Table 1 Characteristics of 938 chronic hepatitis C genotype 1 patients treated with a combination of pegylated interferon plus ribavirin according to age (mean ± SD)

	Group A (age < 65 yr) (*n* = 685)	Group B (age ≥ 65 yr) (*n* = 253)	*P*-value
Age (yr)	53.1 ± 8.9	68.6 ± 3.1	< 0.001
Male/female	374/311	122/131	0.090
Body mass index (kg/m^2)	23.7 ± 3.3	22.8 ± 2.7	< 0.001
Prior IFN monotherapy, *n* (%)	163 (23.8)	76 (30.0)	0.052
Prior combined IFN plus RBV treatment, *n* (%)	51 (7.4)	20 (7.9)	< 0.001
Alanine aminotransferase (IU/L)	80.2 ± 62.0	67.9 ± 46.6	0.004
γ-glutamyltranspeptidase (IU/L)	60.2 ± 56.6	57.1 ± 49.2	0.708
Albumin (g/dL)	4.1 ± 0.4	4.0 ± 0.4	< 0.001
White blood cell count (/mm^3)	5200.0 ± 1476.7	4756.3 ± 1458.9	< 0.001
Hemoglobin (g/dL)	14.1 ± 1.4	13. 5 ± 1.4	< 0.001
Platelet count (10^9/L)	16.6 ± 5.3	15.0 ± 5.2	< 0.001
Creatinine (mg/dL)	0.7 ± 0.6	0.8 ± 1.4	0.107
Creatinine clearance (mL/min)	105.5 ± 28.7	75.8 ± 17.5	< 0.001
Serum HCV-RNA level (kIU/mL)	1776.1 ± 1500.0	1986.9 ± 1604.5	0.125
Histological fibrosis			0.008
F0/F1/F2/F3/F4	36/155/121/61/30	9/46/49/31/17	

IFN: Interferon; RBV: Ribavirin; HCV: Hepatitis C virus.

Table 2 Characteristics of 313 chronic hepatitis C genotype 2 patients treated with a combination of pegylated interferon plus ribavirin according to age (mean ± SD)

	Group C (age < 65 yr) (*n* = 252)	Group D (age ≥ 65 yr) (*n* = 61)	*P*-value
Age (yr)	47.7 ± 10.4	69.2 ± 3.4	< 0.001
Male/female	124/128	28/33	0.671
Body mass index (kg/m^2)	23.1 ± 3.5	22.8 ± 2.9	0.577
Prior IFN monotherapy, *n* (%)	47 (18.7)	16 (26.2)	< 0.001
Prior combined IFN plus RBV treatment, *n* (%)	5 (2.0)	4 (6.6)	0.056
Alanine aminotransferase (IU/L)	79.9 ± 78.7	68.9 ± 52.9	0.821
γ-glutamyltranspeptidase (IU/L)	55.8 ± 64.7	44.3 ± 34.7	0.937
Albumin (g/dL)	4.2 ± 0.4	3.9 ± 0.5	< 0.001
White blood cell count (/mm^3)	5276.3 ± 1636.3	4958.0 ± 1495.6	0.005
Hemoglobin (g/dL)	14.1 ± 1.4	13.4 ± 1.3	< 0.001
Platelet count (10^9/L)	18.9 ± 6.3	15.6 ± 4.7	< 0.001
Creatinine (mg/dL)	0.8 ± 1.5	0.7 ± 0.2	0.581
Creatinine clearance (mL/min)	112.1 ± 31.4	74.6 ± 17.2	< 0.001
Serum HCV-RNA level (kIU/mL)	1588.3 ± 1628.7	1195.4 ± 1645.5	0.038
Histological fibrosis			< 0.001
F0/F1/F2/F3/F4	30/77/39/10/10	1/21/9/2/12	

IFN: Interferon; RBV: Ribavirin; HCV: Hepatitis C virus.

Informed consent was obtained from all patients before enrollment in this study. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization of guidelines for good clinical practice.

Table 1 (genotype 1) and Table 2 (genotype 2) show the baseline characteristics of the enrolled patients, who were further classified into four groups according to age and genotype status: group A, genotype 1 aged less than 65 years (*n* = 685); group B, genotype 1 aged 65 years or older (*n* = 253); group C, genotype 2 aged less than 65 years (*n* = 252); and group D, genotype 2 aged 65 or older (*n* = 61). In group B, body mass index, prior combined IFN plus RBV treatment, alanine aminotransferase, albumin, white blood cell count, hemoglobin, platelet count, and creatinine clearance calculated using the Modification of Diet in Renal Disease equation[15] were significantly lower than in group A ($P < 0.010$). In group D, albumin, hemoglobin, platelet count, creatinine clearance and serum HCV RNA level were significantly lower than in group C ($P < 0.010$). The percentage of patients with platelet counts below 10 × 10^{10}/L was significantly higher in group B (36 of 253, 14.2%) than in group A (56 of 685, 8.2%) ($P = 0.006$), however, there was no significant difference between group C (16 of 252, 6.3%) and group D (7 of 61, 11.5%).

Liver histology

Liver biopsy was performed in 555 patients (59.2%) with genotype 1 and 209 patients (66.8%) with genotype 2. The other patients refused liver biopsy. Fibrosis was staged on a 0-4 scale as follows: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis. Liver fibrosis was more advanced in group B than in group A

and was more advanced in group D than in group C (P = 0.008, $P < 0.001$, respectively).

Treatment regimen

All patients were treated with a weight-based, 1.5 μg/kg weekly dose of subcutaneous PEG-IFN α-2b (PegIntron, Schering-Plough, Osaka, Japan), in combination with RBV (Rebetol, Schering-Plough), which was given orally at a daily dose of 600-1000 mg based on body weight (600 mg for patients weighing less than 60 kg, 800 mg for those weighing 60-80 kg, and 1000 mg for those weighing 80 kg or over). The length of treatment was 48 wk for patients with HCV genotype 1 and 24 wk for patients with genotype 2. The above duration and dosage are those approved by the Japanese Ministry of Health, Labor and Welfare. Patients were considered to have RBV-induced anemia if the hemoglobin level decreased to less than 100 g/L. In such cases, a reduction in the dose of RBV was required. Patients aged 65 years or older had a significantly higher frequency of RBV dose reduction during the treatment period than those aged less than 65 years old (HCV genotype 1: group A *vs* group B, 41.2% *vs* 49.0%, $P = 0.032$, genotype 2: group C *vs* group D, 28.6% *vs* 54.1%, $P < 0.001$). Some patients also had PEG-IFN α-2b-induced psychological adverse effects or a decrease in white blood cell and platelet counts. In such cases, a reduction in the dosage of PEG-IFN α-2b was required. Both PEG-IFN α-2b and RBV were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 85 g/L, 1×10^9/L, and 25×10^9/L, respectively. The treatment was discontinued if severe general fatigue, hyperthyroidism, interstitial pneumonia, or severe hemolytic disorders developed, continuation of treatment was judged not to be possible by the attending physician, or if the patient desired discontinuation of treatment.

Determination of baseline HCV RNA level and HCV genotype

The pretreatment, baseline, serum HCV RNA level was measured by a quantitative HCV RNA polymerase chain reaction (PCR) assay (COBAS Amplicor HCV Monitor Test v 2.0 using the 10-fold dilution method; Roche Diagnostics, Tokyo, Japan), which has a lower limit of quantitation of 5000 IU (13 500 copies)/mL (5 kIU/mL) and an outer limit of quantitation of 5 100 000 IU/mL (5100 kIU/mL). The HCV genotype was determined by type-specific primers of the core region of the HCV genome. The protocol for genotyping was carried out as previously described[3].

Efficacy of treatment

End of treatment (EOT) response and SVR were defined as serum HCV RNA undetectable at the end of treatment and at 24-wk follow-up after the end of treatment, respectively. EOT response and SVR were defined as non-detectable HCV-RNA as measured by qualitative COBAS Amplicor HCV Monitor Test v 2.0, with the results labeled as positive or negative. The lower limit of detection was 50 IU/mL (0.5 kIU/mL). The analysis of EOT and SVR was performed on an intention-to-treat basis.

Statistical analysis

Continuous data are expressed as mean ± SD. The statistics were carried out using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA, USA) for the IBM 3090 system computer. The χ^2 test, Fisher's exact test and Kruskal-Wallis test were used to determine the differences in baseline clinical characteristics, safety, efficacy of the combination therapy, adherence to the total dose, and the association between the adherence and SVR. Logistic regression analysis was used to identify the association between age and SVR. A $P < 0.05$ was considered significant.

RESULTS

EOT response rate by intention-to-treat analysis

Among patients with genotype 1, the EOT response rate was significantly higher in group A (497 of 685, 72.5%) than in group B (129 of 253, 45.0%) ($P < 0.001$). Among patients with genotype 2, there was no significant difference between groups C (239 of 252, 94.8%) and D (55 of 61, 90.1%).

SVR rate by intention-to-treat analysis

Of 1251 patients, 631 (50.4%) achieved SVR in the intention-to-treat analysis. The SVR rate was significantly higher for genotype 2 (249 of 313, 79.6%) than for genotype 1 patients (382 of 938, 40.7%) ($P < 0.001$). Among patients with genotype 1, the SVR rate was significantly higher in group A (324 of 685, 47.3%) than in group B (58 of 253, 22.9%) ($P < 0.001$). Among patients with genotype 2, SVR was also significantly higher in group C (209 of 252, 82.9%) than in group D (40 of 61, 65.6%) ($P = 0.004$). The rate of SVR was significantly higher for females (113 of 128, 88.3%) than for males (96 of 124, 77.4%) in group C only (Figure 1). Furthermore, we analyzed whether or not the SVR rate differed according to the age at which the combination treatment of PEG-IFN α-2b plus RBV was started. The results showed that the SVR rate decreased significantly with age for both genotype 1 and 2. SVR was achieved by 5.6%-26.3% of genotype 1 patients aged 70 years or older, and by 57.1%-100% of genotype 2 patients aged 70 years or older (Figure 2).

We previously reported a minimum acceptable dose of at least 80% or more of the target dosage of PEG-IFN α-2b and 60% or more of the target dosage of RBV for the successful treatment of Japanese patients with genotype 1[8]. Therefore, we analyzed the SVR rates in patients with genotype 1 by the dosage they actually received during treatment (a total dose of at least 80% or more of PEG-IFN α-2b and 60% or more of RBV) (Table 3). The number who received at least this minimum acceptable dosage during treatment were 278 (40.6%) of 685 patients in group A and 62 (24.5%) of 253 in group B, significantly lower in group B than in group A ($P < 0.001$). Compared with patients who received less than the minimum acceptable dosage, in patients who received at least this minimum dosage, the SVR rates increased from 34.2% to 66.5% in group A patients and from 15.7% to 45.2%

Table 3 The comparison of the rate of sustained virological response of patients with genotype 1 receiving a dose of 80% or more of pegylated interferon α-2b plus 60% or more of ribavirin and the reduced dosage group *n* (%)

	Male		Female		Total	
	n	SVR	*n*	SVR	*n*	SVR
Group A						
Minimum acceptable	168	116 (69.0)	110	69 (62.7)	278	185 (66.5)
Reduced	206	73 (35.4)	201	66 (32.8)	407	139 (34.2)
Total	374	189 (50.5)	311	135 (43.4)	685	324 (47.3)
Group B						
Minimum acceptable	31	15 (48.4)	31	13 (41.9)	62	28 (45.2)
Reduced	91	18 (19.8)	100	12 (12.0)	191	30 (15.7)
Total	122	33 (27.0)	131	25 (19.1)	253	58 (22.9)

Minimum acceptable: patients who received 80% or more of the target dose of pegylated interferon (IFN) α-2b and 60% or more of ribavirin (RBV). Reduced: Patients who received less than 80% of pegylated IFN α-2b and less than 60% of RBV. SVR: Sustained virological response.

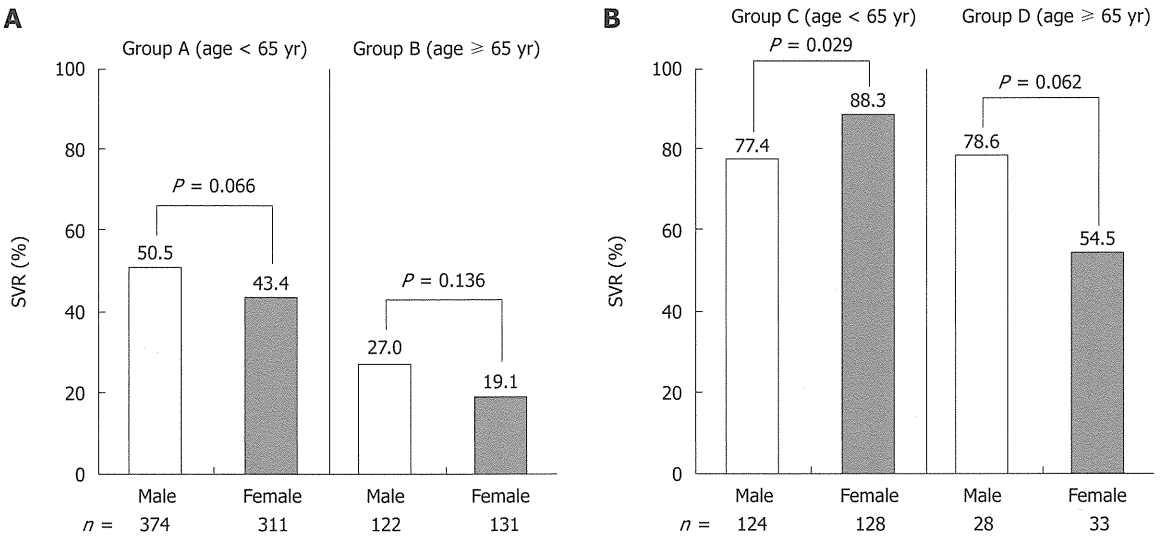

Figure 1 Virological response to the combination treatment by age and sex of patients with genotype 1 (A) and genotype 2 (B). SVR: Sustained virological response.

($P < 0.001$) in group B patients. No significant difference between groups C and D was observed. On comparing patients whose platelet count was under 10×10^{10}/L, the SVR rate for genotype 1 was significantly lower in group B (2 of 36, 5.6%) than in group A (16 of 56, 28.6%) ($P < 0.001$). Among the patients with genotype 2, SVR was not significantly different between group C (9 of 16, 56.3%) and group D (2 of 7, 28.6%).

In a comparison of the SVR rate in patients with or without one or more previous courses of IFN plus RBV, there was no significant difference between the genotypes (genotype 1: 118 of 310, 38.1% *vs* 264 of 628, 42.0%, genotype 2: 44 of 72, 61.1% *vs* 141 of 241, 58.5%). Furthermore, we compared the EOT response rate and SVR rate of cirrhosis patients whose liver fibrosis was F4, and found no significant difference between groups A (EOT: 16 of 30, 53.3%, SVR: 7 of 30, 23.3%) and B (EOT: 6 of 17, 35.3%, SVR: 2 of 17, 11.8%). In addition, no significant difference was found between groups C (EOT: 8 of 10, 80.0%, SVR: 6 of 10, 60.0%) and D (EOT: 9 of 12, 75.0%, SVR: 5 of 12, 41.7%).

Discontinuation of PEG-IFN α-2b plus RBV treatment and adverse effects

Of 1251 patients, 314 (25.1%) did not complete PEG-IFN α-2b plus RBV treatment due to adverse effects or other reasons. The discontinuation rate was significantly higher in patients with genotype 1 (273 of 938, 29.1%) than in those with genotype 2 (41 of 313, 13.1%) ($P < 0.001$) (Tables 4 and 5). Furthermore, the rate of discontinuation due to adverse effects was significantly higher in patients with genotype 1 (135 of 938, 14.4%) than in those with genotype 2 (23 of 313, 7.3%) ($P < 0.010$). The rates of discontinuation due to lack of treatment efficacy and for economic reasons (loss of job, inability to pay the medical costs) were also significantly higher in patients with genotype 1 (55 of 938, 5.9%, 15 of 938, 1.6%) than in those with genotype 2 (1 of 313, 0.3%, 0 of 938, 0%) ($P < 0.001$ and $P = 0.025$, respectively).

For genotype 1 patients, the discontinuation rate was significantly higher in group B (106 of 253, 42.9%) than in group A (167 of 685, 24.4%) ($P < 0.001$), and the rate of discontinuation due to adverse effects was also significantly

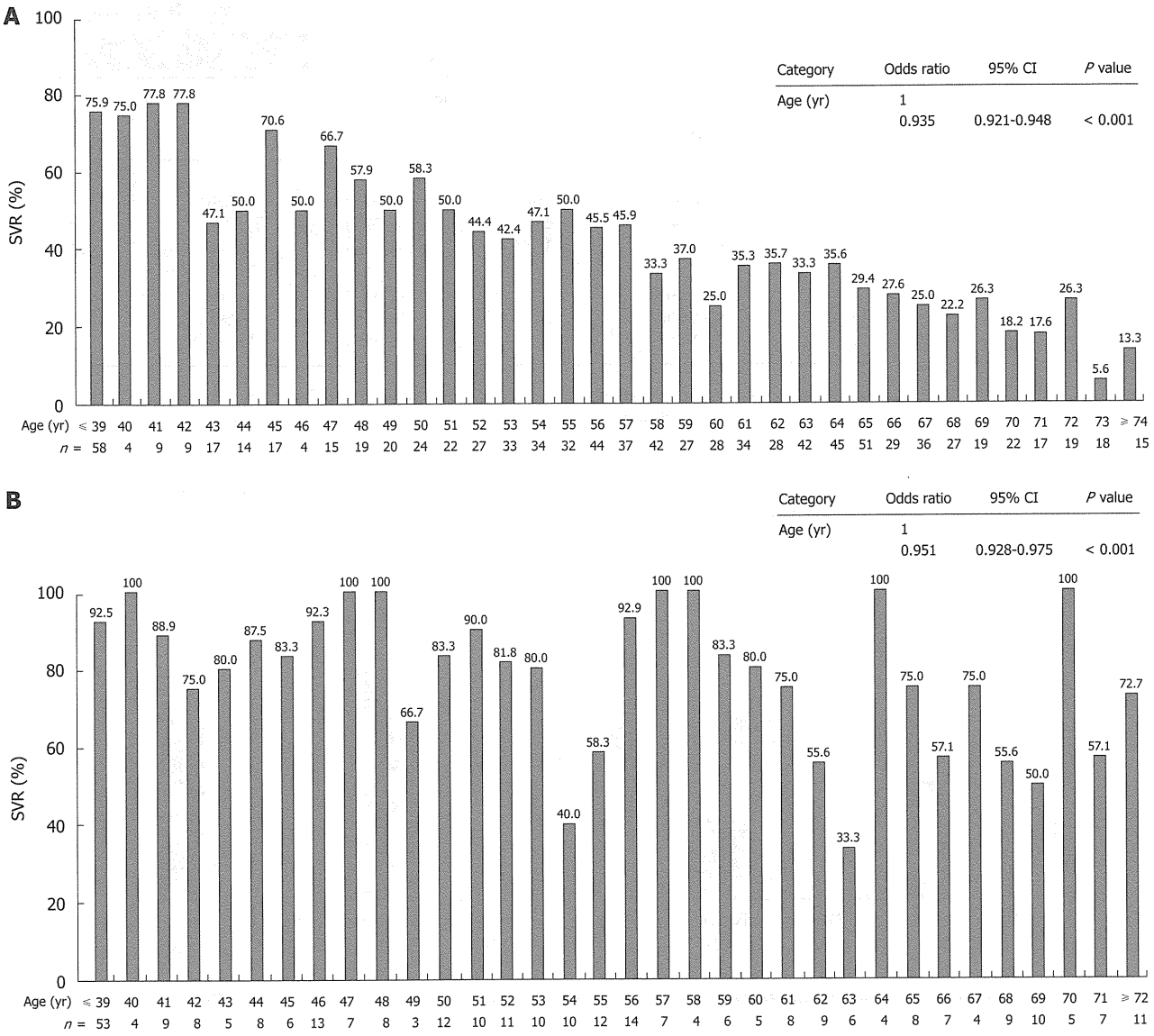

Figure 2 Virological response to the combination treatment by age of patients with genotype 1 (A) and genotype 2 (B). SVR: Sustained virological response; CI: Confidence interval.

higher in group B (61 of 253, 24.1%) than in group A (74 of 685, 10.8%) ($P < 0.001$). General fatigue was the most frequent adverse effect, and was significantly more frequent in group B than in group A ($P < 0.001$). However, in these group 1 patients, RBV was reduced due to anemia in 12.5% (3 of 24) of group A and in 30.4% (7 of 23) of group B. Furthermore, rash and thrombocytopenia were significantly more frequent in group B than in group A ($P = 0.014$ and $P = 0.007$, respectively). In group A, depression was significantly more frequent in females than in males ($P = 0.012$). In genotype 2 patients, treatment discontinuation did not differ between group C (33 of 252, 13.1%) and group D (8 of 61, 13.1%), and the rate of discontinuation due to adverse effects did not differ between these groups (17 of 252, 6.7%, 6 of 61, 9.8%, respectively).

The mean time to discontinuation in group A (21.6 ± 11.9 wk) was not significantly different from group B (21.5 ± 12.6 wk), and the mean time in group C (11.0 ± 6.8 wk) was also not significantly different from group D (11.6 ± 6.0 wk). There was no significant difference between male and female patients in each group (male: 21.0 ± 12.4 *vs* female: 22.1 ± 11.8 in group 1, male: 11.3 ± 7.1 *vs* female: 10.9 ± 6.1 in group 2).

HCC was not seen in genotype 2 patients; only in patients with genotype 1 (29.5 ± 9.9 wk) and was more frequent in group B (5 of 253, 2.0%) than in group A (2 of 685, 0.3%) ($P = 0.008$).

DISCUSSION

In a large, national, multicenter Greek study involving 993 treated and 734 untreated patients with chronic hepatitis C, patients with cirrhosis, showed a protective effect of treatment even among those without SVR. For patients without cirrhosis, the beneficial effect of IFN α treatment was particularly evident in older patients; patients with the worst prognosis if left untreated. Therefore, IFN α-based treatment should be offered to older persons, as these are

Table 4 Reasons for discontinuation of pegylated interferon plus ribavirin treatment by hepatitis C virus genotype 1 patients

	Group A (age < 65 yr)		Group B (age ≥ 65 yr)		Total
	Male (*n* = 374)	Female (*n* = 311)	Male (*n* = 122)	Female (*n* = 131)	
Discontinued number	101	66	52	54	273
Adverse effects	43	31	33	28	135
General fatigue	17	7	12	11	47
Depression	3	11	4	5	23
Appetite loss	1	0	1	0	2
Rash	3	2	3	4	12
Encephalopathy	1	0	0	0	1
Neutropenia	2	0	0	0	2
Anemia	3	2	4	1	10
Thrombocytopenia	1	0	3	1	5
Elevation of ALT	1	0	0	0	1
Hyperthyroidism	3	2	0	1	6
Hypothyroidism	0	1	0	0	1
Retinopathy	1	0	1	0	2
Interstitial pneumonia	2	0	1	1	4
Pulmonary disease (others)[1]	0	1	1	1	3
Psychoneurotic disorder[2]	2	0	2	0	4
Nervous disease[3]	1	1	0	1	3
Autoimmune disease[4]	0	2	0	1	3
Metabolic disease[5]	0	2	0	0	2
Digestive disorder[6]	2	0	1	1	4
Hepatocellular carcinoma	2	0	4	1	7
Malignancy (extra-liver)	0	1	1	0	2
No effect of treatment	22	18	7	8	55
Economic problem	9	3	0	3	15
Others[7]	25	13	7	14	59

[1]Includes pulmonary tuberculosis (*n* = 1), pneumonia (*n* = 1), tuberculous pleuritis (*n* = 1); [2]Includes psychiatric disorder (*n* = 2), disquiet (*n* = 1), insomnia (*n* = 1); [3]Includes nerve paralysis (*n* = 1), cerebral infarction (*n* = 1); [4]Includes rheumatoid arthritis (*n* = 2), myasthenia gravis (*n* = 1); [5]Includes diabetes mellitus (*n* = 1), hypertriglycemia (*n* = 1); [6]Includes cholecystitis (*n* = 3), pancreatitis (*n* = 1); [7]Includes 25, 13, 6 and 13 drop-outs from groups A, B, C and D, respectively: One for excessive alcohol consumption in group C and one was nursing in group D. ALT: Alanine aminotransferase.

the patients with the greatest potential benefit and may achieve SVR[16]. In Japan, the prevalence of chronic HCV infection increases with age, however, the optimal management of older patients has not yet been accurately defined. Whether or not to treat patients older than 65 years with antiviral treatment is highly debated, especially in terms of cost/benefit ratio. In addition, the natural history of chronic hepatitis C in elderly patients is not accurately known, as the presence of comorbidity can affect illness progression and life expectancy. HCV became more prevalent in Japan decades before the United States[17]. Japanese patients with chronic hepatitis C treated with IFN are currently 10 to 15 years older than corresponding patients in the United States and European countries, where patients treated with antiviral treatment tend to average 45 years of age[18-20]. Therefore, our results can serve as a world-wide model for the treatment of older chronic hepatitis C patients.

It has been well documented that the combination therapy of PEG-IFN α-2b plus RBV is more effective than previous IFN monotherapy in chronic hepatitis C patients[7,8]. There have been four studies on the efficacy of PEG-IFN plus RBV therapy in patients 65 years or older with genotype 1, which revealed low rates of SVR (31.1%-51.9%)[21-24]. However, these studies were too small (11-93 patients) for conclusive recommendations to be made. Because the present study was a large multicenter design, it is useful for clarifying the efficacy and safety of PEG-IFN plus RBV combination therapy in older patients. The present study confirmed the results of our previous study which showed that the SVR rate was significantly higher for genotype 2 than for genotype 1 patients[8]. Another important result was that the ability to take at least a minimum acceptable dosage during treatment increased the SVR rate by about three times in older patients with genotype 1. This result also confirmed previous studies which indicated the importance of giving at least the minimum acceptable treatment dosage in patients infected with HCV genotype 1, especially older patients[23,24].

Secondly, we compared discontinuation of treatment by genotype and sex. In genotype 1 patients, adverse effects were seen more often in older than in younger patients. This was the most important reason why the rate of treatment discontinuation was higher in older than in younger patients, and affected the outcome of PEG-IFN α-2b plus RBV combination therapy. General fatigue was the most common adverse effect in older patients. Because older patients often have impaired renal function, they have increased blood levels of RBV[25,26]. They are also inclined to be anemic and to have general fatigue. However, only a small number of older patients in the present study had reduced RBV due to anemia. Therefore, general fatigue is probably a direct adverse effect of PEG-IFN α-2b. We previously reported that herbal medicine

Table 5 Reasons for discontinuation of pegylated interferon plus ribavirin treatment by hepatitis C virus genotype 2 patients

	Group C (age < 65 yr)		Group D (age ≥ 65 yr)		Total
	Male (*n* = 124)	Female (*n* = 128)	Male (*n* = 28)	Female (*n* = 33)	
Discontinued number	18	15	4	4	41
Adverse effects	6	11	3	3	23
General fatigue	1	3	1	0	5
Depression	0	2	0	0	2
Appetite loss	0	0	0	0	0
Rash	2	1	0	2	5
Encephalopathy	0	0	0	1	1
Neutropenia	0	2	0	0	2
Anemia	0	0	2	0	2
Thrombocytopenia	2	0	0	0	2
Elevation of ALT	0	0	0	0	0
Hyperthyroidism	0	1	0	0	1
Hypothyroidism	0	1	0	0	1
Retinopathy	0	0	0	0	0
Interstitial pneumonia	0	0	0	0	0
Pulmonary disease(others)	0	0	0	0	0
Psychoneurotic disorder	0	0	0	0	0
Nervous disease[1]	1	1	0	0	2
Autoimmune disease	0	0	0	0	0
Metabolic disease	0	0	0	0	0
Digestive disorder	0	0	0	0	0
Hepatocellular carcinoma	0	0	0	0	0
Malignancy (extra-liver)	1	0	0	0	1
No effect of treatment	1	0	0	0	1
Economic problem	0	0	0	0	0
Others[2]	10	4	1	1	16

[1]Includes nerve paralysis (*n* = 1), tetany (*n* = 1); [2]All patients were drop out. ALT: Alanine aminotransferase.

relieved the adverse effects of IFN, including general fatigue[27]. Herbal medicine may be useful for mitigating general fatigue during PEG-IFN α-2b plus RBV combination treatment, especially in older patients.

The rate of discontinuation was lower in patients with genotype 2 than in patients with genotype 1, and there was no difference between the older and the younger patients with genotype 2. These results are possibly a consequence of the shorter term of treatment in genotype 2 and the many genotype 1 patients who discontinued due to lack of efficacy.

Two of the characteristics of older patients in the present study were that both hemoglobin and platelet count were significantly lower than in younger patients. The SVR rate was significantly lower when the platelet count was less than 10×10^{10}/L. Furthermore, the older genotype 1 patients were often forced to discontinue treatment due to thrombocytopenia and the occurrence of HCC. These findings appear to result from advanced liver fibrosis in older chronic hepatitis C patients. Therefore, the possibility of HCC during long-term IFN treatment in older patients must be considered.

We previously reported that older female patients had a low response to IFN-α monotherapy[9], and other investigators have reported that older female patients have a poor response to PEG-IFN α-2b plus RBV[22,28]. Although our data showed that sex was not related to SVR, the reason for this finding was not fully elucidated. In any case, studies have conclusively shown that it is important to begin treatment with PEG-IFN α-2b plus RBV combination therapy as soon as possible. Our data suggest that age may be a more important factor than sex for increasing the rate of SVR. Resistance to treatment in older patients may be due to IFN-immunomodulation, advanced liver fibrosis, or reduced dosage.

To maximize adherence to the optimal treatment regimen, the treatment schedule can be modified or other therapeutic modalities added, such as hematopoietic growth factors[29] or the new thrombopoietin-receptor agonist, eltrombopag, for the antiviral treatment of older patients with chronic hepatitis C[30]. A further individualized treatment protocol based on viral kinetics might be more practical[31].

In conclusion, PEG-IFN α-2b plus RBV treatment was effective in the treatment of older chronic hepatitis C patients when they received at least the minimum acceptable treatment dosage. However, there were frequent adverse effects and treatment discontinuation. It is necessary to control for adverse effects that might interrupt treatment and to begin this combination therapy as soon as possible, especially in older patients.

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COMMENTS

Background

Whether or not to treat patients older than 65 years with antiviral treatment is highly debated, especially in terms of cost/benefit ratio. However, there is little data concerning the response and safety of combination treatment for a large number of older patients with chronic hepatitis C virus infection. Therefore, in an attempt to ameliorate these problems, the authors decided to treat older patients with pegylated interferon (PEG-IFN) α-2b plus ribavirin (RBV) combination therapy.

Research frontiers

The combination treatment of PEG-IFN α-2b plus RBV improved the sustained virological response rate in chronic hepatitis C patients. However, the current issue is whether or not to treat older patients because of low response and high dropout rate.

Innovations and breakthroughs

There have been four studies on the efficacy of PEG-IFN plus RBV therapy in patients 65 years or older with genotype 1. However, these studies were too small (11-93 patients) for conclusive recommendations to be made. This study is very useful for clarifying the efficacy and safety of PEG-IFN plus RBV combination therapy in older patients, because of its large scale, multicenter design.

Applications

The study demonstrated that PEG-IFN α-2b plus RBV treatment was effective in chronic hepatitis C patients 65 years or older who completed treatment with at least the minimum required treatment dosage. Furthermore, this study suggested that the combination treatment and beginning this therapy as soon as possible are important, especially in older patients.

Peer review

The study has been well conducted and includes a large number of patients. Results have been described in a lucid and informative manner and are of clinical relevance.

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The longitudinal quantitative assessment by transient elastography of chronic hepatitis C patients treated with pegylated interferon alpha-2b and ribavirin

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ABSTRACT

The aim of this study was to assess the association between liver stiffness measured by transient elastography (FibroScan®) and the efficacy of pegylated interferon alpha-2b plus ribavirin combination treatment for patients with chronic hepatitis C virus (HCV) infection. We prospectively studied 145 Japanese patients with chronic HCV infection. FibroScan was done at baseline, at the end of treatment, and at 48 and 96 weeks after the end of treatment. The FibroScan values were significantly decreased for sustained virological response (SVR) patients (the mean rate of change; −16.2%, −32.2% and −43.5%) in comparison with non-SVR patients (−7.2%, −2.1% and +17.3%) at the end of treatment ($P = 0.0127$), and 48 weeks ($P < 0.0001$) and 96 weeks ($P < 0.0001$) after the end of treatment. Among the non-SVR patients, the FibroScan values were significantly decreased for patients with biochemical response (BR) (−17.9%, −30.0% and −27.1%) in comparison with non-BR (−4.1%, +6.4% and +30.6%) at the end of treatment ($P = 0.0270$), and 48 weeks ($P < 0.0001$) and 96 weeks ($P < 0.0001$) after the end of treatment. The FibroScan values may predict a progressively better clinical outcome for patients with successful virological and biochemical responses.

1. Introduction

Hepatitis C virus (HCV) infection is a main cause of chronic viral hepatitis worldwide. Chronic hepatitis can lead to cirrhosis and hepatocellular carcinoma (HCC) (Seeff, 2002; Hayashi et al., 2000). Antiviral treatment with interferon (IFN) for chronic HCV infection can induce viral clearance and biochemical and histological improvement (Davis et al., 1989; Hayashi et al., 1994). Pegylated interferon (PEG-IFN) alpha in combination with ribavirin (RBV), which aims at viral eradication (Poynard et al., 2002a; Furusyo et al., 2008), has contributed to a reduction in the relapse rate and a significant increase in the rate of sustained virological response (SVR) compared with standard IFN monotherapy (Hayashi et al., 1994; Zeuzem et al., 2000; Lindsay et al., 2001). IFN treatment has been reported to be responsible for the regression of liver fibrosis in patients with SVR (Shiratori et al., 2000; Furusyo et al., 1997). Even if a virological response with IFN treatment was not obtained, the deterioration of compensated cirrhosis was prevented and the development of HCC was inhibited (Nishiguchi et al., 1995; Veldt et al., 2004; Kashiwagi et al., 2003), thereby increasing the survival rate (Poynard et al., 2002a).

Liver biopsy had long remained the gold standard for staging fibrosis. However, liver biopsy is no more considered as a perfect methodology because of the invasive nature of the procedure, sampling error and inter-observer variability (Regev et al., 2002; Manning and Afdhal, 2008). Therefore, further testing strategies are needed for assessment of the liver status of patients with liver diseases.

Transient elastography (FibroScan®; Echosens, Paris, France) has been proposed as a promising, rapid, noninvasive and reproducible method for measuring liver stiffness (Sandrin et al., 2002). We previously reported a clinical assessment of FibroScan among patients with chronic hepatitis B and C (Ogawa et al., 2007). The values measured by FibroScan (FibroScan values) have been significantly correlated with histopathological staging of percutaneous liver biopsy and have been shown to be more accurate than biochemical scores such as the aspartate aminotransferase (AST)-to-platelet ratio (APRI) and markers of liver fibrosis (e.g. hyaluronic acid and type IV collagen), which are products of the degradation or synthesis of the extracellular matrix.

It has been repeatedly observed that at least two biomarkers FibroTest® (Biopredictive, Paris, France) and FibroScan (Manning and Afdhal, 2008; Calès et al., 2008; Friedrich-Rust et al., 2008; Poynard et al., 2008; Shaheen et al., 2007) have the same

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diagnostic value as a 20–25 mm liver biopsy. In case of discordance between FibroTest and liver biopsy, half of failures are attributable to FibroTest and half to the biopsy (Poynard et al., 2004; Ngo et al., 2006). Moreover, FibroTest has already demonstrated a similar prognostic value as biopsy (Ngo et al., 2006). In a few countries, biomarkers are recommended by health authorities as first line test in patients with chronic hepatitis C, and biopsy only if biomarkers are not interpretable (Manning and Afdhal, 2008).

The aim of the present long-term prospective study was to evaluate the association between liver stiffness measured by FibroScan and the efficacy of combined PEG-IFN alpha-2b plus RBV treatment for patients with chronic HCV infection.

2. Materials and methods

2.1. Patients

We prospectively studied a total of 145 patients infected with chronic HCV infection. Of the 145 patients, 19 (13.1%) refused antiviral treatment because of financial problems or anxiety about the possibility of adverse effects and low initial fibrosis degree (low FibroScan values); however, 4 (21.1%) of them underwent liver biopsy. The 19 untreated patients were followed for the same 3-year period as the treated patients. Of the remaining 126 patients, 118 (93.7%) underwent liver biopsy, all of whom were treated with PEG-IFN alpha-2b plus RBV treatment between January 2005 and July 2006 and followed up for 2 years after the end of treatment.

All patients satisfied the following criteria: (1) chronically infected with HCV and (2) a history of an increased alanine aminotransferase (ALT) level for over 6 months. Exclusion criteria for the study were: (1) positivity for antibody to human immunodeficiency virus (HIV) or positivity for both hepatitis B surface antigen and anti-HCV; (2) clinical or biochemical evidence of hepatic decompensation; (3) excessive active alcohol (i.e. ethanol) consumption (>60 g/day) or drug abuse; (4) suspected hepatocellular carcinoma; or (5) treatment with antiviral or immunosuppressive agents prior to enrollment. Patients who fulfilled the above criteria were recruited at Kyushu University Hospital.

Informed consent was obtained from all patients before enrollment. The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and the International Conference on Harmonization of guidelines for good clinical practice.

2.2. Clinical and laboratory assessment

Clinical parameters included aspartate aminotransferase, ALT, platelet count, type IV collagen, prothrombin time, HCV genotype and HCV RNA. We also calculated APRI, using 45 IU/L as the upper limit of the normal AST range (ULN), as previously recommended for evaluating liver fibrosis (Wai et al., 2003):

$$\text{APRI} = \frac{\text{AST (/ULN)} \times 100}{\text{platelet count } (\times 10^9/\text{L})}$$

Body mass index (BMI) was calculated as weight in kilograms/height in square meters. Serum levels of AST, ALT, type IV collagen and HCV RNA, platelet counts, prothrombin time and HCV genotype were measured by standard laboratory techniques at a commercial laboratory (MBC Laboratory, Tokyo, Japan).

2.3. Transient elastography (FibroScan)

FibroScan was done in the right lobe of the liver through the intercostal spaces with the patient lying in the dorsal decubitus position with the right arm in maximal position. The tip of the probe transducer was covered with coupling gel and placed on the skin, between the ribs at the level of the right lobe of the liver. The operator, assisted by an ultrasonic time-motion image, located a liver portion at least 6 cm thick and free of large vascular structures. Once the measurement area had been located, the operator pressed the probe button to start acquisition. The elasticity was automatically calculated by the apparatus and the data were shown as kiloPascal (kPa). All examinations were performed by four accomplished operators (EO, KT, HT, and SO) of our department who individually experienced over 100 examinations. Only liver stiffness measurements obtained with at least six successful acquisitions and a success rate of at least 60% were considered reliable. The validity of FibroScan values depends on an interquartile range of all successful measurements (IQR/M) of less than 30% of median values (Poynard et al., 2008; Lucidarme et al., 2008). The mean IQR/M of the present study was 21.6% and no case with IQR/M > 30% was found. The first measurement of liver stiffness by FibroScan was performed within 2 weeks before liver biopsy examination.

2.4. Liver histology and quantification of liver biopsy

Liver biopsy was done for 122 (84.1%) of the 145 chronic HCV infected patients and was performed by experienced hepatologists with a 16-G disposable needle (Bard® Monopty®; C.R. Bard, Covington, GA) under ultrasound guidance. The median liver biopsy length was 18 mm (minimal length was 15 mm). Liver biopsy specimens were fixed in formalin and paraffin was embedded. All biopsy specimens were analyzed by two experienced pathologists who were blinded to the clinical data. For each specimen, the stage of fibrosis and the grade of activity were established according to the following criteria (Bedossa and Poynard, 1996). Fibrosis was staged on a 0–4 scale as follows: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis and few septa, F3 = numerous septa without cirrhosis, and F4 = cirrhosis. The grading of activity, including the intensity of the necroinflammation, was scored as follows: A0 = no histological activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity.

2.5. Therapeutic protocol

All patients were treated with a weight-based, 1.5 μg/kg weekly dose of subcutaneous PEG-IFN alpha-2b (PegIntron A®; Schering-Plough, Osaka, Japan). In combination with PEG-IFN alpha-2b, RBV (Rebetol®; Schering-Plough) was given orally at a daily dose of 600–1000 mg based on bodyweight (600 mg for patients weighing < 60 kg, 800 mg for those weighing 60–80 kg, and 1000 mg for those weighing > 80 kg). The length of treatment was 48 weeks for genotype 1b and 24 weeks for genotype 2. The above durations and dosages are those approved by the Japanese Ministry of Health, Labor and Welfare. Patients were considered to have RBV-induced anemia if the hemoglobin level decreased to < 100 g/L. In such cases, a reduction in the dose of RBV was required. Some patients also had PEG-IFN alpha-2b induced psychological adverse effects or a decrease of white blood cell and platelet count. In such cases, a reduction in the dosage of PEG-IFN alpha-2b was required. Both PEG-IFN alpha-2b and RBV were discontinued if the hemoglobin level, white blood cell count, or platelet count fell below 85 g/L, 1×10^9/L, or 2.5×10^9/L, respectively. The treatment was discontinued if severe general fatigue, hyperthyroidism, interstitial pneumonia, or severe hemolytic problems developed, continuation of treatment was judged not to be possible by the attending physician, or the patient desired discontinuation of treatment. All patients received at least 80% or more of the target dosage of PEG-IFN alpha-2b and 60% or over of the RBV, because the condition under the sufficient dosage were needed to estimate between treatment response and liver disease progression accurately.

Table 1
Baseline characteristics of 145 patients with chronic HCV infection.

Characteristics	Non-treated (n = 19)	PEG-IFN alpha-2b combination with RBV treatment		
		SVR (n = 57)	Non-SVR (n = 69)	P-value*
Male/Female (n)	9/10	30/27	25/44	0.0655
Age (years)	63.8 ± 9.2	52.7 ± 13.2	60.3 ± 9.3	0.0003
Body mass index (kg/m^2)	22.2 ± 3.4	23.1 ± 3.1	22.8 ± 3.1	0.5912
Aspartate aminotransferase (AST) (IU/L)	61.2 ± 29.5	65.0 ± 39.2	65.8 ± 42.4	0.9192
Alanine aminotransferase (IU/L)	69.7 ± 50.9	88.3 ± 73.7	72.7 ± 53.2	0.1962
Platelet count (10^9/L)	154 ± 52	155 ± 45	154 ± 51	0.9294
Type IV collagen (ng/mL)	180 ± 68	172 ± 69	192 ± 88	0.1750
AST/Platelet count	1.39 ± 0.89	1.37 ± 0.99	1.35 ± 1.19	0.9100
Prothrombin time (%)	94.4 ± 15.9	90.2 ± 8.7	88.2 ± 13.1	0.3414
HCV genotypes 1b/2 n	19/0	34/23	65/4	<0.0001
Serum HCV RNA level (kIU/mL)	3207 ± 1542	1565 ± 1645	2014 ± 1455	0.1062
Liver histology				
Stage of fibrosis				0.7225
F0 (%)	1 (25.0)	7 (13.2)	7 (10.8)	
F1 (%)	0 (0.0)	10 (18.9)	17 (26.2)	
F2 (%)	1 (25.0)	19 (35.8)	16 (24.6)	
F3 (%)	2 (50.0)	11 (20.8)	15 (23.1)	
F4 (%)	0 (0.0)	6 (11.3)	10 (15.4)	
Grade of activity				0.7243
A0 (%)	0 (0.0)	0 (0.0)	0 (0.0)	
A1 (%)	1 (25.0)	17 (32.1)	21 (32.3)	
A2 (%)	2 (50.0)	34 (64.2)	39 (60.0)	
A3 (%)	1 (25.0)	2 (3.8)	5 (7.7)	
Not determined	15	4	4	

Data are shown by the mean ± standard deviation. PEG-IFN, pegylated interferon; RBV, ribavirin.
* P-values were analyzed between SVR and non-SVR patients.

2.6. Efficacy of treatment

SVR was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. This efficacy variable, SVR, was defined as non-detectable HCV RNA as measured by the COBAS® Amplicor® HCV Monitor Test (version 2.0), and the results were labeled as positive or negative. The lower limit of detection was 50 IU/mL (0.5 kIU/mL) (Lee et al., 2000).

2.7. Assessment of biochemical response (BR) among non-SVR patients

We evaluated BR among non-SVR patients after the end of treatment. Patients who had continuous ALT levels under 30 IU/L every month for 96 weeks after the end of treatment were defined as BR.

2.8. Determination of HCV RNA level and HCV genotype

During the treatment period, HCV RNA was analyzed by the COBAS® Amplicor® HCV Monitor assay (version 2.0; Roche Diagnostics, Tokyo, Japan), with a lower limit of quantitation of 5000 IU/mL and an outer limit of quantitation of 5,100,000 IU (5100 kIU)/mL. The COBAS® Amplicor® HCV Monitor assay (version 2.0) is a semi-automated nucleic acid amplification assay, consisting of manual sample preparation and automated reverse transcription (RT), amplification and detection steps on the COBAS® Amplicor® Analyzer. HCV genotype was determined by RT-PCR using universal and type-specific primers from the putative C gene of the HCV genome, according to Okamoto et al. (1992) and the genotype was classified into the type 1b or type 2a or 2b based on Simmonds et al. (1994).

2.9. Statistical analysis

Statistical analysis was done with BMDP statistical software for the IBM 3090 system computer (BMBD Statistical Software, Inc., Los Angeles, CA). Continuous data were expressed as mean values, mean ± standard deviation (SD) of the mean. The paired t-test, unpaired t-test, Mann–Whitney U-test or Kruskal–Wallis non-parametric analysis of variance was used for the analysis. The area under the receiver operating characteristic curve (AUROC) analysis was done to evaluate the relationship between histological findings and FibroScan values. The cutoff values were selected from the receiver operating characteristic (ROC) curve to maximize total sensitivity and specificity. A P-value less than 0.05 was regarded as statistically significant.

3. Results

3.1. Characteristics of patients

The major clinical and biochemical parameters of the patients at entry (baseline) are summarized in Table 1. The mean FibroScan values were significantly higher in the treated group (10.2 kPa) than in the non-treated group (7.6 kPa) (P = 0.0406). Of the 126 treated patients, 57 (45.2%) achieved SVR. The median age was significantly younger in the SVR group (52.7 years) than in the non-SVR group (60.3 years) (P = 0.0003). The rate of SVR was higher for patients with genotype 2 (85.2%) than for those with genotype 1b (34.3%) (P < 0.0001). No significant differences between the SVR and non-SVR groups were found for gender, BMI, AST, ALT, platelet count, type IV collagen, APRI, prothrombin time, or serum HCV RNA level.

3.2. Relationship between liver fibrosis and FibroScan values

Fig. 1a and b shows the distribution of FibroScan values according to fibrosis stage and activity grade, respectively. The median values of the patients were 4.8 kPa, 7.1 kPa, 8.5 kPa, 12.8 kPa and 19.5 kPa for F0, F1, F2, F3 and F4, respectively. The FibroScan values were significantly correlated with fibrosis stage (r = 0.807, P < 0.0001) and were also significantly increased in accordance with

Table 2
Optimal cutoff of FibroScan values for the determination of histological fibrosis stage in 122 biopsy-received patients with chronic HCV infection.

	Histological fibrosis stage by liver biopsy			
	$F=1$ (F0 vs. F1)	$F=2$ (F1 vs. F2)	$F=3$ (F2 vs. F3)	$F=4$ (F3 vs. F4)
Number	15/27	27/36	36/28	28/16
Cutoff value[a] (kPa)	6.1	7.2	10.3	14.9
AUROC	0.81	0.66	0.88	0.94
Sensitivity (%)	63.0	66.7	89.3	100.0
Specificity (%)	86.7	66.7	77.8	82.1
Positive predictive value (%)	89.5	66.7	75.8	76.2
Negative predictive value (%)	56.5	60.0	90.3	100.0
Positive likelihood ratio	4.72	2.00	4.02	5.60

AUROC, area under the receiver operating characteristic curve.
[a] The opitimal cutoff value is the one that gives the higher total sensitivity and specificity.

the grade of activity for the patients ($r=0.343$, $P<0.0001$). Table 2 shows the optimal liver stiffness cutoff values obtained for sensitivity and specificity. In analyses of adjacent fibrosis stages (F0 vs. F1, F1 vs. F2, F2 vs. F3 and F3 vs. F4), four threshold FibroScan values were identified: ≥6.1 kPa for F1 (sensitivity 63.0%, specificity 86.7%); ≥7.2 kPa for F2 (sensitivity 66.7%, specificity 66.7%); ≥10.3 kPa for F3 (sensitivity 89.3%, specificity 77.8%) and 14.9 kPa for F4 (sensitivity 100%, specificity 82.1%). The corresponding AUROC were 0.81 for F0 vs. F1, 0.66 for F1 vs. F2, 0.88 for F2 vs. F3 and 0.94 for F3 vs. F4.

3.3. The longitudinal FibroScan values for the PEG-IFN alpha-2b plus RBV combination treatment (Fig. 2a and b)

The baseline mean FibroScan values were 10.3 ± 4.8 kPa, 10.0 ± 5.5 kPa, and 7.6 ± 3.9 kPa for SVR ($n=57$), non-SVR ($n=69$), and non-treated patients ($n=19$), respectively. For SVR patients, the mean FibroScan values were 8.3 kPa, 6.6 kPa and 5.4 kPa at week 0 and at 48 and 96 weeks after the end of treatment, respectively. For non-SVR patients, the mean FibroScan values were 9.0 kPa, 9.5 kPa and 11.4 kPa at week 0 and at 48 and 96 weeks after the end of treatment, respectively.

The changes of the FibroScan values of each patient at the end of treatment, 48 weeks and 96 weeks after the end of treatment were calculated with the entry values as the estimated standard. Significant differences were found between SVR (−16.2%, −32.2% and −43.5%) and non-SVR patients (−7.2%, −2.1% and +17.3%) in the mean rate of change of FibroScan values between each testing point ($P=0.0127$, $P<0.0001$ and $P<0.0001$, respectively). For the untreated patients, the mean FibroScan values increased to 8.3 kPa, 9.8 kPa and 10.6 kPa (+12.9%, +38.0% and +49.1%, respectively) at each testing point. Significant differences were also found between treated patients (SVR and non-SVR) and untreated patients in the mean rate of change of FibroScan values.

3.4. The longitudinal FibroScan values classified according to the BR and non-BR of non-SVR patients (Fig. 3a and b)

Of the 69 non-SVR group patients, 16 (23.2%) achieved BR. The baseline mean FibroScan values were 9.3 ± 5.2 kPa and 10.2 ± 5.6 kPa for BR ($n=16$) and non-BR ($n=53$) patients, respectively. For BR patients, the mean FibroScan values were 7.4 kPa, 6.2 kPa and 6.7 kPa at week 0 and at 48 and 96 weeks after the end of treatment, respectively. For non-BR patients, the mean FibroScan

Table 3
Differences of the mean rate of changes of FibroScan values after PEG-IFN alpha-2b plus RBV treatment, classified by age group and gender.

	n	PEG-IFN alpha-2b combination with RBV treatment					
		Week 0	P-value*	Week 48	P-value*	Week 96	P-value*
SVR patients							
Age group			0.7995		0.4128		0.3874
<60 years	22	−16.3		−30.7		−42.1	
≥60 years	35	−15.9		−34.6		−45.9	
Gender			0.4517		0.6278		0.1740
Men	30	−17.7		−31.1		−46.3	
Women	27	−14.4		−33.4		−40.5	
Fibrosis stage			0.6653		0.2369		0.0927
≤F2	36	−16.1		−31.1		−42.0	
≥F3	17	−18.3		−37.4		−50.0	
Non-SVR patients							
Age group			0.7995		0.3478		0.3998
<60 years	27	−6.4		−6.9		11.5	
≥60 years	42	−7.8		1.1		20.9	
Gender			0.3218		0.1625		0.4507
Men	25	−10.8		−9.7		11.8	
Women	44	−5.3		2.3		20.4	
Fibrosis stage			0.6720		0.4893		0.4309
≤F2	40	−7.3		−5.0		13.9	
≥F3	25	−9.7		1.1		23.2	

PEG-IFN, pegylated interferon; RBV, ribavirin; SVR, sustained vilorogical response; week 0, at the end of treatment; week 48, 48 weeks after the end of treatment; week 96, 96 weeks after the end of treatment. The rates of changes of FibroScan values of each patient at the end of treatment, 48 weeks and 96 weeks after the end of treatment were calculated by the entry values as the estimated standard.
* P-values were analyzed at each point between age groups, between genders and between fibrosis stages.

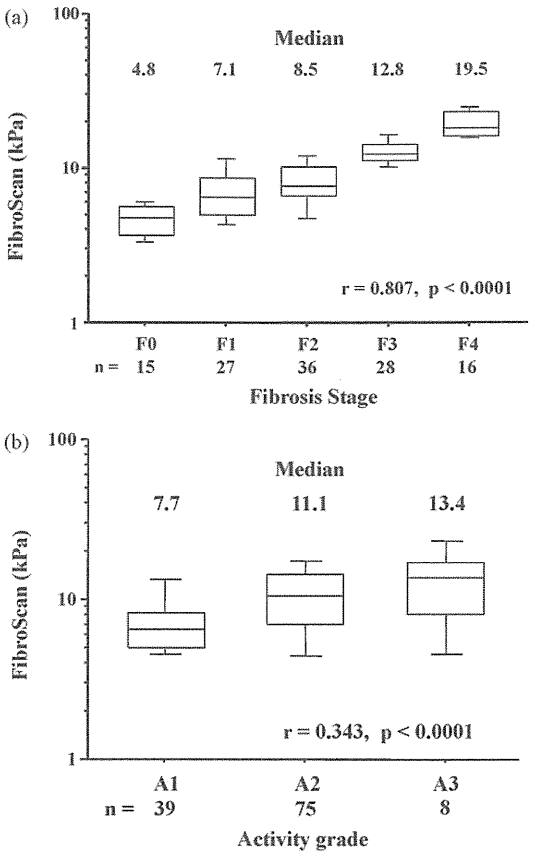

Fig. 1. FibroScan values for each fibrosis stage (a) and activity grade (b) of 122 biopsy-received patients with chronic hepatitis C virus infection. The vertical axis is a logarithmic scale. The bottom and the top boxes are the first and third quartiles, respectively. The length of the box represents the interquartile range within which 50% of the values are located. The lines through the middle of the boxes represent the median. The error bars are the minimum and maximum values.

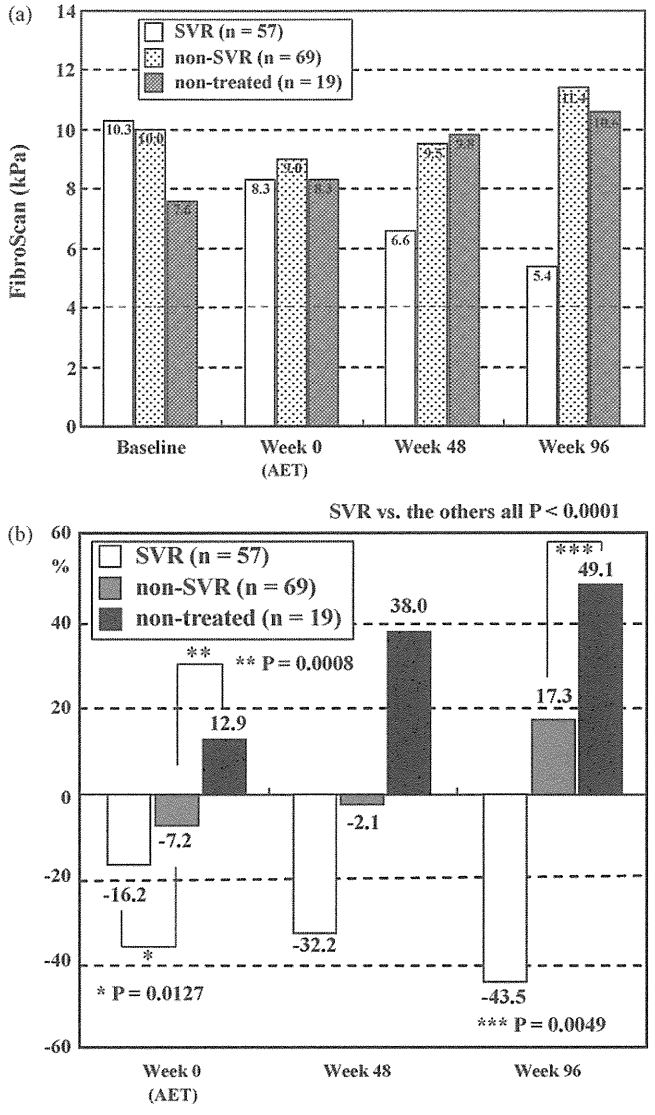

Fig. 2. The longitudinal mean FibroScan values (a) and the mean rate of change of FibroScan values (b) of 126 treated patients after pegylated interferon alpha-2b plus ribavirin combination treatment and 19 non-treated patients. The rates of change of the FibroScan values of each patient at the end of treatment and at 48 and 96 weeks after the end of treatment were calculated using the entry values as the estimated standard. The length of treatment was 48 and 24 weeks for HCV genotypes 1 and 2 patients, respectively. Weeks 0, 48 and 96 for the treated patients indicate the end of treatment and 48 and 96 weeks after the end of treatment, respectively. Weeks 0, 48 and 96 for the non-treated patients indicate 48, 96 and 144 weeks from entry, respectively. SVR, sustained virological response; AET, at the end of treatment.

values were 9.5 kPa, 10.5 kPa and 12.8 kPa at week 0 and at 48 and 96 weeks after the end of treatment, respectively. For BR patients, the mean rate of change of FibroScan values indicated improvement (−17.9%, −30.0% and −27.1% at weeks 0, 48 and 96 after the end of treatment, respectively) with antiviral treatment, in spite of there not being a virological effect. On the other hand, for non-BR patients, the mean rate of change of FibroScan values showed worsening over time (−4.1%, +6.4% and +30.6% at weeks 0, 48 and 96 after the end of treatment, respectively). A significant difference was found between BR and non-BR patients in the mean rate of change of FibroScan values in each period ($P = 0.0270$, $P < 0.0001$ and $P < 0.0001$, respectively). Moreover, although a significant difference was found between non-BR (+6.4%) and non-treated patients (+38.0%) at week 48 after the end of treatment ($P < 0.0001$), no significant difference was found between non-BR (+30.6%) and non-treated patients (+49.1%) at week 96 after the end of treatment ($P = 0.0835$) in the mean rate of change of FibroScan values. In other analyses, no significant difference was found between the longitudinal rate of change of FibroScan values and age (<60 and ≥60), gender or fibrosis stage (≤F2 and ≥F3) for both SVR and non-SVR patients, as shown in Table 3.

4. Discussion

The present prospective study consists of a demonstration of the association between liver stiffness measured by FibroScan and the efficacy of PEG-IFN alpha-2b plus RBV treatment. Recent reports have shown that treatment for chronic hepatitis C is associated with an improvement of FibroScan values at the end of treatment and 6 months later, whatever the virological response (Vergniol et al., 2008, 2009). However, we investigated the association between an efficacy of the antiviral treatment and FibroScan values related to both virological and biochemical response for a longer period after the treatment than the previous reports. Consequently, we demonstrated that the liver elasticity of SVR patients markedly improved over time and that the liver elasticity of non-SVR patients with biochemical response also improved.

In PEG-IFN alpha plus RBV treatment for chronic hepatitis C, we previously reported that it was necessary to administer ≥80% of the target dosage of PEG-IFN alpha-2b plus ≥60% of the target dosage of RBV throughout the treatment in order to achieve virological efficacy in Japanese patients (Furusyo et al., 2008). Each patient in the present study was analyzed under this treatment dosage.

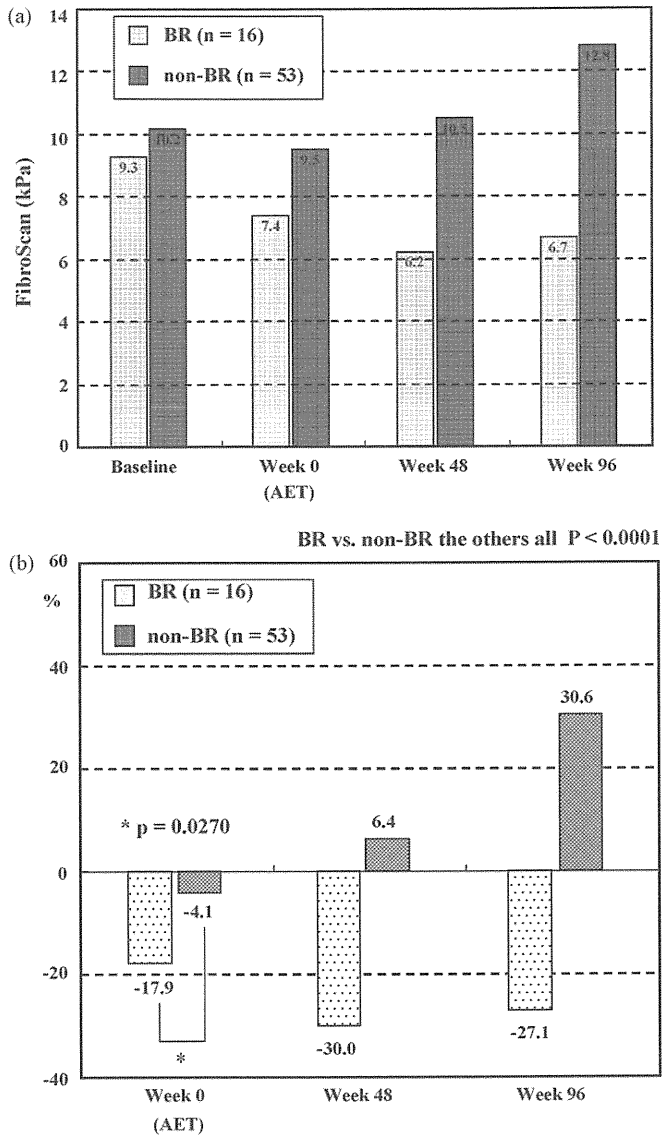

Fig. 3. The longitudinal mean FibroScan values (a) and the mean rate of change of FibroScan values (b) of the non-sustained virological response patients after pegylated interferon alpha-2b plus ribavirin combination treatment, classified by biological response (BR). The rates of change of the FibroScan values of each patient at the end of treatment and at 48 and 96 weeks after the end of treatment were calculated using the entry values as the estimated standard. Weeks 48 and 96 indicate 48 and 96 weeks after the end of treatment, respectively. AET, at the end of treatment.

FibroScan represents a novel clinical methodology based on ultrasound. This can survey a larger sample of the liver parenchyma than liver biopsy and thus more accurately estimates liver fibrosis across a wide range of liver disorders (Ziol et al., 2005; Ogawa et al., 2007). According to meta-analysis, FibroScan could be performed with excellent diagnostic accuracy at distinguishing liver cirrhosis (F4) or no liver cirrhosis (≤F3), with a mean AUROC of 0.94, and at distinguishing ≥F2 and ≤F1 with a mean AUROC of 0.84 (Friedrich-Rust et al., 2008). The optimal cutoff values for the diagnosis of F4 and ≥F2 suggested from the summary ROC are 13.01 kPa and 7.65 kPa, respectively (Friedrich-Rust et al., 2008). Thus, prior studies have already shown that FibroScan can distinguish absent or mild fibrosis from advanced fibrosis in both HCV-mono-infected and HIV-/HCV-co-infected individuals, but it seems to be less accurate at differentiating between intermediate stages. However, Macías et al. (2008) suggested that the usefulness of FibroScan could be enhanced using two different cutoff values (6.0 kPa and 9.0 kPa) to identify with ≤F1 and ≥F2, respectively, in HIV- and HCV-co-infected patients.

Biomarkers such as FibroTest demonstrated similar results as FibroScan in patients treated with IFN and RBV (Poynard et al., 2002b, 2003; Ngo et al., 2006; Patel et al., 2009) and the combination of FibroTest and ActiTest® (which is a modification of the FibroTest) give not only the fibrosis estimate but also the activity estimate (Poynard et al., 2003). FibroScan has never been demonstrated better than FibroTest for F4 vs. ≤F3 according to evidence-based data (Castéra et al., 2005; Shaheen et al., 2007; Manning and Afdhal, 2008; Poynard et al., 2008; Calès et al., 2008).

While natural history of liver fibrosis progression can vary depending on gender and alcohol consumption, age is the main risk factor for liver fibrosis (Poynard et al., 1997). We recommended that all our patients stop drinking alcohol while under treatment and during follow-up. In our study, the baseline mean value of FibroScan was 7.6 kPa, almost the same as the F2 stage, with a value of 10.5 kPa, nearly equal to the F3 stage, after 3 years in non-treated patients (median age 63.8 years). Poynard et al. (1997) suggested that the rate of liver fibrosis progression of untreated patients was highest in individuals older than 50 years (at a rate of 0.333 stage/year). The findings obtained in the present study were similar to those of the above report.

Many previous reports have shown that IFN treatment achieves biochemical and histological improvement with viral suppression by patients with chronic hepatitis C (Poynard et al., 2002a; Furusyo et al., 1997; Cammá et al., 1998; Bruno et al., 2007; Furusyo et al., 2008). Several potential mechanisms have been hypothesized for the anti-fibrotic effect of IFN, including that IFN alpha can directly reduce fibrogenesis. Shiratori et al. (2000) showed that the fibrosis stage improved from −0.60 at <3 years of follow-up to −0.88 at >3 years follow-up (a rate of −0.28/years) for SVR patients treated with non-pegylated IFN monotherapy. However, Everson et al. (2008) showed that the fibrosis stage improved −1.00 between before treatment and 6 months after the end of treatment of SVR patients receiving PEG-IFN alpha monotherapy. Poynard et al. (2002a) showed that the mean fibrosis stage 6 months after the end of treatment was 1.9 ± 0.9 (SD) for SVR patients with compensated cirrhosis treated with PEG-IFN alpha monotherapy or in combination with RBV. There was no significant difference between PEG-IFN alpha plus RBV and PEG-IFN alpha alone for the 48-week regimen as to an anti-fibrotic effect (Poynard et al., 2002a). Therefore, PEG-IFN alpha itself may have a stronger anti-fibrotic effect than non-pegylated IFN and RBV, due to a pharmaco-dynamic advantage.

In the present study, we demonstrated a dramatic reduction of FibroScan values in both SVR and BR patients. Firstly, our results confirmed, from the viewpoint of liver fibrosis, that IFN treatment is of long-term benefit for chronic hepatitis C patients. Moreover, such reduction was observed at an early stage of PEG-IFN alpha plus RBV treatment by SVR and BR patients. It is probable that such early reduction of FibroScan values means not only the improvement of fibrosis but also of inflammation in the liver as a result of treatment, because FibroScan values were significantly correlated with the grade of activity in the liver in the present study. Compared with the rate of change of FibroScan values between non-advanced fibrosis ($F \leq 2$) and advanced fibrosis ($F \geq 3$), no significant differences were found at each testing point. Secondly, another important result was that FibroScan values decreased even for BR patients without HCV clearance by treatment. The findings of the present study can explain why the rate of development of HCC is lower in BR patients treated with IFN. Because HCC tends to develop in patients with advanced liver fibrosis (cirrhosis), BR patients who have a reduction of fibrosis probably will not as quickly develop HCC in the future. Thirdly, although the virological treatment itself may produce good

results in terms of a short-term anti-fibrotic effect of the liver, the FibroScan values of non-BR patients became progressively worse in the long-term course after the antiviral treatment. In the case of a high serum ALT level for non-SVR patients, an additional antiviral retreatment has to be considered. In fact, we recommended antiviral treatment for the studied non-treated patients after the completion of this study, and then most of them have started to receive IFN treatment. A long-term IFN treatment regimen was effective in a smaller trial of the anti-fibrotic effect (Arase et al., 2004); therefore, we believe that treatment with careful attention to IFN-related adverse effects could be usefully introduced to help patients avoid progressing to liver fibrosis and HCC. Thus, the FibroScan is a very interesting tool for the follow-up of chronic HCV carriers, as it is easier to use than liver biopsy in clinical settings. These results are of great interest for the understanding of the effects of IFN treatment and of HCV-related liver disease.

In conclusion, our study shows that transient elastography (FibroScan) is a useful tool for the longitudinal assessment of IFN treatment of chronic hepatitis C patients.

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Prolonged recurrence-free survival following OK432-stimulated dendritic cell transfer into hepatocellular carcinoma during transarterial embolization

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Summary

Despite curative locoregional treatments for hepatocellular carcinoma (HCC), tumour recurrence rates remain high. The current study was designed to assess the safety and bioactivity of infusion of dendritic cells (DCs) stimulated with OK432, a streptococcus-derived anti-cancer immunotherapeutic agent, into tumour tissues following transcatheter hepatic arterial embolization (TAE) treatment in patients with HCC. DCs were derived from peripheral blood monocytes of patients with hepatitis C virus-related cirrhosis and HCC in the presence of interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor and stimulated with 0·1 KE/ml OK432 for 2 days. Thirteen patients were administered with 5×10^6 of DCs through arterial catheter during the procedures of TAE treatment on day 7. The immunomodulatory effects and clinical responses were evaluated in comparison with a group of 22 historical controls treated with TAE but without DC transfer. OK432 stimulation of immature DCs promoted their maturation towards cells with activated phenotypes, high expression of a homing receptor, fairly well-preserved phagocytic capacity, greatly enhanced cytokine production and effective tumoricidal activity. Administration of OK432-stimulated DCs to patients was found to be feasible and safe. Kaplan–Meier analysis revealed prolonged recurrence-free survival of patients treated in this manner compared with the historical controls ($P = 0{\cdot}046$, log-rank test). The bioactivity of the transferred DCs was reflected in higher serum concentrations of the cytokines IL-9, IL-15 and tumour necrosis factor-α and the chemokines CCL4 and CCL11. Collectively, this study suggests that a DC-based, active immunotherapeutic strategy in combination with locoregional treatments exerts beneficial anti-tumour effects against liver cancer.

Keywords: dendritic cells, hepatocellular carcinoma, immunotherapy, recurrence-free survival, transcatheter hepatic arterial embolization

Introduction

Many locoregional therapeutic approaches including surgical resection, radiofrequency ablation (RFA) and transcatheter hepatic arterial embolization (TAE) have been taken in the search for curative treatments of hepatocellular carcinoma (HCC). Despite these efforts, tumour recurrence rates remain high [1,2], probably because active hepatitis and cirrhosis in the surrounding non-tumour liver tissues causes *de novo* development of HCC [3,4]. One strategy to reduce tumour recurrence is to enhance anti-tumour immune responses that may induce sufficient inhibitory effects to prevent tumour cell growth and survival [5,6]. Dendritic cells (DCs) are the most potent type of antigen-presenting cells in the human body, and are involved in the regulation of both innate and adaptive immune responses [7]. DC-based immunotherapies are believed to contribute to the eradication of residual and recurrent tumour cells.

To enhance tumour antigen presentation to T lymphocytes, DCs have been transferred with major histocompatibility complex (MHC) class I and class II genes [8] and co-stimulatory molecules, e.g. CD40, CD80 and CD86 [9,10], and loaded with tumour-associated antigens, including tumour lysates, peptides and RNA transfection [11]. To induce natural killer (NK) and natural killer T (NK T) cell activation, DCs have been stimulated and modified to

Table 1. Patient characteristics.

Patient no.	Gender	Age (years)	HLA	TNM stages	No. of tumours	Largest tumour (mm)	Child–Pugh	KPS	Post-TAE Rx
1	M	60	A11 A33	III	5	35	B	100	RFA
2	M	57	A11 A24	III	1	21	B	100	RFA
3	M	57	A11 A31	III	2	39	B	100	RFA
4	M	77	A2 A24	III	2	35	A	100	RFA
5	F	83	A11 A24	III	3	29	B	100	RFA
6	F	74	A2 A24	II	1	35	A	100	RFA
7	F	72	A24 A33	III	3	41	B	100	RFA
8	F	65	A2 A11	II	4	12	B	100	RFA
9	M	71	A2 A11	II	4	16	A	100	RFA
10	M	79	A11 A24	III	2	40	A	100	RFA
11	M	71	A2 A24	II	1	28	A	100	RFA
12	M	56	A2 A26	III	2	25	B	100	RFA
13	M	64	A2 A33	III	2	37	B	100	RFA

M, male; F, female; TNM, tumour–node–metastasis; Child–Pugh, Child–Pugh classification; KPS, Karnofsky performance scores; TAE, transcatheter arterial embolization; Rx, treatment; HCC, hepatocellular carcinoma; HLA, human leucocyte antigen; RFA, percutaneous radiofrequency ablation.

produce larger amounts of cytokines, e.g. interleukin (IL)-12, IL-18 and type I interferons (IFNs)[10,12]. Furthermore, DC migration into secondary lymphoid organs could be induced by expression of chemokine genes, e.g. C-C chemokine receptor-7 (CCR7) [13], and by maturation using inflammatory cytokines [14], matrix metalloproteinases and Toll-like receptor (TLR) ligands [15].

DCs stimulated with OK432, a penicillin-inactivated and lyophilized preparation of *Streptococcus pyrogenes*, were suggested recently to produce large amounts of T helper type 1 (Th1) cytokines, including IL-12 and IFN-γ and enhance cytotoxic T lymphocyte activity compared to a standard mixture of cytokines [tumour necrosis factor-α (TNF-α), IL-1β, IL-6 and prostaglandin E_2 (PGE_2)] [16]. Furthermore, because OK432 modulates DC maturation through TLR-4 and the β_2 integrin system [16,17] and TLR-4-stimulated DCs can abrogate the activity of regulatory T cells [18], OK432-stimulated DCs may contribute to the induction of anti-tumour immune responses partly by reducing the activity of suppressor cells. Recently, in addition to the orchestration of immune responses, OK432-activated DCs have themselves been shown to mediate strong, specific cytotoxicity towards tumour cells via CD40/CD40 ligand interactions [19].

We have reported recently that combination therapy using TAE together with immature DC infusion is safe for patients with cirrhosis and HCC [20]. DCs were infused precisely into tumour tissues and contributed to the recruitment and activation of immune cells *in situ*. However, this approach by itself yielded limited anti-tumour effects due probably to insufficient stimulation of immature DCs (the preparation of which seems closely related to therapeutic outcome [21,22]). The current study was designed to assess the safety and bioactivity of OK432-stimulated DC infusion into tumour tissues following TAE treatment in patients with cirrhosis and HCC. In addition to documenting the safety of this approach, we found that patients treated with OK432-stimulated DCs displayed unique cytokine and chemokine profiles and, most importantly, experienced prolonged recurrence-free survival.

Patients and methods

Patients

Inclusion criteria were a radiological diagnosis of primary HCC by computed tomography (CT) angiography, hepatitis C virus (HCV)-related HCC, a Karnofsky score of $\geq$ 70%, an age of $\geq$ 20 years, informed consent and the following normal baseline haematological parameters (within 1 week before DC administration): haemoglobin $\geq$ 8·5 g/dl; white cell count $\geq$ 2000/μl; platelet count $\geq$ 50 000/μl; creatinine $<$ 1·5 mg/dl and liver damage A or B [23].

Exclusion criteria included severe cardiac, renal, pulmonary, haematological or other systemic disease associated with a discontinuation risk; human immunodeficiency virus (HIV) infection; prior history of other malignancies; history of surgery, chemotherapy or radiation therapy within 4 weeks; immunological disorders including splenectomy and radiation to the spleen; corticosteroid or anti-histamine therapy; current lactation; pregnancy; history of organ transplantation; or difficulty in follow-up.

Thirteen patients (four women and nine men) presenting at Kanazawa University Hospital between March 2004 and June 2006 were enrolled into the study, with an age range from 56 to 83 years (Table 1). Patients with verified radiological diagnoses of HCC stage II or more were eligible and enrolled in this study. In addition, a group of 22 historical controls (nine women and 13 men) treated with TAE without DC administration between July 2000 and September 2007 was included in this study. All patients received RFA therapy to increase the locoregional effects 1 week later [24].

They underwent ultrasound, computed tomography (CT) scan or magnetic resonance imaging (MRI) of the abdomen about 1 month after treatment and at a minimum of once every 3 months thereafter, and tumour recurrences were followed for up to 360 days. The Institutional Review Board reviewed and approved the study protocol. This study complied with ethical standards outlined in the Declaration of Helsinki. Adverse events were monitored for 1 month after the DC infusion in terms of fever, vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

Preparation and injection of autologous DCs

DCs were generated from blood monocyte precursors, as reported previously [25]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). For generating DCs, PBMCs were plated in six-well tissue culture dishes (Costar, Cambridge, MA, USA) at $1{\cdot}4 \times 10^7$ cells in 2 ml per well and allowed to adhere to plastic for 2 h. Adherent cells were cultured in serum-free media (GMP CellGro® DC Medium; CellGro, Manassas, VA, USA) with 50 ng/ml recombinant human IL-4 (GMP grade; CellGro®) and 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (GMP grade; CellGro®) for 5 days to generate immature DC, and matured for a further 2 days in 0·1 KE/ml OK432 (Chugai Pharmaceuticals, Tokyo, Japan) to generate OK-DC. On day 7, the cells were harvested for injection, 5×10^6 cells were suspended in 5 ml normal saline containing 1% autologous plasma, mixed with absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn, Peapack, NJ, USA) and infused through an arterial catheter following Lipiodol (iodized oil) (Lipiodol Ultrafluide, Laboratoire Guerbet, Aulnay-Sous-Bois, France) injection during selective TAE therapy. Release criteria for DCs were viability > 80%, purity > 30%, negative Gram stain and endotoxin polymerase chain reaction (PCR) and negative in process cultures from samples sent 48 h before release. All products met all release criteria, and the DCs had a typical phenotype of $CD14^-$ and human leucocyte antigen (HLA)-DR^+.

Flow cytometry analysis

The DC preparation was assessed by staining with the following monoclonal antibodies for 30 min on ice: anti-lineage cocktail 1 (lin-1; CD3, CD14, CD16, CD19, CD20 and CD56)-fluorescein isothiocyanate (FITC), anti-HLA-DR-peridinin chlorophyll protein (PerCP) (L243), anti-CCR7-phycoerythrin (PE) (3D12) (BD PharMingen, San Diego, CA, USA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA, USA). Cells were analysed on a fluorescence activated cell sorter (FACS0Calibur™ flow cytometer. Data analysis was performed with CELLQuest™ software (Becton Dickinson, San Jose, CA, USA).

DC phagocytosis

Immature DCs and OK432-stimulated DCs were incubated with 1 mg/ml FITC dextran (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C and the cells were washed three times in FACS buffer before cell acquisition using a FACS-Calibur™ cytometer. Control DCs (not incubated with FITC dextran) were acquired at the same time to allow background levels of fluorescence to be determined.

Enzyme-linked immunosorbent assay (ELISA)

DCs were seeded at 200 000 cells/ml, and supernatant collected after 48 h. IL-12p40 and IFN-γ were detected using matched paired antibodies (BD Pharmingen) following standard protocols.

Cytotoxicity assays

The ability of DCs to exert cytotoxicity was assessed in a standard ^{51}Cr release assay [19]. We used the HCC cell lines Hep3B and PLC/PRF/5 [American Type Culture Collection (ATCC), Manassas, VA, USA] and a lymphoblastoid cell line T2 that expresses HLA-A*0201 (ATCC) as target cells. Target cells were labelled with ^{51}Cr. In a 96-well plate, $2{\cdot}5 \times 10^3$ target cells per well were incubated with DCs for 8 h at different effector/target (E/T) ratios in triplicate. Percentage of specific lysis was calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Spontaneous release was always < 20% of the total.

NK cell activity

NK cell cytotoxicity against K562 erythroleukemia target cells was measured by using ^{51}Cr-release assay, according to previously published methods [26], with PBMCs obtained from the patients. All experiments were performed in triplicate. Percentage of cytotoxicity was calculated as follows: {[experimental counts per minute (cpm) – spontaneous cpm]/[total cpm – spontaneous cpm]} × 100.

Intracellular cytokine expression

Freshly isolated PBMCs were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) at 37°C in humidified 7% CO_2 for 4 h. To block cytokine secretion, brefeldin A (Sigma) [27] was added to a final concentration of 10 μg/ml. After addition of stimuli, the surface staining was performed with anti-CD4-PC5 (13B8·2), anti-CD8-PerCP (SK1) and anti-CD56-PC5 (N901) (Beckman

Coulter). Subsequently, the cells were permeabilized, stained for intracellular IFN-γ and IL-4 using the FastImmune™ system (BD Pharmingen), resuspended in phosphate-buffered saline (PBS) containing 1% paraformaldehyde (PFA), and analysed on a flow cytometer (≈ 10 000 gated events acquired per sample).

IFN-γ enzyme-linked immunospot (ELISPOT) assay

ELISPOT assays were performed as described previously with the following modifications [28–30]. HLA-A24 restricted peptide epitopes, squamous cell carcinoma antigen recognized by T cells 2 $(SART2)_{899}$ (SYTRLFLIL), $SART3_{109}$ (VYDYNCHVDL), multi-drug resistance protein 3 $(MRP3)_{765}$ (VYSDADIFL), $MRP3_{503}$ (LYAWEPSFL), $MRP3_{692}$ (AYVPQQAWI), alpha-fetoprotein $(AFP)_{403}$ (KYIQESQAL), AFP_{434} (AYTKKAPQL), AFP_{357} (EYSRRHPQL), human telomerase reverse transcriptase $(hTERT)_{167}$ (AYQVCGPPL) (unpublished), $hTERT_{461}$ (VYGFVRACL) and $hTERT_{324}$ (VYAETKHFL) were used in this study. Negative controls consisted of an HIV envelope-derived peptide ($HIVenv_{584}$). Positive controls consisted of 10 ng/ml PMA (Sigma) or a CMV pp65-derived peptide ($CMVpp65_{328}$). The coloured spots were counted with a KS ELISPOT Reader (Zeiss, Tokyo, Japan). The number of specific spots was determined by subtracting the number of spots in the absence of antigen from the number of spots in its presence. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than the number of spots in the absence of antigen.

Cytokine and chemokine profiling

Serum cytokine and chemokine levels were measured using the Bioplex assay (Bio-Rad, Hercules, CA, USA). Briefly, frozen serum samples were thawed at room temperature, diluted 1:4 in sample diluents, and 50 μl aliquots of diluted sample were added in duplicate to the wells of a 96-well microtitre plate containing the coated beads for a validated panel of 27 human cytokines and chemokines (cytokine 27-plex antibody bead kit) according to the manufacturer's instructions. These included IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, G-CSF, GM-CSF, IFN-γ, interferon gamma-induced protein (IP)-10, monocyte chemoattractant protein (MCP)-1, MIP-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated upon activation normal T cell-expressed and secreted (RANTES), TNF-α and vascular endothelial growth factor (VEGF). Eight standards (ranging from 2 to 32 000 pg/ml) were used to generate calibration curves for each cytokine. Data acquisition and analysis were performed using Bio-Plex Manager software version 4.1.1.

Arginase activity

Serum samples were tested for arginase activity by conversion of L-arginine to L-ornithine [31] using a kit supplied by the manufacturer (BioAssay Systems, Hayward, CA, USA). Briefly, sera were treated with a membrane filter (Millipore, Billerica, MA, USA) to remove urea, combined with the sample buffer in wells of a 96-well plate, and incubated at 37°C for 2 h. Subsequently, the urea reagent was added to stop the arginase reaction. The colour produced was read at 520 nm using a microtitre plate reader.

Statistical analysis

Results are expressed as means ± standard deviation (s.d.). Differences between groups were analysed for statistical significance by the Mann–Whitney *U*-test. Qualitative variables were compared by means of Fisher's exact test. The estimated probability of tumour recurrence-free survival was determined using the Kaplan–Meier method. The Mantel–Cox log-rank test was used to compare curves between groups. Any *P*-values less than 0·05 were considered statistically significant. All statistical tests were two-sided.

Results

Preparation of OK432-stimulated DCs

Adherent cells isolated from PBMCs of patients with cirrhosis and HCC (Table 1) were differentiated into DCs in the presence of IL-4 and GM-CSF. The cells were stimulated with 0·1 KE/ml OK432 for 3 days; 54·6 ± 9·5% (mean ± s.d.; $n = 13$) of OK432-stimulated cells showed high levels of MHC class II (HLA-DR) and the absence of lineage markers including CD3, CD14, CD16, CD19, CD20 and CD56, in which 30·9 ± 14·2% were CD11c-positive (myeloid DC subset) and 14·8 ± 11·2 were CD123-positive (plasmacytoid DC subset), consistent with our previous observations [20]. As reported [32,33], greater proportions of the cells developed high levels of expression of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86) and an activation marker (CD83) compared to DCs prepared without OK432 stimulation (Fig. 1a). Furthermore, the chemokine receptor CCR7 which leads to homing to lymph nodes [13,34] was also induced following OK432 stimulation.

To evaluate the endocytic and phagocytic ability of the OK432-stimulated cells, uptake of FITC-dextran was quantitated by flow cytometry (Fig. 1b). The cells showed lower levels of uptake due to maturation compared to DCs prepared without OK432 stimulation, while the OK432-stimulated cells derived from HCC patients preserved a moderate uptake capacity. As expected, the OK432-stimulated cells produced large amounts of cytokines IL-12 and IFN-γ (Fig. 1c). In addition, they displayed high cyto-