show in this paper the development of reverse genetics system of genotypes 1a and 2a after intrahepatic injection of transcribed RNA and intravenous injection of cell culture-produced virus, respectively. We also show here that HCV in these mice can be transmitted to naïve mice. Interferon treatment of these mice resulted in a greater reduction of HCV titer in genotype 2a clone infected mice than in genotype 1a infected mice. As these results are consistent with our clinical experience, we consider this model suitable for the study of resistance of HCV against IFN and other drugs.

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator

2. Materials and methods

2.1. Generation of human hepatocyte chimeric mice and quantification of human serum albumin

Generation of the uPA$^{+/+}$/SCID$^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group [11,12]. All mice used in this study were transplanted with frozen

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human hepatocytes obtained from one donor. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index [11], and were measured as described previously [12]. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Graduate School of Biomedical Sciences, Hiroshima University.

2.2. HCV RNA transcription and inoculation into chimeric mice

A plasmid containing the full-length genotype 1a HCV cDNA clone, pCV-H77C, was kindly provided by Dr. Robert H. Purcell (National Institutes of Health). Ten micrograms of plasmid DNA, linearized by *XbaI* (Promega, Madison, WI) digestion, was transcribed in a 100-μl reaction volume with T7 RNA polymerase (Promega) at 37 °C for 2 h [13], and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μl of phosphate-buffered saline (PBS) and injected into the liver of chimeric mice. Transcripts of plasmid pJFH-1 containing the full-length HCV genotype 2a were transfected into Huh7 cells as described previously [6]. Seventy-two hours after transfection, 200 μl of the culture medium was injected intravenously into the chimeric mice. IFN-treatment was also performed by intramuscular injection of diluted IFN solutions. IFN-alpha was a kind gift from Hayashibara Biochemical Labs, Inc. (Okayama, Japan). Serum samples collected every 2 weeks after inoculation were frozen at −80 °C until further analysis.

2.3. Human serum samples

For control infection experiments, human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis after obtaining a written informed consent. The individual serum samples were divided into small aliquots and separately stored in liquid nitrogen until use.

2.4. RNA extraction and amplification

RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μl RNase-free H_2O, and reverse transcribed by using a random primer (Takara Bio, Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in a 20 μl reaction mixture according to the instructions provided by the manufacturer. One microliter of cDNA solution was amplified by Light Cycler (Roche Diagnostic, Japan, Tokyo) for quantitation of HCV. The primers used for amplification were 5′-TTTATCCAAGAAAGGACCC-3′ and 5′-TTCACGCAGAAAGCGTCTAGC-3′. The amplification conditions included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 5 s, and extension at 72 °C for 6 s. The lower detection limit of this assay is 10^3 copies/ml. Nested PCR was used with the outer primers NC1 (5′-CAACACTACTCGGCTAGCAGT-3′) and NC2 (5′-CCTGTGAGGAACTACTGTC-3′) and inner primers cc6 (5′-TTTATCCAAGAAAGGACCC-3′) and cc7 (5′-TTCACGCAGAAAGCGTCTAGCttc-3′). The amplification condition included 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s, and 72 °C for 1 min after 5 min of initial denaturation at 94 °C followed by 7 min of final extension using Gene Taq (Wako Pure Chemicals, Tokyo) with anti-Taq high according to the instructions provided by the manufacturer (TOYOBO).

2.5. Histochemical analysis of mouse liver

Histopathological analysis and immunohistochemical staining using an antibody against HSA (Bethyl Laboratories Inc.) were performed as described previously [12].

3. Results

3.1. High serum HCV RNA titer in human hepatocyte chimeric mice after inoculation of serum samples obtained from HCV-infected patient

We inoculated 50 μl of genotype 1b serum samples into five chimeric mice intravenously to test their susceptibility to HCV infection. All mice became positive for HCV RNA by nested PCR at 2 weeks after inoculation (Fig. 1). The viremia reached a plateau level at 6–8 weeks after infection, and persisted for more than 12 weeks.

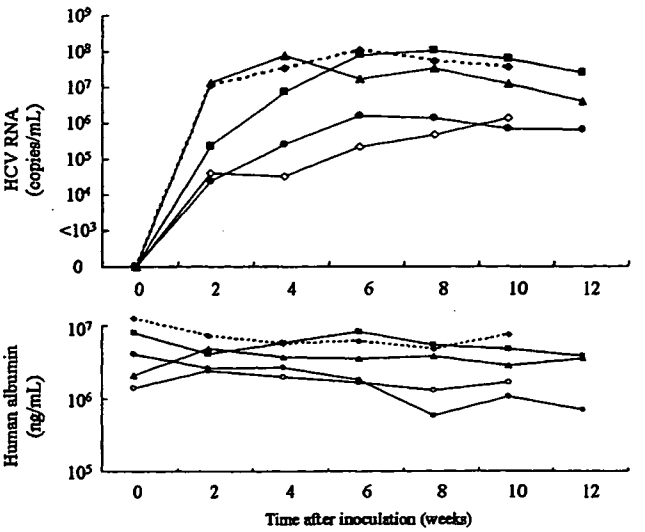

Fig. 1. Serial changes in HCV RNA and human serum albumin in sera of mice inoculated with human serum samples positive for genotype 1b HCV. Fifty microliter serum samples were injected intravenously into each mouse. Mice serum samples were obtained every 2 weeks after injection, and HCV RNA titer was analyzed.

3.2. Infection with in vitro-transcribed genotype 1a HCV RNA and cell culture generated genotype 2a HCV

In the next step, we tried to establish infection of cloned HCV using infectious genotype 1a and genotype 2a clones. In these experiments, we used two different strategies to establish infection using these two clones because genotype 1a has not been confirmed to replicate in cell culture system. We used genotype 1a HCV RNA (CV-H77C), which has been reported to be infectious to chimpanzee [13]. In vitro-transcribed HCV RNA was directly injected intrahepatically in three chimeric mice. We also infected three chimeric mice by intravenous injection of Huh7 cell-produced genotype 2a HCV after transfection of in vitro transcribed RNA from an infectious clone JFH-1. This clone has been shown to be infectious to a chimpanzee [6] and a chimeric mouse [7]. All mice developed measurable viremia 2 weeks after inoculation. At 6 weeks after inoculation, HCV RNA titer was 2.4×10^7 copies/ml (range: 8.8×10^6–2.9×10^7 copies/ml) in genotype 1a HCV-infected mice, and 2.5×10^5 copies/ml (range: 1.4×10^5–3.7×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 2).

3.3. Passage experiment of HCV to naïve chimeric mice

We then performed passage experiments using naïve mice. Each of three mice was inoculated intravenously with 10 μl serum samples obtained from the above genotype 1a and genotype 2a HCV-infected mice at week 6. Two weeks after injection, all mice developed measurable viremia, and the titer was 8.5×10^6 copies/ml (range: 1.4×10^6–2.4×10^7 copies/ml) in genotype 1a, and 1.7×10^5 copies/ml (range: 1.5×10^5–2.5×10^5 copies/ml) in genotype 2a HCV-infected mice (Fig. 3).

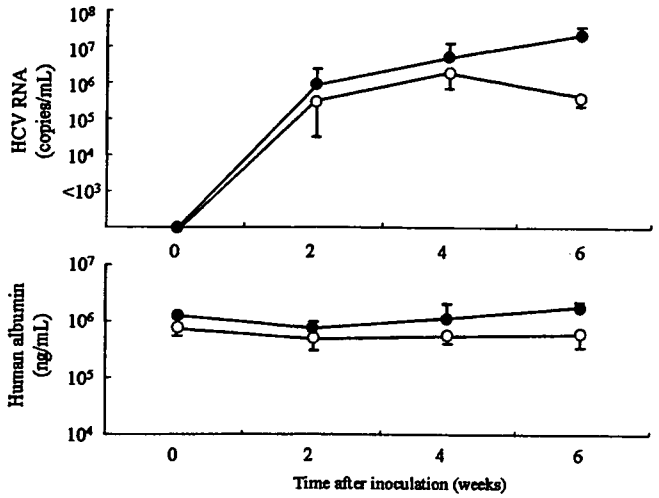

Fig. 2. Changes in HCV RNA and human albumin concentrations in serum of mice infected with clonal HCV. Each of three mice were inoculated intrahepatically with in vitro transcribed genotype 1a HCV RNA (closed circles) or intravenously with a culture medium collected from Huh7 cells transfected with JFH-1 genome intravenously (open circles). Data are mean ± S.D.

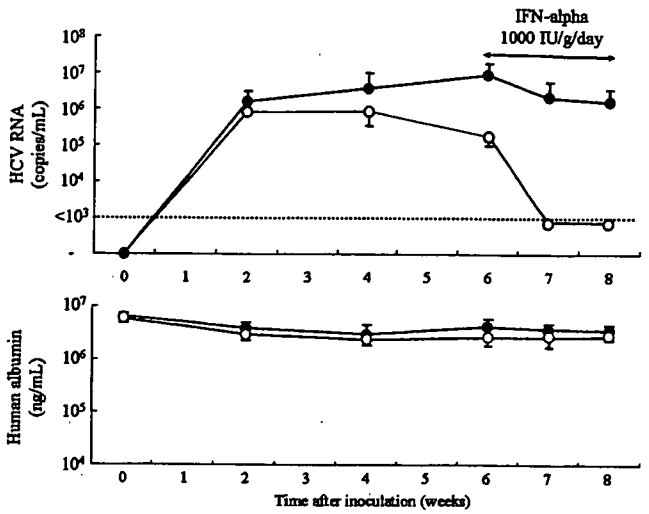

Fig. 3. Passage experiment and response to IFN-alpha therapy in mice infected with HCV genotypes 1a and 2a clones. Serum samples (10 μl) obtained from genotype 1a and 2a clonal HCV-infected mice sera (see Fig. 2) were inoculated intravenously into each of three naïve chimeric mice. Six weeks after infection, all six mice were injected intramuscularly with 1000 IU/g/day of IFN-alpha daily for 2 weeks. Closed circles: genotype 1a HCV-infected mice, open circles: genotype 2a HCV-infected mice. Data are mean ± S.D.

3.4. Variable susceptibility of HCV clones to IFN therapy

We treated each of the three mice infected with genotype 1a and 2a clones by passage experiments with 1000 IU/g of IFN-alpha daily for 2 weeks. Such treatment induced only a slight decrease in HCV in genotype 1a-infected mice; the viral load decreased only 0.6 and 0.7 log after 1 and 2 weeks of treatment, respectively (Fig. 3). In contrast, the same treatment reduced HCV genotype 2a RNA to undetectable levels after 1 and 2 weeks of IFN therapy. During IFN-treatment, serum HSA levels did not decrease in mice infected with genotype 1a or 2a HCV. Histopathological examination showed no morphological changes or apoptotic hepatocytes in replaced

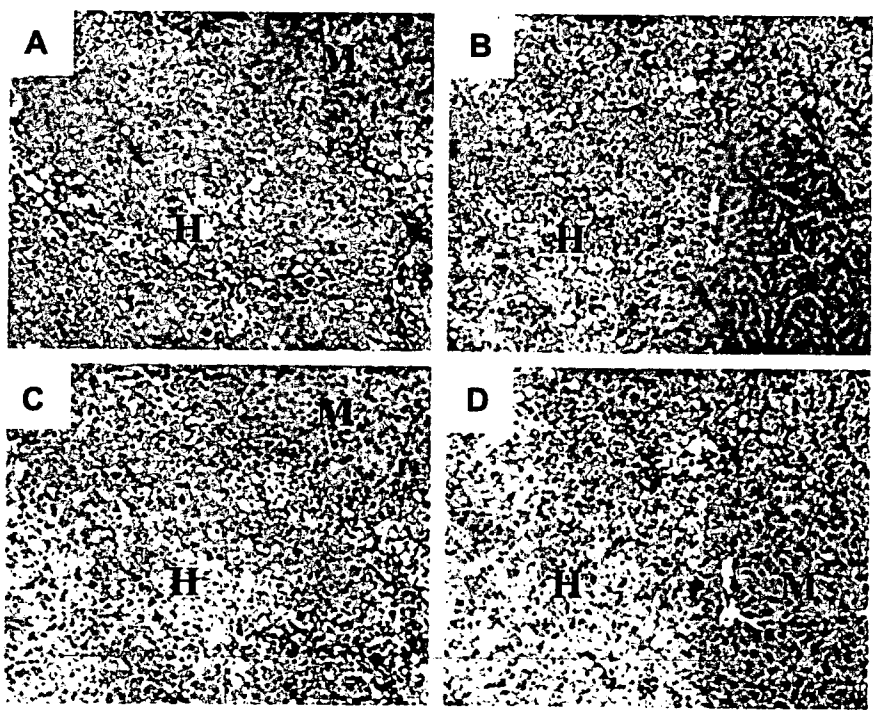

Fig. 4. Histochemical analysis of the tissues of infected chimeric mice. Liver samples obtained from mice infected with genotype 1a (A, C) and genotype 2a (B, D) stained with hematoxylin–eosin staining (A, B) or by immunohistochemical staining with anti-human serum albumin antibody (C, D). Regions are shown as human (H) and mouse (M) hepatocytes, respectively. (Original magnification, ×100.)

human hepatocytes in mice infected with each genotype after 2-week IFN-treatment (Fig. 4). These results suggest that the decrease in HCV is due to the direct anti-viral effect of IFN and not induced by liver cell damage. The difference in the virus titer and susceptibility to IFN are considered to be due to the characteristics of the genotypes.

4. Discussion

In this study, we established a reverse genetics system of HCV genotype 1a and 2a clones using human hepatocyte chimeric mice. The HCV genotype 2a clone, JFH-1, has remarkable features, i.e., infects cultured Huh7 cell line as well as establish infection in chimeric mouse [7]. It has been reported that HCV genotype 1a clone, H77-S, also infects Huh7 cell line and produces infectious virion [14]. In the present study, we intrahepatically inoculated genotype 1a infectious clone, CV-H77C. As reported in chimpanzee [13,15–17], we were able to establish genotype 1a infection using human hepatocyte chimeric mice. Using this technique, it is hoped that we can conduct further experiments in the future using genetically engineered HCV clones. Experiments using chimeric clone described by Lindenbach et al. [7] should also provide further information regarding the variable replication property of HCV genomes. Modifying genomes with nucleotide substitutions allowed examination of the functions of HCV peptides as we showed with HBV [12].

As reported recently by Kneteman et al. [10], the mouse model system is useful for evaluating the effect of anti-HCV drugs such as IFN, protease inhibitors and polymerase inhibitors. As we showed in this study, the response to IFN therapy varied according to HCV genotype. Further experiments are necessary to determine whether differences in response to IFN are due to the different replication ability (replication level of genotype 2a clone was slightly lower than that of genotype 1b, see Figs. 2 and 3) or differences in genotypes, as has been reported in clinical studies [18]. As we showed in this study (Fig. 4), there is no hepatocyte damage or inflammation in the liver of the infected chimeric mouse. Thus, this model is suitable for the study of mechanisms involved in HCV replication and IFN resistance.

The intrahepatic injection method used in this study simplified our experiments using genetically engineered virus. This is particularly important in studies of protease inhibitors and polymerase inhibitors because HCV will easily develop resistance against these small molecule agents.

Previous studies identified amino acid sequences that correlate with different susceptibilities of genotype 1b HCV against IFN therapy, namely, interferon sensitivity determining region [19] and the PKR-eIF2 phosphorylation homology domain [20,21]. To elucidate such issues, we are currently trying to establish genotype 1b infection system using the method described in this paper.

In summary, we showed in the present study the successful application of a genetically engineered HCV in human hepatocyte chimeric mice. Using this mouse model, we showed that genotypes 1a and 2a HCV clones exhibit different susceptibilities to IFN-alpha therapy. Our mouse model seems useful for the study of HCV virology and resistance of HCV against IFN and for the development of new anti-HCV therapy.

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References

[1] WHO. (1999) Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J. Viral. Hepat. 6, 35–47.

[2] Kiyosawa, K., Sodeyama, T., Tanaka, E., Gibo, Y., Yoshizawa, K., Nakano, Y., Furuta, S., Akahane, Y., Nishioka, K. and Purcell, R.H. (1990) Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 12, 671–675.

[3] Niederau, C., Lange, S., Heintges, T., Erhardt, A., Buschkamp, M., Hurter, D., Nawrocki, M., Kruska, L., Hensel, F., Petry, W. and Haussinger, D. (1998) Prognosis of chronic hepatitis C: results of a large, prospective cohort study. Hepatology 28, 1687–1695.

[4] Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales Jr., F.L., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J. and Yu, J. (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347, 975–982.

[5] Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T. and Chisari, F.V. (2005) Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA 102, 9294–9299.

[6] Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R. and Liang, T.J. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

[7] Lindenbach, B.D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A.J., McKeating, J.A., Lanford, R.E., Feinstone, S.M., Major, M.E., Leroux-Roels, G. and Rice, C.M. (2006) Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc. Natl. Acad. Sci. USA 103, 3805–3809.

[8] Shimizu, Y.K., Weiner, A.J., Rosenblatt, J., Wong, D.C., Shapiro, M., Popkin, T., Houghton, M., Alter, H.J. and Purcell, R.H. (1990) Early events in hepatitis C virus infection of chimpanzees. Proc. Natl. Acad. Sci. USA 87, 6441–6444.

[9] Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L. and Kneteman, N.M. (2001) Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 7, 927–933.

[10] Kneteman, N.M., Weiner, A.J., O'Connell, J., Collett, M., Gao, T., Aukerman, L., Kovelsky, R., Ni, Z.J., Zhu, Q., Hashash, A., Kline, J., His, B., Schiller, D., Douglas, D., Tyrrell, D.L. and Mercer, D.F. (2006) Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. Hepatology 43, 1346–1353.

[11] Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., Asahara, T., Yokoi, T., Furukawa, T. and Yoshizato, K. (2004) Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am. J. Pathol. 165, 901–912.

[12] Tsuge, M., Hiraga, N., Takaishi, H., Noguchi, C., Oga, H., Imamura, M., Takahashi, S., Iwao, E., Fujimoto, Y., Ochi, H., Chayama, K., Tateno, C. and Yoshizato, K. (2005) Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. Hepatology 42, 1046–1054.

[13] Yanagi, M., Purcell, R.H., Emerson, S.U. and Bukh, J. (1997) Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. Proc. Natl. Acad. Sci. USA 94, 8738–8743.

[14] Yi, M., Villanueva, R.A., Thomas, D.L., Wakita, T. and Lemon, S.M. (2006) Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA 103, 2310–2315.
[15] Kolykhalov, A.A., Agapov, E.V., Blight, K.J., Mihalik, K., Feinstone, S.M. and Rice, C.M. (1997) Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 277, 570–574.
[16] Yanagi, M., StClaire, M., Emerson, S.U., Purcell, R.H. and Bukh, J. (1999) In vivo analysis of the 3′ untranslated region of the hepatitis C virus after in vitro mutagenesis of an infectious cDNA clone. Proc. Natl. Acad. Sci. USA 96, 2291–2295.
[17] Beard, M.R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D.V. and Lemon, S.M. (1999) An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. Hepatology 30, 316–324.
[18] McHutchison, J.G., Gordon, S.C., Schiff, E.R., Shiffman, M.L., Lee, W.M., Rustgi, V.K., Goodman, Z.D., Ling, M.H., Cort, S. and Albrecht, J.K. (1998) Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N. Engl. J. Med. 339, 1485–1492.
[19] Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Ogura, Y., Izumi, N., Marumo, F. and Sato, C. (1996) Mutations in the non-structural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N. Engl. J. Med. 334, 77–81.
[20] Taylor, D.R., Shi, S.T., Romano, P.R., Barber, G.N. and Lai, M.M. (1999) Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 285, 107–110.
[21] Chayama, K., Suzuki, F., Tsubota, A., Kobayashi, M., Arase, Y., Saitoh, S., Suzuki, Y., Murashima, N., Ikeda, K., Takahashi, N., Kinoshita, M. and Kumada, H. (2000) Association of amino acid sequence in the PKR-eIF2 phosphorylation homology domain and response to interferon therapy. Hepatology 32, 1138–1144.

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

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

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

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

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

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

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

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

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

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

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

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

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

SUBJECTS, MATERIALS, AND METHODS

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

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

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

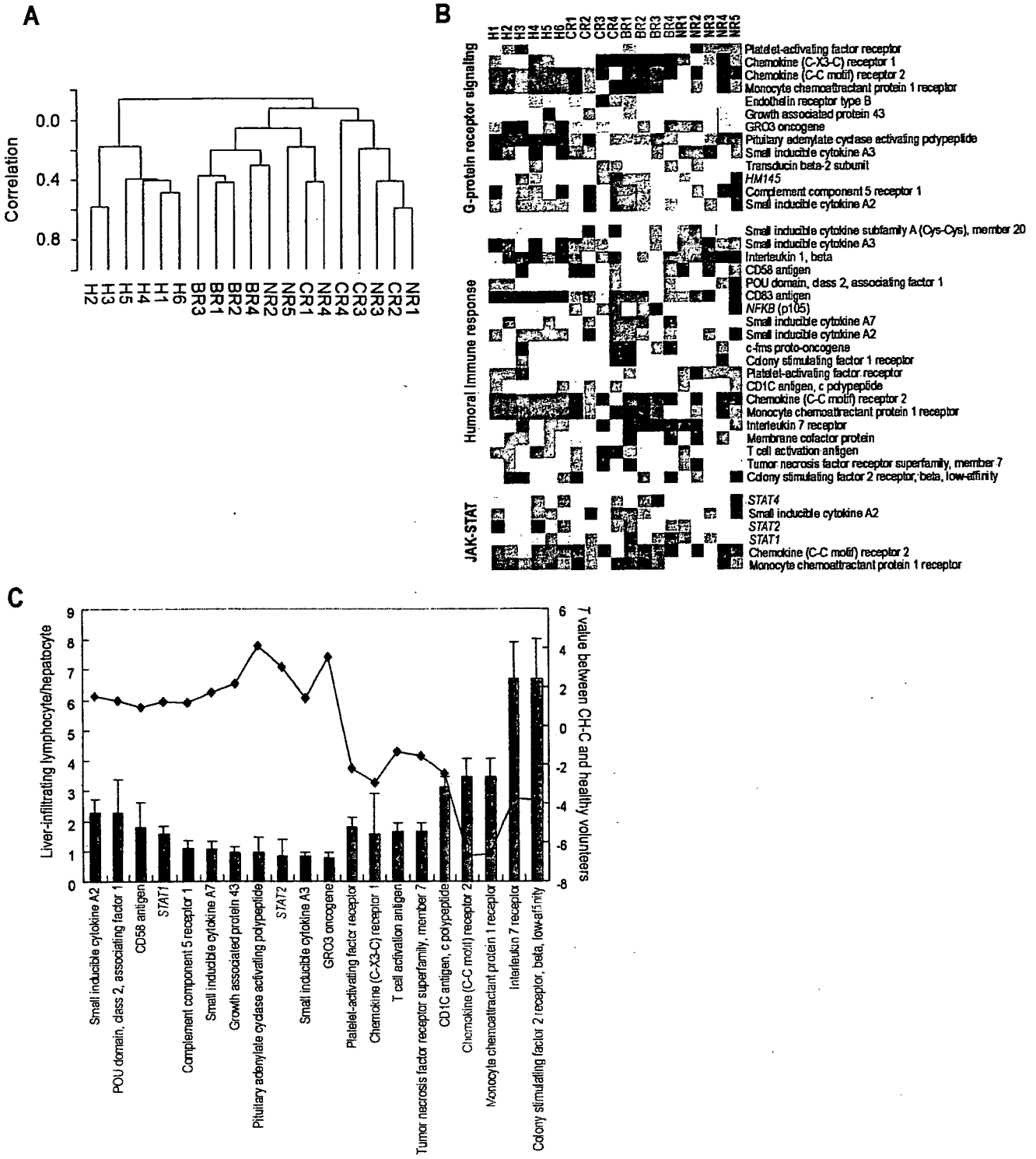

Figure 1. *A*, Hierarchical clustering analysis of gene-expression profiles of peripheral-blood mononuclear cell (PBMC) samples from 13 patients with chronic hepatitis C (CH-C; complete responders [CRs] 1–4, biochemical responders [BRs] 1–4, and nonresponders [NRs] 1–5) and 6 healthy volunteers (H1–H6)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

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

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

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

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

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

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

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

Gene-expression profiles of liver-infiltrating lymphocytes in

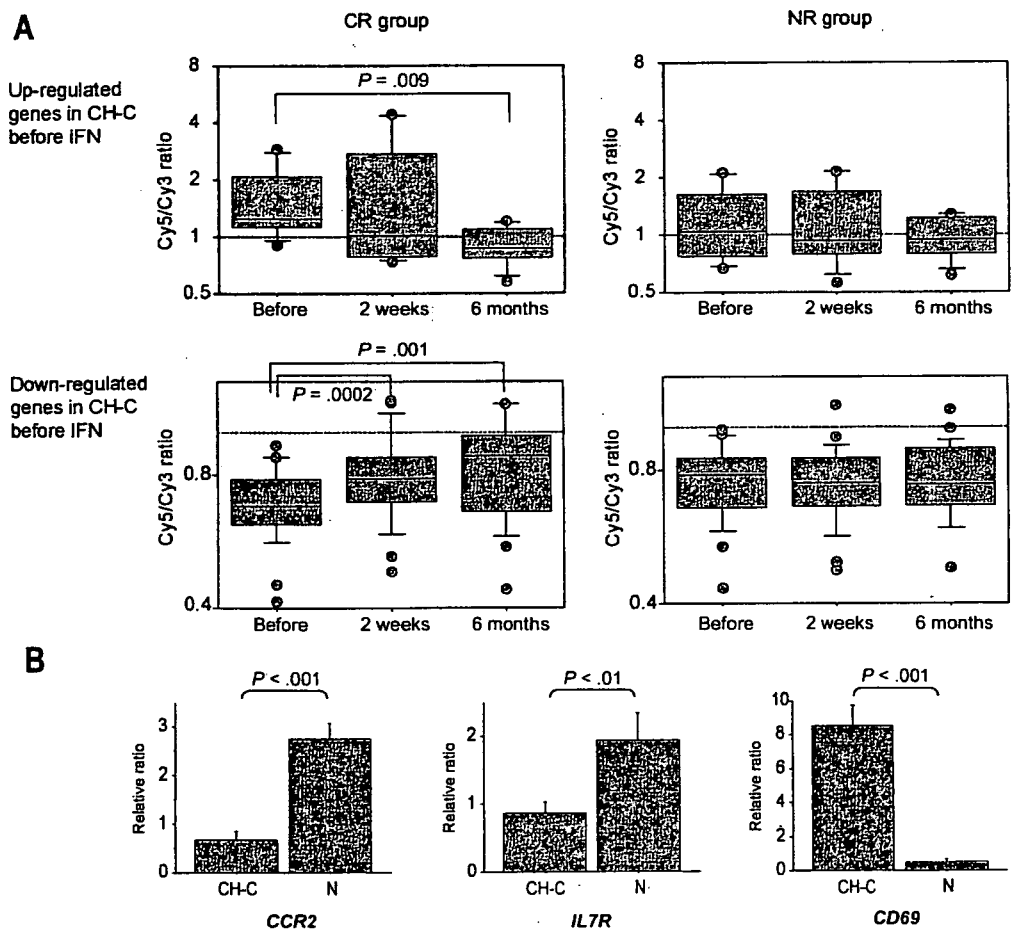

Figure 2. *A*, Changes in gene-expression profiles over the course of interferon (IFN) therapy (as shown in table 2) distinguishing patients with chronic hepatitis C (CH-C) from healthy volunteers before the start of IFN therapy. Box charts show average rates of change in relation to healthy volunteers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

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

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

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

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

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

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

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

RESULTS

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

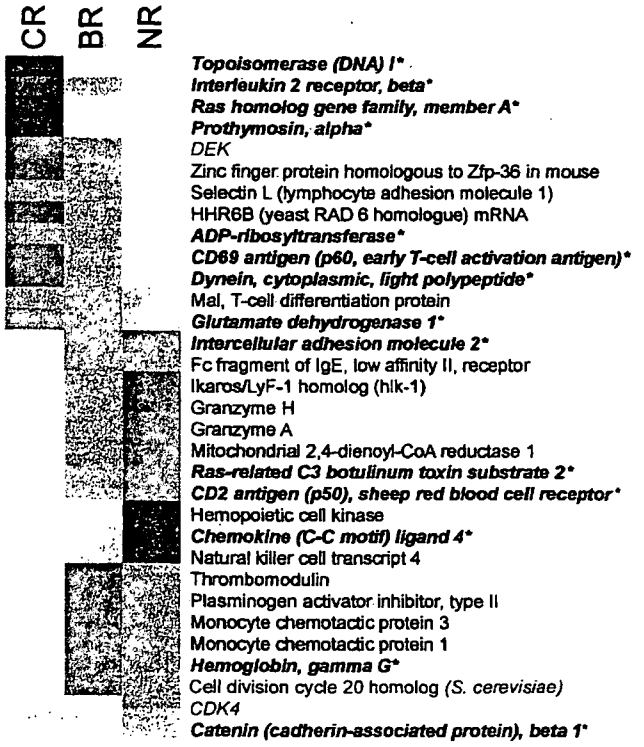

Figure 3. Thirty-two genes screened for gene-expression data before interferon (IFN) therapy by projective adaptive resonance theory. Red indicates up-regulated genes, and blue indicates down-regulated genes. Asterisks indicate genes that are included in table 4. BR, biochemical responder; CR, complete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

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

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

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

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

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