relationships are little known and still controversial [10]. The aim of this study was to investigate whether amino acid substitutions in the core region and ISDR among patients with HCV genotype 1b affect the response to pegylated-IFN-alpha 2b and ribavirin combination therapy.

MATERIAL AND METHODS

A total of 891 patients with chronic hepatitis C genotype 1b and high viral load who were treated at Nagoya University Hospital and Affiliated Hospitals were enrolled; 213 patients who completed IFN treatment were randomly selected for this study. The patients' clinical characteristics are summarized in Table 1. Patients whose HCV-RNA levels were <100 KIU/mL were excluded. The core region (aa 30–110) and ISDR (aa 2209–2248) were examined by direct sequencing. All patients received subcutaneous injections of pegylated-IFN-alpha 2b (1.5 μg/kg) once each week plus oral ribavirin daily for 48 weeks. HCV-RNA in serum samples was examined at 12 weeks, at the end of IFN therapy and at 6 months after the end of treatment. Serum was stored at −80 °C for virologic examination. Early virologic response (EVR) was defined as HCV-negative at 12 weeks. Patients who were persistently negative for serum HCV-RNA and who had a normal serum alanine aminotransferase (ALT) level at 24 weeks after withdrawal of IFN treatment were considered to have sustained virologic response (SVR). Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Virologic analysis

HCV-RNA quantitative viremia load was determined by polymerase chain reaction (PCR). HCV was genotyped by direct sequencing of the 5′-untranslated region and/or E1 regions as described previously [11,12]. Genotypes were classified according to the nomenclature proposed by Simmonds *et al.* [13]. Direct sequencing of the core and NS5A-ISDR region was carried out as reported previously, but with modifications [7,14]. In brief, RNA was extracted from 140 μL serum with a commercial kit (QIAamp Viral RNA Kit; Qiagen, Valencia, CA, USA) and dissolved in 50 μL diethylpyrocarbonate-treated water. RNA (10 ng) was used for reverse transcription with oligo and random hexamer primers with a commercial kit (iScript cDNA Synthesis Kit; Bio-Rad, Hercules, CA, USA). HCV core region and NS5A-ISDR were amplified by nested PCR. In brief, each 50-μL PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μL GeneAmp 10 × PCR buffer, 2 μL dNTPs and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Primers for core region were sense 5′-GGGAGGTCTCGTAGACCGTGCACCATG-3′ and antisense 5′-GAGMGGKATRTACCCCATGAGRTCGGC-3′ and primers for the NS5A-ISDR were sense 5′-TGGATGGAGTGCGGTTGCACAGGTA-3′ and antisense 5′-TCTTTCTCCGTGGAGGTGGTATTG-3′. Amplification conditions consisted of 10 min at 94 °C, followed by 40 cycles of 94 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). The second PCR was performed in the same reaction buffer with the first-round PCR product as template, and the following sets of primers: for the core region, sense primer 5′-AGACCGTGCACCATGAGCAC-3′ and antisense 5′-TACGCCGGGGGTCAKTRGGGCCCCA-3′; and for the NS5A-ISDR, sense 5′-CAGGTACGCTCCGGCGTGCA-3′ and antisense 5′-GGGGCCTTGGTAGGTGGCAA-3′. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. PCR products were then purified and sequenced with the second-round PCR primers with a dye terminator sequencing kit (BigDye Terminator v1.1 Cycle Sequencing Kit; Applied Biosystems) and an ABI 310 DNA Sequencer (Applied Biosystems). A mutation mixture was defined as viral mutants that constituted 50% or more of the total viral population.

Table 1 Clinical characteristics

Clinical characteristics	N =2 13
Age (years)	55.2 ± 10.6
Sex: male/female	120/93
AST(IU/L)	58.5 ± 37.7
ALT(IU/L)	66.0 ± 53.9
Platelet count (10^4/uL)	17.1 ± 5.1
HCV RNA level (KIU/mL)	1720 (100–7200)
Treatment: naive/retreatment	117/96
Body weight (kg)	55.3 ± 19.9

Data are expressed as mean ± standard deviation HCV RNA level was shown by median (range). AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus.

Statistical analysis

Data are expressed as means ± standard deviation (SD). The paired *t*-test, the chi-square and the Fisher's exact tests were used to analyze differences in variables. A *P*-value of <0.05 was considered statistically significant. Multiple logistic regression models were used to identify factors predictive of EVR and SVR. Statview 5.0 software (SAS Institute, Inc., Cary, NC, USA) was used for all analyses.

RESULTS

Genetic heterogeneity in NS5A-ISDR and core regions of the HCV genome

The mutations in the HCV core region were measured by direct sequencing. The core region of HCV is well conserved,

Table 2 Prevalence of amino acid substitutions at 70, 75, and 91

Core 70	
Histidine	$n = 6$
Glutamine	$n = 46$
Glutamine/Histidine	$n = 1$
Arginine	$n = 160$
Core 75	
Alanine	$n = 112$
Alanine/Serine	$n = 1$
Alanine/Threonine	$n = 2$
Glutamine	$n = 1$
Serine	$n = 5$
Threonine	$n = 91$
Valine	$n = 1$
Core 91	
Leucine	$n = 162$
Methionine	$n = 51$

but substitutions of aa 70, aa 75 and aa 91 were frequently found, as previously reported. The distribution of mutations in the HCV core region at aa 70, aa 75 and aa 91 is shown in Table 2. The sequence of the HCVJ strain was defined as the consensus sequence, and the approach of counting the number of mutations to the chosen consensus sequence in ISDR was used to analyze the ISDR system. The number of NS5A-ISDR mutations was as follows: none ($n = 102$), 1 ($n = 63$), 2 ($n = 14$), 3 ($n = 8$), 4 ($n = 8$), 5 ($n = 7$), 6 ($n = 2$), 7 ($n = 4$) and 8 ($n = 5$). The relationships between substitutions of amino acids in the HCV core region and NS5A-ISDR are shown in Fig. 1. There were no significant relationships between the two regions. Thus, the HCV core region and the NS5A-ISDR were independent factors.

Virological response

Of 213 patients, 117 (54.9%) showed EVR, with HCV-negativity, at 12 weeks, and 76 became HCV-negative after 12 weeks; overall, 187 patients became HCV-negative at the end of treatment (87.8%). However, 85 patients continued to be HCV-positive after withdrawal of IFN treatment, and 102 of 213 (47.9%) patients were defined as achieving a SVR. Of 117 patients with EVR, 87 (74.4%) achieved SVR. Of 96 patients without EVR, 81 became non-SVR (84.4%). Thus, EVR was strongly associated with SVR.

Factors associated with early virologic response

The results of univariate analysis for factors predictive of EVR are shown in Table 3. The EVR rate according to amino acid substitutions of ISDR are shown in Table 4. The EVR rate of patients with more than two mutations in the ISDR (mutant-type) was 68.9%. Of 166 patients without glutamine (Gln) at aa 70 in the core region, 100 achieved EVR. The EVR rate of patients with Leu91 in the core region was 61.1%. The results of multivariate analysis for factors predictive of EVR are shown in Table 5. Factors related to EVR on multivariate analysis were non-Gln70, Leu91 and ISDR mutant-type.

Factors associated with sustained virologic response

The results of univariate analysis for factors predictive of SVR are shown in Table 6. The SVR rate according to amino acid substitutions of ISDR are shown in Table 4. SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) (odds ratio, 0.217; 95% confidence interval (CI), 0.101–0.466; $P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% with mutant-type ISDR (odds ratio, 0.465; 95% CI, 0.240–0.899; $P = 0.0227$). Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type, as shown in Table 7.

The virological response according to amino acid substitutions in the 70 core region and ISDR

The SVR and EVR rates according to amino acid substitutions in the 70 core region and ISDR are shown in Table 8. The best response for both SVR and EVR was achieved in patients with non-Gln70 and mutant-type ISDR, and the

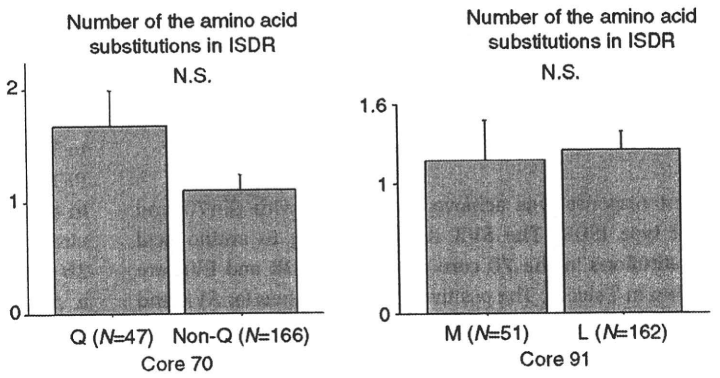

Fig. 1 The association between amino acid substitutions in core region and ISDR. ISDR, interferon sensitivity-determining region; Q, glutamine; L, leucine; M, methionine; NS, not significant.

Table 3 Univariate analysis: Factors predictive of EVR

Factors	EVR (n = 117)	Non-EVR (n = 96)	P-value
Age (years)	54.7 ± 11.3	55.9 ± 9.7	0.4511
Gender: male/female	63/54	57/39	0.7830
ALT (IU/L)	69.6 ± 64.8	61.5 ± 36.2	0.3002
AST (IU/L)	59.4 ± 40.9	57.3 ± 33.5	0.7026
PLT ($\times 10^4/mm^3$)	17.4 ± 5.1	16.9 ± 5.18	0.4955
HCV RNA level (KIU/mL)	2051.3 ± 1373.4	2006.1 ± 1462.7	0.8216
Core 70:non-Q/Q	100/17	66/30	0.0046
Core 75: A/non-A	58/59	54/42	0.3387
Core 91: L/M	99/18	63/33	0.0020
ISDR: wild/mutant	84/33	81/15	0.0327

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine; ISDR, interferon sensitivity-determining region

Table 4 Amino acid substitutions of ISDR and virologic response

ISDR; number of the amino acid substitutions	0 N = 102	1 N = 63	2 N = 14	3 N = 8	4 N = 8	5 N = 7	6 N = 2	7 N = 4	8 N = 5
EVR rate (%)	51 (50.0)	33 (52.4)	10 (71.4)	4 (50.0)	7 (87.5)	4 (80.0)	0 (0)	3 (75.0)	5 (100)
SVR rate (%)	41 (40.2)	31 (49.2)	10 (71.4)	4 (50.0)	4 (50.0)	5 (71.4)	0 (0)	3 (75.0)	4 (80.0)

EVR, early virologic response; SVR, sustained virologic response.

Table 5 Multivariate analysis: Factors predictive of EVR

Factors	P-value	Risk ratio	95% CI	
Gender: male	0.3760	0.754	0.403	1.410
Age: <60 years	0.8247	0.915	0.416	2.012
AST: <60 IU/L	0.3301	1.525	0.652	3.569
ALT: <60 IU/L	0.2484	0.613	0.267	1.407
PLT: $<17 \times 10^4/mm^3$	0.0666	0.530	0.269	1.044
Core 70: nonQ	0.0242	2.406	1.121	5.165
Core 91: A	0.0022	3.409	1.557	7.463
Core 75: M	0.0683	1.863	0.954	3.635
ISDR: mutant	0.0085	0.338	0.151	0.759

EVR, early virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; ISDR, Interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M; methionine.

worst response was achieved in patients with Gln70 and wild type ISDR. The SVR rates according to amino acid substitutions in the 70 core region and ISDR and EVR are shown in Table 9. The positive predictive values for SVR and non-SVR improved to 88.9% and 90.9%, respectively, when EVR was considered with the 70 core region and ISDR.

DISCUSSION

Peginterferon and ribavirin combination therapy has been standard treatment for patients with chronic hepatitis C. However, the SVR rate was almost 50% for HCV genotype 1b, which is a refractory strain. The standard doses and duration of peginterferon plus ribavirin may be suboptimal for half of the patients; patients need a new approach for eradicating HCV. Peginterferon and ribavirin therapy has been a useful treatment, but cost and adverse events have been problems. To select patients who could attain cure from HCV by current standard treatment, it is necessary to predict the response before therapy. Current guidelines for HCV treatment recommend that the selection of IFN treatment regimen depends on HCV genotypes and viral loads. Several studies have focused on sequence variation of the HCV genome and response to IFN therapy, but prediction of IFN responsiveness has been less well characterized. NS5A-ISDR heterogeneity is an important factor that may affect response to IFN, especially in Asia [6,7,9]. The ISDR interacts with PKR and regulates replication of HCV *in vitro* [5]. Mutations in the ISDR affect the interaction with PKR and may inhibit viral replication. Therefore, ISDR of not only HCV genotype 1b but also 2a and 2b could also play an important role as a predictor of IFN responsiveness in clinical research of standard IFN or Peg-IFN monotherapy [15,16]. The differences in HCV 1b subtype and race affect the utility of ISDR

Table 6 Univariate analysis: factors predictive of SVR

Factors	SVR (n = 102)	Non-SVR (n = 111)	P-value
Age (years)	53.6 ± 10.8	56.7 ± 10.2	0.0319
Gender: male/female	57/45	63/48	0.7830
ALT (IU/L)	69.6 ± 66.7	62.6 ± 38.5	0.3606
AST (IU/L)	58.8 ± 40.9	58.3 ± 34.8	0.9469
PLT ($\times 10^4/mm^3$)	17.7 ± 5.1	16.7 ± 5.0	0.1563
HCV RNA level (KIU/mL)	2111.1 ± 1504.9	1956.4 ± 1319.8	0.4386
Core 70:non-Q/Q	92/10	74/37	0.0001
Core 75: A/non-A	50/52	62/49	0.3388
Core 91: L/M	82/20	80/31	0.1984
ISDR: wild/mutant	72/30	93/18	0.0227

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count; HCV, hepatitis C virus; Q, glutamine; A, alanine; L, leucine; M, methionine, ISDR, Interferon sensitivity-determining region.

Table 7 Multivariate analysis: factors predictive of SVR

Factors	P-value	Risk ratio	95% CI	
Age: <60 years	0.5219	0.770	0.346	1.714
Gender: male	0.6775	1.140	0.614	2.116
AST: <60 IU/L	0.1017	0.487	0.206	1.153
ALT: <60 IU/L	0.1690	1.799	0.779	4.157
PLT: $<17 \times 10^4/mm^3$	0.4067	1.324	0.682	2.573
HCV RNA levels: <106 IU/mL	0.6409	0.841	0.405	1.743
Core70: nonQ	0.0004	0.220	0.094	0.512
Core91: M	0.5643	0.799	0.373	1.711
Core75: A	0.3993	0.757	0.396	1.446
ISDR: mutant	0.0096	2.879	1.294	6.407

SVR, sustained virologic response; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PLT, platelet count, HCV, hepatitis C virus; ISDR, interferon sensitivity-determining region; Q, glutamine; A, alanine; L, leucine; M, methionine.

Table 8 The SVR and EVR rate according to amino acid substitutions in 70 core region and ISDR

Core70/ISDR	SVR (n = 102)	EVR (n = 117)
Q/wild (n = 33)	6 (18.2%)	11 (33.3%)
Q/mutant (n = 14)	4 (28.6%)	6(42.9%)
Non-Q/wild (n = 132)	66 (50.0%)	73 (55.3%)
Non-Q/mutant (n = 34)	26 (76.5%)	27 (79.4%)

SVR, sustained virologic response; EVR, early virologic response; SDR, interferon sensitivity-determining region; Q, Glutamine; ISDR, interferon sensitivity-determining region.

sequences for predicting IFN responsiveness [7,17,18]. Thus, ISDR was found to be good for predicting IFN outcome of patients in Asian countries rather than of patients in Western countries. The approach of counting the number of mutations to the HCV-J strain in the ISDR was used in the original report by Enomoto *et al.*, [6] and they classified the mutations into three groups: wild type (no mutation), intermediate (1–3 mutations) and mutant-type (more than four mutations). SVR did not occur in any of the 30 patients with wild type ISDR in the original report using standard IFN monotherapy. In the present study, 41 of 102 patients (40.2%) with the wild type ISDR (no mutation) achieved SVR because of improvement of Peg-IFN plus RBV combination therapy. We examined the association between the number of mutations and SVR with adjustment for current standard treatment. We were unable to identify a significant relation between no mutation and one mutation in ISDR and SVR. Thus, sequences of the HCV-J strain and HCV-J strain with single substitutions were defined as the wild-type, and ISDR sequences with more than two mutations were defined as the mutant-type. SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ISDR in this study. ISDR alone was insufficient to predict IFN responsiveness in patients who received peginterferon plus ribavirin combination therapy. We speculated that the other region would explain differences in IFN sensitivity in patients infected with wild type ISDR. HCV core, E2-PePHD and NS5A-V3 regions were reported to be associated with IFN response [8,10,19,20]. The HCV core interacts with several cell factors and modulates numerous gene expressions, including down-regulating transcription of IFN-induced antiviral genes, and it affects the inhibition of the antiviral action of IFN. Several studies indicated that the HCV core region could predict IFN responsiveness [8,10]. Therefore, the utility of substitutions of amino acids in the HCV core region combined with NS5A-ISDR sequences for predicting

Table 9 The SVR rate according to EVR amino acid substitutions in 70 core region and ISDR

Core70/ISDR	SVR of patients with EVR (n = 87)	Non SVR of patients with EVR (n = 30)	SVR of patients without EVR (n = 15)	Non SVR of patients without EVR (n = 81)
Q/wild (n = 33)	4 (40%*)	7	2	20 (90.9%**)
Q/mutant (n = 14)	3 (50%*)	3	1	7 (87.5%**)
Non-Q/wild (n = 132)	56 (76.7%*)	17	10	49 (83.1%**)
Non-Q/mutant (n = 34)	24 (88.9%*)	3	2	5 (71.4%**)

*Positive predictive value for SVR. **Positive predictive value for non-SVR. SVR, sustained virologic response; EVR, early virologic response; ISDR, interferon sensitivity-determining region; Q, glutamine.

IFN responsiveness was investigated. The non-Gln70 amino acid substitution in the HCV core region was related to SVR on univariate and multivariate analysis. SVR occurred more frequently in patients without Gln70 (50.6%) than with Gln70 (14.3%). SVR was not associated with aa 75 and aa 91 in the core region. When core 70 was considered in the analysis of ISDR, the SVR rates varied widely according to amino acid substitutions in core region 70 and ISDR. For instance, only 18.1% of patients with Gln70 and wild type ISDR achieved SVR compared with 76.4% in those with non-Gln70 and mutant-type ISDR. Despite having genotype 1b, patients with non-Gln70 and mutant-type ISDR responded to IFN as well as those with genotypes 2 and 3. Pegylated-IFN-alpha 2b and ribavirin combination therapy was suitable for treatment of Japanese patients with HCV genotype 1b, particularly those with non-Gln70 and mutant-type ISDR. Optimal duration of IFN therapy in some patients with non-Gln70 and mutant-type ISDR could be shorter than 48 weeks; and in these patients, costs and side effects could be reduced without reducing the efficacy of IFN therapy by using a shorter regimen. On the other hand, patients with Gln70 and wild type ISDR resistant to pegylated-IFN-alpha 2b and ribavirin combination therapy should receive much more powerful treatment, such as triple therapy including the new protease inhibitor, peginterferon alfa and ribavirin as their first regimen [21,22]. This is an important consideration to achieve optimal therapy and avoid unnecessary treatment. The effects of amino acid substitutions in core 70 on gene expression and core protein function were unclear, and further studies are needed to determine their mechanism. Although the effects of amino acid substitutions of the core region and ISDR were unclear, the mutation at core 70 and the ISDR system could be clinically used as a simple diagnostic tool to predict SVR in patients infected with genotype 1b. It is not easier to routinely measure the HCV sequence to determine the core 70 and ISDR sequence. Virologic response, as rapid virologic response and EVR, could be easy to measure by commercial kits in clinical practice and would be useful for prediction of achieving SVR for chronic hepatitis C patients. The present study also confirmed that EVR has been associated with SVR, but virologic response cannot be assessed before treatment. HCV sequencing analysis will become a convenient method because of progression of sequencing technology and cost reduction. In this respect, the core region and ISDR were useful predictors of virologic response. Analysis of EVR in combination with the core region and ISDR revealed that 24 of 34 patients with non-Gln70 and mutant-type ISDR and EVR achieved SVR. EVR, core region and ISDR are considered strong indicators of SVR for patients with HCV genotype 1b. Although validation of these observations in larger cohorts is required, amino acid substitutions in the core region of HCV and ISDR were useful for predicting the response to pegylated-IFN-alpha 2b and ribavirin combination therapy in patients with chronic hepatitis C genotype 1b. Combining amino acid substitutions in the core region and ISDR could improve the predictive value of SVR in patients with genotype 1b, but the efficacy is still not satisfactory. The explanation for the lack of SVR in patients with non-Gln70 and mutant-type ISDR remains unclear. The other regions of HCV or host factors are candidates for a third factor for improving the prediction of SVR [23,24].

CONCLUSION

Amino acid substitutions in the 70 core region of HCV and ISDR were useful for predicting the response to pegylated-IFN-alpha 2b and ribavirin combination therapy in patients with chronic hepatitis C genotype 1b.

Data of this study were presented in part at the 59th annual meeting of the American association for the study of liver diseases (AASLD), October 31-November 4, 2008, San Francisco, CA, USA.

DISCLOSURE

All people have nothing to disclose.

REFERENCES

1 Seeff LB. Natural history of chronic hepatitis C. *Hepatology* 2002; 36: S35–S46.

2 Reed KE, Rice CM. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. *Curr Top Microbiol Immunol* 2000; 242: 55–84.
3 Large MK, Kittlesen DJ, Hahn YS. Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. *J Immunol* 1999; 162: 931–938.
4 Gale M Jr, Foy EM. Evasion of intracellular host defence by hepatitis C virus. *Nature* 2005; 436: 939–945.
5 Gale M Jr, Blakely CM, Kwieciszewski B *et al.* Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol* 1998; 18: 5208–5218.
6 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334: 77–81.
7 Nakano I, Fukuda Y, Katano Y, Nakano S, Kumada T, Hayakawa T. Why is the interferon sensitivity-determining region (ISDR) system useful in Japan? *J Hepatol* 1999; 30: 1014–1022.
8 Akuta N, Suzuki F, Kawamura Y *et al.* Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007; 46: 403–410.
9 Yen YH, Hung CH, Hu TH *et al.* Mutations in the interferon sensitivity-determining region (nonstructural 5A amino acid 2209–2248) in patients with hepatitis C-1b infection and correlating response to combined therapy of pegylated interferon and ribavirin. *Aliment Pharmacol Ther* 2008; 27: 72–79.
10 Okanoue T, Itoh Y, Hashimoto H *et al.* Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study. *J Gastroenterol* 2009; 44: 952–963.
11 Otagiri H, Fukuda Y, Nakano I *et al.* Evaluation of a new assay for hepatitis C virus genotyping and viral load determination in patients with chronic hepatitis C. *J Virol Methods* 2002; 103: 137–143.
12 Hayashi K, Fukuda Y, Nakano I *et al.* Prevalence and characterization of hepatitis C virus genotype 4 in Japanese hepatitis C carriers. *Hepatol Res* 2003; 25: 409–414.
13 Simmonds P, Bukh J, Combet C *et al.* Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005; 42: 962–973.
14 Ohno O, Mizokami M, Wu RR *et al.* New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *Clin Microbiol* 1997; 35: 201–207.
15 Murakami T, Enomoto N, Kurosaki M, Izumi N, Marumo F, Sato C. Mutations in nonstructural protein 5A gene and response to interferon in hepatitis C virus genotype 2 infection. *Hepatology* 1999; 30: 1045–1053.
16 Hayashi K, Katano Y, Honda T *et al.* Mutations in the interferon sensitivity-determining region of hepatitis C virus genotype 2a correlate with response to pegylated-interferon-alpha 2a monotherapy. *J Med Virol* 2009; 81: 459–466.
17 Reddy KR, Hoofnagle JH, Tong MJ *et al.* Racial differences in responses to therapy with interferon in chronic hepatitis C. Consensus Interferon Study Group. *Hepatology* 1999; 30: 787–793.
18 Missiha S, Heathcote J, Arenovich T, Khan K; Canadian Pegasys Expanded Access Group. Impact of asian race on response to combination therapy with peginterferon alfa-2a and ribavirin in chronic hepatitis C. *Am J Gastroenterol* 2007; 102: 2181–2188.
19 Muñoz de Rueda P, Casado J, Patón R *et al.* Mutations in E2-PePHD, NS5A-PKRBD, NS5A-ISDR, and NS5A-V3 of hepatitis C virus genotype 1 and their relationships to pegylated interferon-ribavirin treatment responses. *J Virol* 2008; 82: 6644–6653.
20 El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. *Hepatology* 2008; 48: 38–47.
21 Hézode C, Forestier N, Dusheiko G *et al.* Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009; 360: 1839–1850.
22 McHutchison JG, Everson GT, Gordon SC *et al.* Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 2009; 360: 1827–1838.
23 Shirakawa H, Matsumoto A, Joshita S *et al.* Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. *Hepatology* 2008; 48: 1753–1760.
24 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; 41: 1105–1109.

Hepatology Research

Hepatology Research 2010; 40: 261–268 doi: 10.1111/j.1872-034X.2009.00602.x

Original Article

Advantage of IFN-β/α2b same-day administration for ribavirin-intolerant patients with chronic hepatitis C

Eiichi Tomita,[1] Kazuki Ando,[2] Jun-ichi Sugihara,[1] Youichi Nishigaki,[1] Tetsuya Yamada,[3] Ryoko Ando,[1] Masao Takemura[4] and Mitsuru Seishima[4]

[1]*Gastroenterology and* [3]*Clinical Laboratory, Gifu Municipal Hospital, Gifu,* [2]*Goto Clinic, Ogaki, and* [4]*Department of Laboratory Medicine, Gifu University Graduate School of Medicine, Gifu, Japan*

Aim: Although interferon (IFN)/ribavirin is the mainstream combination treatment for chronic hepatitis C in patients with a high viral load, ribavirin is problematic for women of childbearing age and patients with anemia. Therefore we needed to establish a new regimen without ribavirin.

Methods: We devised a new regimen (same-day β/α2b) to administer IFN-β and α2b on the same-day, and compared it with IFN-α2b alone and IFN-α2b plus ribavirin. The cases were 36 patients (26.1%) in whom ribavirin could not be used (young women, anemia, etc.) among 138 patients who underwent IFN treatment after ribavirin release.

Results: The percentages of patients withdrawing due to side-effects were 6.8%, 18.8% and 17.0% in the treatment with same-day β/α2b, IFN-α2b alone and IFN-α2b plus ribavirin groups, respectively. In genotype 1b, the sustained viral response (SVR) was 28.6% (4/14), 13.6% (3/22) and 25.0% (8/32) with a high viral load, and 91.7% (11/12), 27.3% (3/11) and 57.1% (4/7) with a low viral load for the respective groups. According to a study on viral half-life during the early phase of IFN therapy, there was no difference among the regimens of same-day IFN-β/α2b, β alone, α2b alone and twice-daily treatment with IFN-β. Same-day β/α2b treatment showed a significantly higher SVR rate in moving type patients with a genotype 1b/high viral load.

Conclusions: Same-day β/α2b treatment resulted in few cases where therapy was discontinued and showed a high SVR rate. This regimen is especially appropriate in cases where ribavirin has been deemed unsuitable.

Key words: chronic hepatitis C, interferon treatment, new regimen, ribavirin, sustained viral response

INTRODUCTION

AT PRESENT, ONLY interferon (IFN) treatment enables the elimination of HCV-RNA for cases of chronic hepatitis C (CH-C). However, the rate of disappearance of HCV RNA is determined by factors such as genotype, quantity of HCV RNA, and the number of the NS5A mutations.[1–3] In particular, a sustained viral response (SVR) to IFN monotherapy in patients with genotype 1b and a high viral load is quite low, which is the main problem with IFN treatment. In addition, SVR in genotype 1b patients with a low viral load and genotype 2a or 2b patients with a high viral load is not nearly sufficient.

Correspondence: Dr Eiichi Tomita, Department of Gastroenterology, Gifu Municipal Hospital, 7-1 Kashima-cho, Gifu City, Gifu 500-8513, Japan. Email: etomita_jp@yahoo.co.jp
Received 30 March 2009; revision 24 August 2009; accepted 31 August 2009.

In recent years, the therapeutic effect in patients with a high viral load has been significantly improved by the use of ribavirin.[4–8] However, because ribavirin is not recommended for women of childbearing age and patients with anemia, IFN/ribavirin combination therapy is inappropriate in these cases. Therefore it was necessary to devise a protocol to treat such cases that would deliver an equivalent therapeutic effect without using ribavirin. In this study, we considered the duration, dosage, and method of administration.

Here, we devised a new regimen to give IFN-β and α on the same-day for the purpose of raising the clinical efficacy without exacerbating the side-effects.

PATIENTS AND METHODS

Patients

WE EXAMINED 174 CH-C patients for whom IFN medical treatment was performed for examination of availability. As shown in Table 1, we analyzed

Table 1 Clinical and virological background of 174 patients in the response to three interferon (IFN) treatment regimens

Variables	Same-day β /α2b ($n = 73$)	IFN-α2b monotherapy ($n = 48$)	IFN-α2b with ribavirin ($n = 53$)
Age (mean ± SD)	47.80 ± 13.43	48.40 ± 11.35	52.22 ± 9.92
Sex			
Men/women	45/28	36/12	42/11
		$P = 0.021$	
SVR ratio		$P = 0.002$	
Genotype1b	15/26 (57.7%)	6/33 (18.2%)	12/39 (30.8%)
High viral load	4/14 (28.6%)	3/22 (13.6%)	8/32 (25.0%)
Low viral load	11/12 (91.7%)	3/11 (27.3%)	4/7 (57.1%)
		$P = 0.002$	
Genotype2a+2b	39/47 (83.0%)	8/15 (53.3%)	9/14 (64.3%)
High viral load	17/24 (70.8%)	N.T.	3/6 (50.0%)
Low viral load	22/23 (95.7%)	N.T.	6/8 (75.0%)

N.T., not tested; SD, standard deviation; SVR, sustained viral response

the data by dividing the patients into three groups: (i) same-day administration with IFN-β and α2b (same-day IFN-β/α2b) (73 patients) (ii) IFN-α2b monotherapy (48 patients) (iii) IFN-α2b plus ribavirin (53 patients). (b) For examination of therapy choice, we analyzed the data of 138 patients with a high viral load who received IFN therapy to achieve SVR (Table 2a). Because our purpose in Table 2a was to analyze the frequency of problematic ribavirin use, we targeted all patients (high viral load) who were received IFN after ribavirin release commercially. Thus, the patient group differs from those in Tables 1 and 2b. To examine the frequency of withdrawal and modification in the three IFN regimens above, we examined 174 CH-C patients (the same patient group as Table 1 [Table 2b]). A competitive RT-PCR method was used to determine the quantity of HCV-RNA (analyzed by SRL Inc. Tachikawa, Tokyo) and defined $\geq 10^5$ copies/50 μL as the high virus group and $\leq 10^{4.5}$ copies/50 μL as the low virus group. The genotype assay was conducted according to the method of Ohno *et al.*[9]

Table 2 Patients for whom (a) ribavirin treatment was unsuitable and (b) therapeutic withdrawal and modification during treatment was necessary

a. Impossibility of ribavirin use (high viral load)

Total	36/138 (26.1%)
Old age (≥70 years of age)	13/36 (36.1%)
Invalidity or intolerance at the first IFN therapy	8/36 (22.2%)
Women of child-bearing age	6/36 (16.7%)
Rejection	5/36 (13.9%)
Anemia	4/36 (11.1%)

b. Therapeutic withdrawal and modification

	Withdrawal	Modification		Total		
		Major	Minor			
Same-day IFN-β/α2b	5/73 (6.8%)	1/73 (1.4%)	13/73 (17.8%)	19/73 (26.0%)		
IFN-α2b monotherapy	9/48 (18.8%)	0/48 (0%)	1/48 (2.1%)	10/48 (20.8%)	*	**
IFN-α2b with ribavirin	9/53 (17.0%)	2/53 (3.8%)	11/53 (20.8%)	22/53 (41.5%)		

*$P = 0.014$, **$P = 0.029$. IFN, interferon.

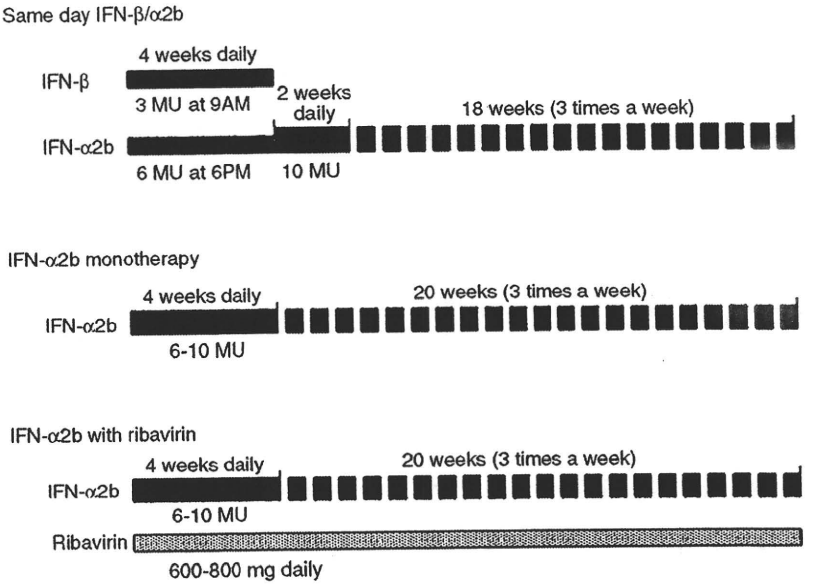

Figure 1 Therapeutic schedule of the group of same-day interferon (IFN)-β/α2b, IFN-α2b monotherapy and IFN-α2b plus ribavirin. The duration of total IFN therapy and daily injection were unified to 24 weeks and 4 weeks, respectively.

IFN Treatment Regimen (see Fig. 1)

Since there were various types of patients according to their genotype and viral load, the term of each standard regimen was different. However, for the comparison of SVR among the groups we unified the terms of IFN therapy to 24 weeks.

Group receiving same-day IFN-β/α2b

Patients received 3 MU of natural IFN-β intravenously (Feron; Toray Industries Inc., Tokyo, Japan) at 0900 hours and 6 MU of IFN-α2b (Intron; Schering-Plough, Kenilworth, NJ) intramuscularly at 1800 hours for the initial 4 weeks of therapy, after which 10 MU of IFN-α2b was given daily for 2 weeks, and then 10 MU of IFN-α2b was given 3 times per week for 18 weeks.

IFN-α2b monotherapy group

Patients received 6–10 MU (10 MU in principle) of IFN-α2b intramuscularly (Intron) daily at 0900 hours for an initial 4 weeks and 6–10 MU (10 MU in principle) of IFN-α2b 3 times a week for 20 weeks.

Group receiving IFN-α2b plus ribavirin

Patients received 6–10 MU of IFN-α2b intramuscularly (Intron) daily at 0900 hours for an initial 4 weeks and 6–10 MU of IFN-α2b 3 times a week for 20 weeks. These patients received ribavirin (Schering-Plough, Kenilworth, NJ) at a daily dosage of 600 or 800 mg according to the body weight (≤ or >60 kg, respectively) for 24 weeks.

IFN-β monotherapy group

Patients received 6 MU of IFN-β intravenously (Feron) daily at 0900 hours for an initial four weeks and 6 MU of IFN-β three times a week for four weeks. Because the duration of administration of IFN in this regimen was different from the others, the data was used only for the analyses of viral half-life.

Group receiving twice-daily treatment with IFN-β

Patients received 6 MU of IFN-β intravenously (Feron) daily at 0900 hours and 1800 hours for an initial 4 weeks and 6–10 MU of IFN-α2b 3 times a week for 20 weeks. Because only a few patients completed IFN therapy without downward modification in this regimen, the data were used only for the analyses of viral half-life.

Analysis for serum level of HCV

In order to examine HCV dynamics, we sampled sera before, 24 h and 1 week after IFN treatment. For the group receiving same-day administration with IFN-β and α2b we also took a sample 8 h after the first administration of IFN-β (i.e. just before IFN-α2b administration). We measured HCV core antigen by chemiluminescence enzyme linked immune assay (CLEIA). For calibration of CLEIA, we quantified RNA

using AMPLICOR Monitor test v2.0 and HCV bDNA-probe method for a number of cases. HCV core antigen was analyzed by CLEIA method using Lumipulse Ortho HCV antigen kit (Ortho-Clinical Diagnostics Inc., Tokyo, Japan) as described previously.[10] The data were analyzed by Lumipulse *f* (Fujirebio Inc., Tokyo, Japan).

Outcome

Patients who were negative for serum HCV-RNA, according to the AMPLICOR Monitor test, during a 6 month period after completion of the IFN therapy were defined as SVR. Patients positive for HCV-RNA during this period were defined as non-responders.

The basis of non-possible ribavirin use is either hemoglobin <12.0 g/dL, age equal to or more than 70 years old or women equal to or less than 40 years old. A downward modification of the administration method was separated into two groups of major and minor: modifications that canceled ribavirin or changed twice-daily treatment with IFN to once daily were classified as major and other changes were classified as minor.

Viral half-life

Viral half-life was calculated according to the equation:

[Half – life = $T_{1/2}$, viral amount = V (V_0 = before IFN treatment, V_{24} = 24 h after the first IFN administration), time = t (day)]

$$V = V_0 e^{-st} \rightarrow V_{24} = V_0 e^{-s}$$

$$\log V_{24} = \log(V_0 e^{-s}) = \log V_0 + \log e^{-s} = \log V_0 - s$$

$$s = \log V_0 - \log V_{24}$$

$$(1/2)V_0 = V_0 e^{-sT1/2}$$

$$1/2 = e^{-sT1/2}$$

$$\log(1/2) = -\log 2 = \log(e^{-sT1\,2}) = -sT_{1\,2}$$

$$T_{1\,2} = (\log 2)/s$$

$$T_{1\,2} = (\log 2)/[\log V_0 - \log V_{24}]$$

Because the competitive RT-PCR method used for groups divided by the quantity of HCV-RNA expresses the amount of HCV using $\log_{10}$, we converted the calculation type from $\log_e$ to $\log_{10}$. The viral half-life used in this study was calculated as follows,

$$T_{1/2} = (\log_{10} 2)/[\log_{10} V_0 - \log_{10} V_{24}]$$

Statistics

Differences between experimental and control groups were analyzed by the Mann–Whitney's *U*-test or the Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical effect

AS SHOWN IN Table 1, the SVR rates for patients with a genotype 1b/ high viral load were 28.6% (4/14), 13.6% (3/22) and 25.0% (8/32) in groups of same-day IFN-β/α2b, IFN-α2b monotherapy and IFN-α2b plus ribavirin, respectively. The SVR rates for patients with a genotype 1b/ low viral load were 91.7% (11/12), 27.3% (3/11) and 57.1% (4/7) in the respective groups. By contrast, the SVR rates for patients with a genotype 2a+2b/high viral load were 70.8% (17/24), not tested and 50.0% (3/6) in the respective groups. There was no difference in the clinical effect between groups of therapy.

Patients with problematic ribavirin use and side-effects

Among the 174 patients who received IFN therapy after ribavirin release, ribavirin was not given to 36 (26.1%) of 138 patients in the high virus group (see Table 2a). The reasons were old age (≥70 years of age; 13 patients), invalidity or intolerance at the first IFN therapy (8 patients), women of childbearing age (6 patients), rejection (5 patients) and low levels of hemoglobin (4 patients). As shown in Table 2b, the cases that became withdrawals or downward modification after IFN therapy were 6.8% or 19.2% (major modification 1.4%, minor modification 17.8%) in the group of same-day IFN-β/α2b (73 patients), 18.8% or 2.1% in that of IFN-α2b monotherapy (48 patients), and 17.0% or 24.6% (major modification 3.8%, minor modification 20.8%) in that of IFN-α2b plus ribavirin (53 patients), respectively. Importantly, the combined rate of withdrawal and modification in a group of IFN-α2b plus ribavirin was significantly higher than that in either group of same-day IFN-β/α2b or IFN-α2b monotherapy.

Dynamics of HCV core antigen during the early phase of IFN therapy

We monitored the level of RNA and core antigen during the early phase of the IFN regimen. Based on the results

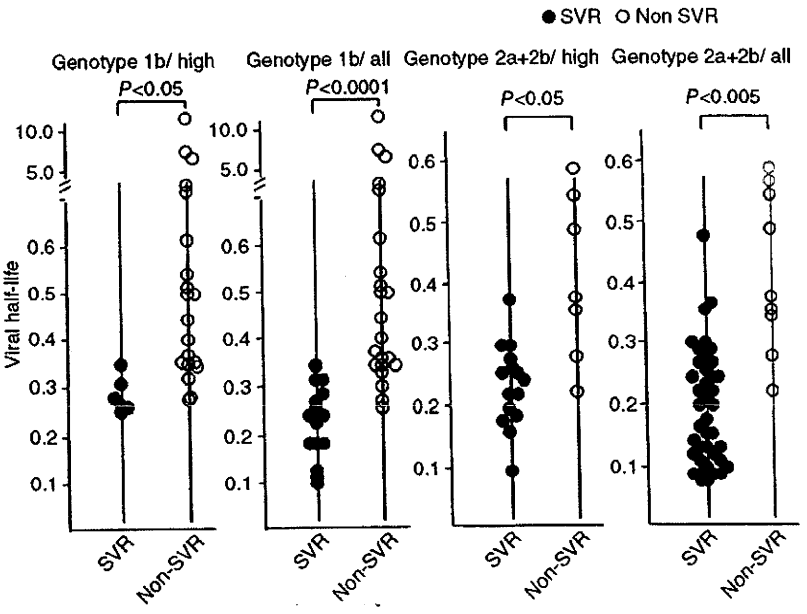

Figure 2 Significant differences can be seen in the early phase of viral half-life between the sustained viral response (SVR) and non-SVR groups. The half-life in patients achieving SVR was significantly lower than that in non-SVR in patients with genotype 1b/high viral load, genotype 1b/all, genotype 2a+2b/high viral load, and genotype 2a+2b/all ($P < 0.05$, $P < 0.0001$, $P < 0.05$ and $P < 0.005$, respectively). The data of three of 20 patients with genotype1b/high were excluded from the analysis because Smirnov's rejection test determined those as abnormal values. The open or closed circles display the half-life in patients achieving non-SVR or SVR, respectively. All patients showing a half-life under 0.2 achieved SVR.

above, HCV core antigen assayed by the CLEIA method appears suitable for the analysis of the dynamics of HCV. As shown in Figure 2, there was a significant difference in the half-life between those patients showing SVR and non SVR in all genotypes of HCV. However, there was no difference in viral half-life among the regimens of same-day IFN-β/α2b, IFN-β alone, IFN-α2b alone and twice-daily treatment with IFN-β (see Fig. 3).

DISCUSSION

THE NUMBER OF patients with hepatocellular carcinoma due to CH-C is still increasing. Currently, IFN treatment is the only effective treatment to eliminate HCV. However, the SVR rate after IFN treatment in CH-C varies according to several factors, such as genotype, quantity of HCV-RNA and the number of muta-

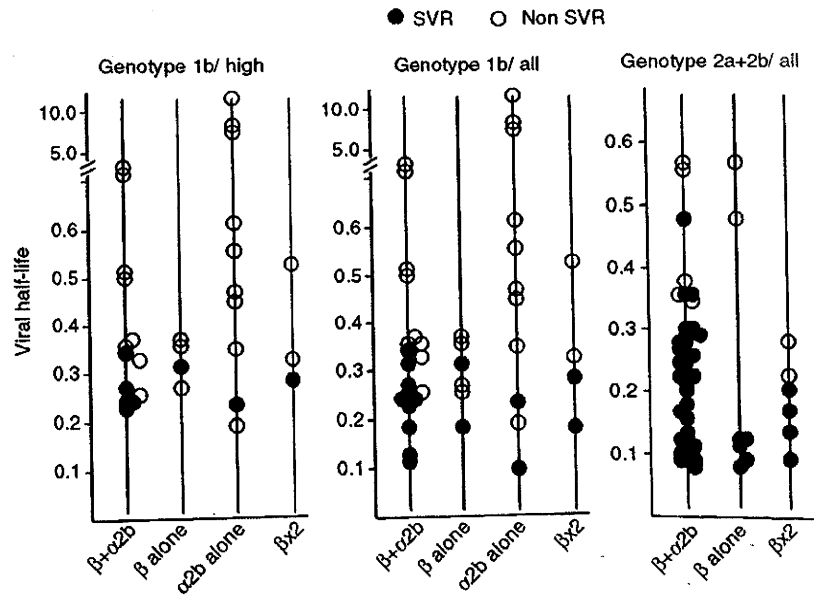

Figure 3 Dynamics of hepatitis C virus core antigen during the early phase of interferon therapy. We calculated the early phase of viral half-life using the viral amount of hepatitis C virus before and 24 h after the first interferon injection. The open or closed circles display the half-life in patients from non-sustained viral response (SVR) or SVR, respectively.

tions in the NS5A region. In particular, the outcome of treatment in genotype1 is poor. Furthermore, the SVR rate with the treatment of IFN alone for the patients of a genotype 1b/high viral load provided particularly poor results (approximately 5%). In addition, the clinical effect for patients of genotype 1 or 2b with a low viral load and of genotype 2a with a high viral load is insufficient.

On this account, various IFN administration methods, such as prolonged interferon treatment[11,12] and combination with ribavirin,[4–8] have been reported to raise the SVR rate. A rise of SVR induced by prolonged interferon treatment is generally acknowledged, although there are problems of patient acceptability and cost.

Since it has been confirmed by various reports that an IFN with ribavirin regimen raises the therapeutic efficacy for CH-C patients with a high viral load, treatment with a combination of IFN and ribavirin is becoming mainstream. However, ribavirin has side-effects, such as anemia and teratogenicity, making it unsuitable for patients with anemia and women of childbearing age. In addition, the side-effects on digestive system are often serious, so it is difficult to use ribavirin for patients aged 70 years or more. As shown in Table 2a, 36 patients (26.1%) were not able to receive ribavirin among 138 patients of the high virus group for whom ribavirin was thought to be desirable. Thus, it is a major problem to determine how to treat patients who are intolerant to ribavirin.

In this study, we attempted to devise an effective IFN regimen without using ribavirin. We anticipated new therapeutic outcomes by treating patients with IFN-β and IFN-α on the same-day. In patients with a genotype 1b/high viral load, the SVR rate of same-day IFN-β/α2b (28.6%) was higher than that in the groups of IFN-α2b monotherapy (13.6%) (see Table 1). In the genotype 1b/ low virus group, regarded as not as effective by IFN monotherapy, the SVR rate for same-day IFN-β/α2b (91.7%) treatment was markedly higher than those in the other two groups.

There was no significant difference among the three therapy groups in terms of withdrawal (Table 2b). However, the combined rate of withdrawal and modification in either group of same-day IFN-β/α2b or IFN-α2b monotherapy was significantly lower than that in a group of IFN-α2b with ribavirin. Although twice-daily treatment with IFN-β established by Okushin *et al.*[13] was similar to same-day IFN-β/α2b regimen in terms of SVR rate for patients of genotype 1b/high and a low viral load, a variety of severe adverse events were caused by the regimen (liver damage, decreased serum albumin, marked thrombocytopenia, and severe proteinuria).[14–16] In our examination, only 4 of 10 patients completed the treatment without downward modification (data not shown), so it is difficult to choose twice-daily treatment with IFN-β as a standard regimen. In our data, the percentage of withdrawal from treatment with IFN-α2b monotherapy seems to be too high. Because we mainly used the IFN-α2b at a dose of 10 MU/day for the group of IFN-α2b monotherapy, some patients became intolerant of such a dose of IFN. On the other hand, since we use 6 MU/day of IFN-α2b at the beginning of treatment for same-day IFN-β/α2b regimen, patients had a period of adjustment. We can draw several conclusions from our data concerning a same-day IFN-β/α2b regimen; (i) almost all cases are able to undergo this treatment, (ii) the SVR rate in whole patients with genotype 1b is higher compared to an IFN-α2b plus ribavirin regimen, (iii) the frequency of stopping and withdrawal of therapy is not high compared to IFN-α2b monotherapy and is significantly lower than IFN-α2b plus ribavirin.

In order to analyze the mechanism by which the same-day IFN-β/α2b regimen is highly effective, we evaluated the dynamics of HCV-RNA and core antigen during the early phase of IFN therapy. As for the AMPLICORE Monitor test, which is generally used for RNA assay, its dynamic range of quantification is 0.6–500 KIU/mL.[17] Therefore, it was thought that the AMPLICORE Monitor test could not be used in an evaluation of dynamics of viral load in this concentration range (data not shown). Under the conditions used in this study the HCV core Ag determined by the CLEIA method shows high assay accuracy ranging from 20 to 20 000 fmol/L. Thus, we concluded that this methodology was a very reliable laboratory procedure to monitor HCV dynamics (data not shown). Based upon these data, we used the HCV core Ag by CLEIA method for analyses of the differential dynamics of HCV amount during the early phase of each IFN therapy regimen. Because there was a significant difference of half-life between patients showing SVR and non-SVR in all genotypes of HCV, we focused on the differential half-life of HCV-RNA by the treatments between same-day IFN-β/α2b and the others. Interestingly, there was no significant difference in the HCV half-life during the early phase of IFN therapy among the regimens of same-day IFN-β/α2b, β alone, α2b alone and twice-daily treatment with IFN-β (see Fig. 3). These data show that the efficacy of same-day

IFN-β/α2b was not through shortening of the viral half-life.

Because both IFN-α or β monotherapy is less effective than our same-day IFN-β/α2b regimen, we next have to consider the mechanism of the differential effects. IFN-α and β together bind the IFN-α receptor (R) 1 and 2, and make a complex to activate Jak1 and Tyk2.[18] These first stages of IFN action appear to be the same. However, IFN-β bind much stronger to IFN-α R1 (α chain) and 2 (β chain), thereby changing the structure of the complex.[19] However, IFN-α and IFN-β binds the same α and β_L subunits of the IFNR, yet differences in signaling and biological effects exist between them. Domanski *et al.* demonstrated that IFN-α and IFN-β utilize different regions of the intracellular domain of the β_L subunit to generate an antiviral state.[20–24] Furthermore, recent studies have revealed intracellular signal transductions by IFN-α and β occurring in a different manner. Although signal transductions by IFN-α were stopped in Tyk2-deficient cells, IFN-β induced the expression of an upstream region of the IFN-regulated human gene without Tyk2.[25] This finding suggests that additional signaling mechanisms should be triggered by IFN-β. Above all, despite using the same receptor, IFN-β has at least another function to activate and generate an antiviral state, compared to IFN-α. The same-day IFN-β/α2b regimen stimulates two or more signaling pathways, which may be why our IFN-β/α2b regimen shows a higher SVR rate compared to other treatment regimens. Next, we focused on immunological modification for the effect of IFN treatments, because immunological response is required in the fight against HCV. Hiroishi *et al.* reported that high- titer infection with HCV may suppress the cytotoxic T lymphocyte responses.[26] We hypothesized that immunological responses against HCV (genotype 1b/high viral load) were more impaired in the patients with stably high viral load. In the same-day β/α2b but not IFN-α2b monotherapy, the SVR rate in patients with genotype 1b/stably high viral load was markedly lower than that with 1b/moving type (moving type patients were defined as individuals with a high viral load who showed a low viral load more than once during a one-year period before the start of IFN treatment; data not shown). These data may indicate the reason for the higher effect of same-day IFN-β/α2b was due to the higher SVR rate, compared to that of IFN-α2b monotherapy.

In conclusion, same-day β/α2b treatment had few cases where therapy was discontinued and showed a high SVR rate. This regimen is an effective treatment, especially for cases where ribavirin is unsuitable.

REFERENCES

1 Shiratori Y, Kato N, Yokosuka O *et al.* Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. *Gastroenterology* 1997; 113: 558–66.

2 Hagiwara H, Hayashi N, Mita E *et al.* Quantitative analysis of hepatitis C virus RNA in serum during interferon therapy. *Gastroenterology* 1993; 104: 877–83.

3 Enomoto N, Sakuma I, Asahina Y *et al.* Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; 334: 77–81.

4 Dusheiko G, Main J, Thomas H *et al.* Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. *J Hepatol* 1996; 25: 591–8.

5 Bellobuono A, Mondazzi L, Tempini S, Silini E, Vicari F, Ideo G. Ribavirin and interferon-alpha combination therapy vs interferon-alpha alone in the retreatment of chronic hepatitis C: a randomized clinical trial. *J Viral Hepat* 1997; 4: 185–91.

6 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *N Engl J Med* 1998; 339: 1485–92.

7 Davis GL, Esteban-Mur R, Rustgi V *et al.* Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. *N Engl J Med* 1998; 339: 1493–9.

8 Reichard O, Norkrans G, Fryden A, Braconier JH, Sonnerborg A, Weiland O. Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. *Lancet* 1998; 351: 83–7.

9 Ohno O, Mizokami M, Wu RR *et al.* New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a. *J Clin Microbiol* 1997; 35: 201–7.

10 Takahashi M, Saito H, Higashimoto M, Atsukawa [illegible] H. Benefit of hepatitis C virus core antigen assay in prediction of therapeutic response to interferon and rebavirin combination therapy. *J Clin Microbiol* 2005; 43: 186–91.

11 Kasahara A, Hayashi N, Hiramatsu N *et al.* Ability of prolonged interferon treatment to suppress relapse after cessation of therapy in patients with chronic hepatitis [illegible] a multicenter randomized controlled trial. *Hepatology* 1995; 21: 291–7.

12 Reichard O, Foberg U, Fryden A *et al.* High sustained response rate and clearance of viremia in chronic hepatitis C after treatment with interferon-α2b for 60 weeks. *Hepatology* 1994; 19: 280–5.

13 Okushin H, Morii K, Kishi F, Yuasa F. Efficacy of the combination therapy using twice-a-day IFN-β followed by IFN-α2b in treatment for chronic hepatitis C [in Japanese, English abstract]. *Acta Hepatol Jap* 1997; 38: 11–18.

14 Kakizaki S, Takagi H, Yamada T *et al.* Evaluation of twice-daily administration of interferon-beta for chronic hepatitis C. *J Viral Hepat* 1999; 6: 315–19.

15 Shiratori Y, Perelson AS, Weinberger L *et al.* Different turnover rate of hepatitis C virus clearance by different treatment regimen using interferon-beta. *J Hepatol* 2000; 33: 313–22.
16 Asahina Y, Izumi N, Uchihara M *et al.* A potent antiviral effect on hepatitis C viral dynamics in serum and peripheral blood mononuclear cells during combination therapy with high-dose daily interferon alfa plus ribavirin and intravenous twice-daily treatment with interferon beta. *Hepatology* 2001; 34: 377–84.
17 Pawlotsky JM. Molecular diagnosis of viral hepatitis. *Gastroenterology* 2002; 122: 1554–68.
18 Pestka S, Krause CD, Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev* 2004; 202: 8–32.
19 Russell-Harde D, Wagner TC, Perez HD, Croze E. Formation of a uniquely stable type I interferon receptor complex by interferon beta is dependent upon particular interactions between interferon beta and its receptor and independent of tyrosine phosphorylation. *Biochem Biophys Res Commun* 1999; 255: 539–44.
20 Domanski P, Witte M, Kellum M *et al.* Cloning and expression of a long form of the beta subunit of the interferon alpha beta receptor that is required for signaling. *J Biol Chem* 1995; 270: 21606–11.
21 Platanias LC, Uddin S, Domanski P, Colamonici OR. Differences in interferon alpha and beta signaling. Interferon beta selectively induces the interaction of the alpha and betaL subunits of the type I interferon receptor. *J Biol Chem* 1996; 271: 23630–3.
22 Domanski P, Fish E, Nadeau OW *et al.* A region of the beta subunit of the interferon alpha receptor different from box 1 interacts with Jak1 and is sufficient to activate the Jak-Stat pathway and induce an antiviral state. *J Biol Chem* 1997; 272: 26388–93.
23 Domanski P, Nadeau OW, Platanias LC *et al.* Differential use of the betaL subunit of the type I interferon (IFN) receptor determines signaling specificity for IFNalpha2 and IFNbeta. *J Biol Chem* 1998; 273: 3144–7.
24 Platanias LC, Domanski P, Nadeau OW *et al.* Identification of a domain in the beta subunit of the type I interferon (IFN) receptor that exhibits a negative regulatory effect in the growth inhibitory action of type I IFNs. *J Biol Chem* 1998; 273: 5577–81.
25 Pellegrini S, John J, Shearer M, Kerr IM, Stark GR. Use of a selectable marker regulated by alpha interferon to obtain mutations in the signaling pathway. *Mol Cell Biol* 1989; 9: 4605–12.
26 Hiroishi K, Kita H, Kojima M *et al.* Cytotoxic T lymphocyte response and viral load in hepatitis C virus infection. *Hepatology* 1997; 25: 705–12.

Hepatology Research

Hepatology Research 2010; 40: 376–382 doi: 10.1111/j.1872-034X.2009.00616.x

Insulin resistance raises the risk for recurrence of stage I hepatocellular carcinoma after curative radiofrequency ablation in hepatitis C virus-positive patients: A prospective, case series study

Kenji Imai,[1] Koji Takai,[1] Yoichi Nishigaki,[2] Shogo Shimizu,[3] Takafumi Naiki,[1] Hideki Hayashi,[2] Takahiro Uematsu,[3] Junichi Sugihara,[3] Eiichi Tomita,[2] Masahito Shimizu,[1] Masahito Nagaki[1] and Hisataka Moriwaki[1]

Departments of Gastroenterology, [1]Gifu University Graduate School of Medicine, [2]Gifu Municipal Hospital and [3]Gifu Prefectural General Medical Center, Gifu, Japan

Aim: Several studies have reported that insulin resistance raises the risk of primary hepatocellular carcinoma (HCC). We conducted a prospective, case series study to test the impact of insulin resistance on the recurrence after curative radiofrequency ablation (RFA) of stage I HCC in HCV-positive patients.

Methods: From January 2006 to December 2007, 226 consecutive patients underwent treatment for primary HCC at our institutions, including 37 stage I cases. Among them, 33 were HCV-positive, and three, six and 24 received curative surgery, transarterial chemoembolization or RFA, respectively. In the 24 patients treated with RFA, recurrence-free survival was analyzed using the Kaplan–Meier method. The factors contributing to recurrence of HCC were subjected to univariate and multivariate analyses using the Cox proportional hazards model. Insulin resistance was estimated by the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR).

Results: Kaplan–Meier analysis showed that the recurrence-free survival was lower in patients with higher HOMA-IR (>2.3, $P = 0.0252$) or with lower serum albumin level (<3.3 g/dL, $P = 0.0004$). In the univariate analysis, HOMA-IR ($P = 0.0420$) and albumin ($P = 0.0036$) were significantly associated with recurrence of HCC. Multivariate analysis revealed albumin (odds ratio = 0.01, 95% confidence interval = 0.0002–0.015, $P = 0.0001$) and HOMA-IR (odds ratio = 3.85, 95% confidence interval = 1.57–14.2, $P = 0.0015$) to be independent predictors for recurrence of HCC.

Conclusion: Serum albumin level and HOMA-IR were independent risk factors for recurrence of stage I HCC after curative RFA in HCV-positive patients. Patients with these factors require closer surveillance.

Key words: hepatitis C virus, hepatocellular carcinoma, Homeostatic Model Assessment of Insulin Resistance, insulin resistance, radiofrequency ablation, recurrence

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is prevalent worldwide, especially in Africa and the Western Pacific Region. HCC is the third most common cause of cancer death in men and the fifth most common in women; every year, more than 600 000 people die from this disease (www.who.int/whosis/).

Risk factors for the development of primary HCC include viral infection such as hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol consumption, aflatoxin and immune-related hepatitis.[1] Regarding risk factors for recurrence, several studies have suggested male sex, presence of cirrhosis, high α-fetoprotein (AFP), large tumor foci, multiplicity of tumors, pathologically high-grade atypia of tumor cells and presence of portal venous invasion of tumor.[2–6] Recently, several epidemiological studies have revealed a close association between diabetes mellitus (DM) and HCC. Wideroff *et al.*[7] described the standardized incidence ratios in Denmark for primary liver cancer in subjects with DM compared with the general population as 4.0 (95% confidence interval [CI] = 3.5–4.6) and 2.1 (95% CI = 1.6–2.7) for men and women, respectively. El-Serag *et al.*[8] reported that DM increased the risk of chronic non-alcoholic liver disease and HCC in male patients without concomitant

Correspondence: Professor Hisataka Moriwaki, Department of Gastroenterology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Email: hmori@gifu-u.ac.jp
Received 13 July 2009; revision 29 August 2009; accepted 17 October 2009.

liver disease in the USA. Furthermore, patients with chronic hepatitis and cirrhosis tend to experience complications with DM or to show insulin resistance.[9] This is particularly the case for patients with HCV infection and non-alcoholic fatty liver disease (NAFLD), including its most severe form, non-alcoholic steatohepatitis (NASH), which can lead directly to HCC.[10–12] The HCV core protein induced insulin resistance by increasing tumor necrosis factor-α which disrupts tyrosine phosphorylation of insulin receptor substrate-1.[13] Thus, DM including insulin resistance seems to be closely associated with various liver diseases that can lead to HCC, although the impact of insulin resistance on the recurrence of HCC has not been evaluated.

In this study, to identify the impact of insulin resistance on recurrence after initial curative treatment for HCC, we designed a prospective, case series analysis to examine recurrence-free survival in consecutive patients with stage I HCC, stratified by Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) level, which is commonly used for measuring insulin resistance.[14,15] In particular, we focused on HCV-positive patients who were treated with radiofrequency ablation (RFA).

METHODS

Patients

FROM JANUARY 2006 to December 2007, 226 primary HCC patients underwent initial treatment at our institutions, and 199 of them were followed to the end of this study (April 2008). Among them, we had 37 consecutive patients with stage I HCC that met all the criteria: a single tumor of 2 cm or less diameter, with no vascular invasion, no lymph-node invasion and no distant metastasis.[16]

Hepatocellular carcinoma nodules were detected by imaging modalities including abdominal ultrasonography, dynamic computed tomography (CT), dynamic magnetic resonance imaging (MRI) and abdominal arteriography. Diagnosis of HCC was made from a typical hypervascular tumor stain on angiography and typical dynamic-study findings of enhanced staining in the early phase and attenuation in the delayed phase. Etiologies for HCC were HCV in 33 patients, HBV in two and others in two.

Treatment, follow up and determination of recurrence

Three patients were treated with surgical resection, six with transarterial chemoembolization (TACE) and 28 with RFA. Among them, we only recruited those who were positive for HCV and treated with RFA ($n = 24$). Therapeutic effect was judged to be curative using dynamic CT or MRI with total disappearance of imaging characteristics of HCC as described above.

Patients were thereafter followed on an out-patient basis using serum tumor markers such as AFP and protein induced by vitamin K absence or antagonists II (PIVKA-II) every month, and by abdominal ultrasound, dynamic CT scan or dynamic MRI every 3 months. Recurrent HCC was diagnosed using the imaging modalities described earlier as the appearance of another lesion different from the primary one. The follow-up period was defined as the interval from the date of initial treatment until the date of diagnosis of recurrence, or until April 2008 if HCC did not recur. We defined the local tumor progression at the initial HCC site as censored.

Statistical analysis

Baseline characteristics were compared using the Student's *t*-test for continuous variables or χ^2-test for categorical variables. Recurrence-free survival was estimated using the Kaplan–Meier method, and differences between curves were examined by log–rank test. There were 13 possible predictors for recurrence of HCC after the initial curative treatment: sex, age, body mass index (BMI), Child–Pugh classification, serum albumin level, total bilirubin level, alanine aminotransferase (ALT) activity, platelet count, prothrombin time, HOMA-IR (defined as fasting plasma glucose [mg/dL] × fasting immunoreactive insulin [μU/mL] / 405), hemoglobin A_{1c} (HbA_{1c}), and serum tumor markers (AFP and PIVKA-II). The parameters, that proved to be significant by log–rank test, were then subjected to the univariate and multivariate analyses using the Cox proportional hazards model. Statistical significance was declared if the P-value was 0.05 or less. In addition, we employed the quantitative insulin sensitivity check index (QUICKI), which directly correlates with the glucose clamp method,[17] to supplement the evaluation of insulin resistance by HOMA-IR: QUICKI = 1 / (log [immunoreactive insulin] + log [fasting plasma glucose]).

RESULTS

Patients' baseline characteristics and laboratory data

THE BASELINE CHARACTERISTICS and laboratory data of 24 patients (15 men and nine women, median age 73 years) are shown in Table 1. Twenty

Table 1 Baseline demographic and clinical characteristics

		Normal range
Sex (male/female)	15/9	
Age (years)	73 (61–82)	
BMI	22.3 (19.5–33.5)	
Child–Pugh classification (A/B/C)	20/4/0	
Follow-up period (days)	365 (60–770)	
ALB (g/dL)	3.75 (2.4–4.4)	3.9–4.9
ALT (IU/L)	48.5 (21–98)	7–40
T-Bil (mg/dL)	0.96 (0.6–2.1)	0.2–1.2
PLT ($\times 10^4/\mu L$)	8.85 (4.1–21)	14.1–32.7
PT (%)	74 (56–118)	70–120
FPG (mg/dL)	107 (75–155)	70–110
FIRI (μg/dL)	10.8 (2.78–32.2)	2–10
HOMA-IR	2.96 (0.76–7.39)	<1.6
HbA1c (%)	5.2 (3.7–7.2)	<5.6
AFP (ng/dL)	26.7 (2.2–203)	<20
PIVKA-II (mAU/mL)	24 (9–127)	<40

Values are median (range).
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

patients were classified into Child–Pugh class A, four patients into class B and none into class C. The median follow-up period was 365 days (range 60–770 days), and no patient died during the study.

Possible risk factors for recurrence of HCC

No local tumor progression was diagnosed in this study period. Seven patients experienced the defined recurrence in the liver, but no one showed distant metastasis. One-year recurrence-free survival in total patients was 64%; Figure 1(a,b) shows Kaplan–Meier curves for recurrence-free survival according to HOMA-IR level (≤2.3 and >2.3), which produced significant difference ($P = 0.0252$). Serum albumin level (≥3.3 and <3.3 g/dL; $P = 0.0004$) was also a significant variable (Fig. 1c).

The Cox proportional hazards model was used to analyze risk factors for recurrence of stage I HCC after the curative RFA, using the 13 variables described earlier (Table 2). HOMA-IR level (odds ratio [OR] = 1.66, 95% CI = 1.01–2.72, $P = 0.0420$), and serum albumin level (OR = 0.08, 95% CI = 0.01–0.45, $P = 0.0036$) were identified as significant risk factors by univariate analysis. Multivariate analysis identified albumin (OR = 0.01,

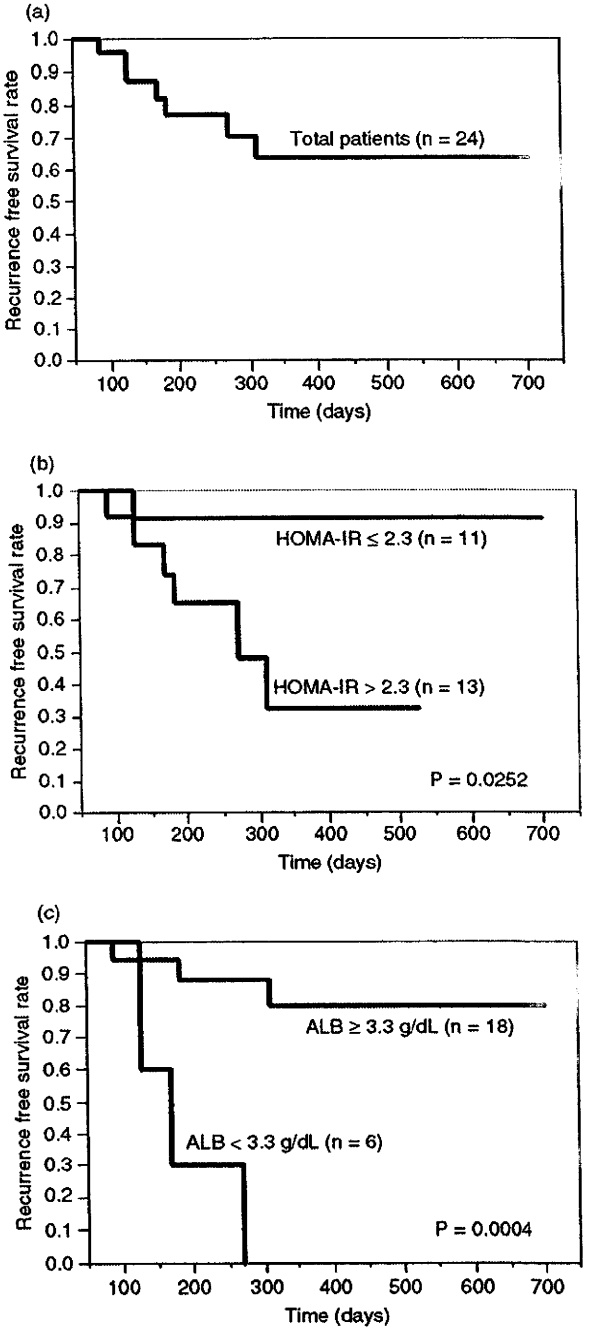

Figure 1 Kaplan–Meier curves for recurrence-free survival in (a) total patients and in subgroups divided according to (b) Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) level or (c) serum albumin level. ALB, albumin.

Table 2 Univariate analyses of possible risk factors for recurrence of hepatocellular carcinoma by Cox proportional hazards model

	95% CI			
	OR	Lower	Upper	*P*-value
Men (vs women)	1.20	0.25	8.41	0.8242
Age (years)	1.06	0.93	1.23	0.3451
BMI	0.90	0.59	1.22	0.6036
Child B (vs A)	4.81	0.60	31.3	0.1253
ALB (g/dL)	0.08	0.01	0.45	0.0036
T-Bil (mg/dL)	2.75	0.27	19.7	0.3603
ALT (IU/L)	0.99	0.95	1.02	0.6923
PLT ($\times 10^4/\mu L$)	0.86	0.65	1.05	0.1770
PT (%)	0.95	0.87	1.01	0.1617
HOMA-IR	1.66	1.01	2.72	0.0420
HbA1c (%)	0.69	0.27	1.53	0.3850
AFP (ng/dL)	1.00	0.99	1.02	0.1242
PIVKA-II (mAU/mL)	1.00	0.96	1.03	0.6172

OR is shown with a unit increase in continuous variables.
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; CI, confidence interval; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; OR, odds ratio; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

95% CI = 0.0002–0.15, $P = 0.0001$) and HOMA-IR level (OR = 3.85, 95% CI = 1.57–14.2, $P = 0.0015$) as significant independent risk factors for recurrence.

Table 3 shows the patients' baseline characteristics and laboratory data divided according to HOMA-IR level (≤2.3 and >2.3). No significant differences were noted between the two subgroups except fasting plasma glucose and fasting immunoreactive insulin. Two patients in the HOMA-IR 2.3 or less subgroup took oral hypoglycemic drugs, sulfonylurea derivatives and voglibose. Three patients in the HOMA-IR more than 2.3 subgroup took oral hypoglycemic drugs; two took sulfonylurea derivatives and one took pioglitazone. No patient received insulin treatment.

We supplementally analyzed the data by excluding the patients under treatment with these oral hypoglycemics and also the patients with fasting plasma glucose above 140 mg/dL, in order to avoid possible unreliability in HOMA-IR evaluation. In nine patients, each remaining in HOMA-IR of 2.3 or less and HOMA-IR of more than 2.3, serum albumin (OR = 0.02, 95% CI = 0.0002–0.40, $P = 0.0060$) and HOMA-IR (OR = 3.49, 95% CI = 1.45–13.8, $P = 0.0033$) were still significant.

In a similar manner, evaluation of insulin sensitivity by QUICKI gave the results that lower QUICKI (≤0.33,

Table 3 Baseline demographic and clinical characteristics of patients classified according to HOMA-IR level

	HOMA-IR ≤2.3 ($n = 11$)	HOMA-IR >2.3 ($n = 13$)	*P*-value
Sex (male/female)	7/4	8/5	0.9157
Age (years)	70 (61–82)	74 (63–80)	0.1846
BMI	23.55 (19.5–33.5)	22.1 (19.5–25.1)	0.1219
Follow-up period (days)	393 (155–701)	337 (60–770)	0.2785
Child–Pugh classification (A/B)	10/1	10/3	0.3483
ALB (g/dL)	3.9 (2.4–4.4)	3.4 (2.7–4.4)	0.3304
ALT (IU/L)	40 (21–98)	53 (25–80)	0.6103
T-Bil (mg/dL)	0.8 (0.7–2.1)	1.0 (0.6–1.7)	0.6655
PLT ($\times 10^4/\mu L$)	8.5 (4.1–21)	8.9 (4.9–13.9)	0.5766
PT (%)	72 (56–95.5)	74 (58–118)	0.9953
FPG (mg/dL)	90 (75–119)	109 (86–155)	0.0151
FIRI (μg/dL)	7.98 (2.78–10.8)	14.1 (7.86–32.2)	0.0005
HOMA-IR	1.79 (0.76–2.27)	3.76 (2.91–7.39)	<0.0001
HbA1c (%)	5.05 (3.7–7.2)	5.3 (4.1–6.8)	0.6848
AFP (ng/dL)	23.2 (2.2–153.2)	28 (8–203)	0.7339
PIVKA-II (mAU/mL)	21 (9–127)	28 (9–67)	0.8071
Presence of oral hypoglycemic drugs (yes/no)	2/9	3/10	0.7678
Presence of insulin treatment (yes/no)	0/11	0/13	1.0000

Values are median (range).
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

i.e. impaired insulin sensitivity) was associated significantly with the increased risk of HCC recurrence (OR = 7.97, 95% CI = 1.32–152, $P = 0.0213$).

DISCUSSION

SEVERAL EPIDEMIOLOGICAL STUDIES have revealed the association of DM with cancer incidence and cancer mortality for various organs such as the liver, biliary tract, pancreas, endometrium, kidney, colon, bladder and breast.[7,18–21] The mechanism by which insulin acts as a carcinogenic factor is currently a focus of interests. First, insulin functions as a growth factor by phosphorylating insulin receptor substrate 1 and activating the downstream mitogen-activated protein kinase cascade, which affects cellular proliferation.[22,23] Second, hyperinsulinemia increases peripheral lipolysis and hepatic accumulation of free fatty acids, and the excess β-oxidation in mitochondria and microsome leads to the production of reactive oxygen species[24,25] that play a significant role in carcinogenesis.[26,27] Adipocyte-secreted cytokines (adipokines) such as tumor necrosis factor-α and interleukin-6 also play a significant role in both insulin residence and carcinogenesis.[28,29] Thus, these factors could cooperatively induce the insulin resistance and carcinogenesis.

We demonstrated in the present study that a higher HOMA-IR level increases the risk of early recurrence after initial curative RFA of stage I HCC in HCV-positive patients. This finding basically agrees with previous studies[7,8,18] that suggested an association between insulin resistance and carcinogenesis as described above, but HbA_{1c} level did not predict recurrence (Table 2). This might be explained by the clinical relevance that HbA_{1c} level in patients with liver cirrhosis is often underestimated because of anemia. Therefore, the results of the present study suggest that the role of hyperinsulinemia is more important than that of hyperglycemia as reflected by HbA_{1c} in the recurrence of HCC. We therefore should pay attention to levels not only of glucose and HbA_{1c} but also of insulin when we follow patients who are at risk for HCC.

Interventional modalities to improve insulin-resistance could be a key to prevent the primary or recurrent HCC in patients complicated with such metabolic disorders. For instance, metformin and thiazolidine derivatives could be potential candidates for this purpose.[30,31] Oral branched-chain amino acid (BCAA) granules might be a candidate for preventing HCC recurrence in DM cases because, in addition to improving hypoalbuminemia,[32,33] this agent improves insulin resistance without stimulating insulin secretion.[34] Improvements of insulin resistance and glucose tolerance by BCAA have been reported in clinical trials.[35,36] Furthermore, Muto *et al.*[37] described that oral supplementation with BCAA granules inhibited liver carcinogenesis in HCV-positive liver cirrhosis with DM and obesity. Such effect of BCAA is also supported in experimental models.[38,39] These reports,[32–39] together with our present findings (Table 2 and Fig. 1b), suggest that insulin resistance is a significant risk factor for early recurrence of HCC and thus might be a critical target to prevent the recurrence and development of second primary HCC.

A limitation of this study is that the therapeutic effect of the primary HCC was judged as curative by imaging diagnosis but not by surgical pathology. Although the recurrent HCC developed apart from the primary tumor, we could not totally differentiate the recurrent lesion and a second primary HCC. A higher recurrence rate in this study (Fig. 1a) might be explained by this fact. Advanced medical imagings such as positron emission tomography would help solving such limitations in future study of this kind. Furthermore, the basic question, if de novo, namely, the first or second primary liver carcinogenesis is regulated by insulin resistance, should be addressed by recruiting HCC-free cirrhotics. However, such study requires a larger sample size and a longer observation period. Instead, we focused on the recurrent HCC, including possible second primary tumors, which develop at a 2–3-fold higher incidence than the first primary one.

Other study limitations are the short observation period, the small number of recruited patients and also the small number of detected events. Such a sample size essentially raises the possibility of β-error, including the absence of the statistical power of AFP and PIVKA-II for predicting the recurrence (Table 2). Previous reports agree that these tumor markers are risk factors of HCC recurrence,[2,6] although some criticisms remain.[4,5] In addition, we should state that the small number of events particularly restricts the reliability of multivariate analysis, while the calculation itself was possible in our study.

In conclusion, we presented for the first time that insulin resistance is significantly associated with the early recurrence of stage I HCC after curative RFA in HCV-positive patients. Increased HOMA-IR, which sensitively reflects insulin resistance, might be a useful biomarker for prediction of high-risk patients who cause early recurrence of HCC.

ACKNOWLEDGMENTS

THIS WORK WAS supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture (no. 17015016 to H. M.) and from the Ministry of Health, Labor and Welfare of Japan (to H. M.).

REFERENCES

1 Gomaa AI, Khan SA, Toledano MB, Waked I, Taylor-Robinson SD. Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. *World J Gastroenterol* 2008; **14**: 4300–8.
2 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.
3 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.
4 Adachi E, Maeda T, Matsumata T *et al.* Risk factors for intrahepatic recurrence in human small hepatocellular carcinoma. *Gastroenterology* 1995; **108**: 768–75.
5 Nagashima I, Hamada C, Naruse K *et al.* Surgical resection for small hepatocellular carcinoma. *Surgery* 1996; **119**: 40–5.
6 Ishii H, Okada S, Nose H *et al.* Predictive factors for recurrence after percutaneous ethanol injection for solitary hepatocellular carcinoma. *Hepatogastroenterology* 1996; **43**: 938–43.
7 Wideroff L, Gridley G, Mellemkjaer L *et al.* Cancer incidence in a population-based cohort of patients hospitalized with diabetes mellitus in Denmark. *J Natl Cancer Inst* 1997; **89**: 1360–5.
8 El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology* 2004; **126**: 460–8.
9 Kingston ME, Ali MA, Atiyeh M, Donnelly RJ. Diabetes mellitus in chronic active hepatitis and cirrhosis. *Gastroenterology* 1984; **87**: 688–94.
10 Allison ME, Wreghitt T, Palmer CR, Alexander GJ. Evidence for a link between hepatitis C virus infection and diabetes mellitus in a cirrhotic population. *J Hepatol* 1994; **21**: 1135–9.
11 Mehta SH, Brancati FL, Strathdee SA *et al.* Hepatitis C virus infection and incident type 2 diabetes. *Hepatology* 2003; **38**: 50–6.
12 Marchesini G, Brizi M, Morselli-Labate AM *et al.* Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 1999; **107**: 450–5.
13 Shintani Y, Fujie H, Miyoshi H *et al.* Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; **126**: 840–8.
14 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412–19.
15 Wallace TM, Matthews DR. The assessment of insulin resistance in man. *Diabet Med* 2002; **19**: 527–34.
16 International Union Against Cancer (UICC). Digestive system tumors, liver. In: Sobin LH, Wittekind CH, eds. *TMN Classification of Malignant Tumours*, 5th edn. New York: Wiley-Liss, 1997; 74–7.
17 Chen H, Sullivan G, Quon MJ. Assessing the predictive accuracy of QUICKI as a surrogate index for insulin sensitivity using a calibration model. *Diabetes* 2005; **54**: 1914–25.
18 Coughlin SS, Calle EE, Teras LR, Petrelli J, Thun MJ. Diabetes mellitus as a predictor of cancer mortality in a large cohort of US adults. *Am J Epidemiol* 2004; **159**: 1160–7.
19 Trevisan M, Liu J, Muti P, Misciagna G, Menotti A, Fucci F. Markers of insulin resistance and colorectal cancer mortality. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 937–41.
20 Silverman DT, Schiffman M, Everhart J *et al.* Diabetes mellitus, other medical conditions and familial history of cancer as risk factors for pancreatic cancer. *Br J Cancer* 1999; **80**: 1830–7.
21 Lindblad P, Chow WH, Chan J *et al.* The role of diabetes mellitus in the aetiology of renal cell cancer. *Diabetologia* 1999; **42**: 107–12.
22 Rose DW, Saltiel AR, Majumdar M, Decker SJ, Olefsky JM. Insulin receptor substrate 1 is required for insulin-mediated mitogenic signal transduction. *Proc Natl Acad Sci USA* 1994; **91**: 797–801.
23 Skolnik EY, Batzer A, Li N *et al.* The function of GRB2 in linking the insulin receptor to Ras signaling pathways. *Science* 1993; **260**: 1953–5.
24 Pessayre D, Berson A, Fromenty B, Mansouri A. Mitochondria in steatohepatitis. *Semin Liver Dis* 2001; **21**: 57–69.
25 Weltman MD, Farrell GC, Hall P, Ingelman-Sundberg M, Liddle C. Hepatic cytochrome P450 2E1 is increased in patients with nonalcoholic steatohepatitis. *Hepatology* 1998; **27**: 128–33.
26 Cerutti PA, Trump BF. Inflammation and oxidative stress in carcinogenesis. *Cancer Cells* 1991; **3**: 1–7.
27 Dreher D, Junod AF. Role of oxygen free radicals in cancer development. *Eur J Cancer* 1996; **32A**: 30–8.
28 Vettor R, Milan G, Rossato M, Federspil G. Review article: adipocytokines and insulin resistance. *Aliment Pharmacol Ther* 2005; **22** (Suppl 2): 3–10.
29 Scott KA, Arnott CH, Robinson SC *et al.* TNF-alpha regulates epithelial expression of MMP-9 and integrin alphavbeta6 during tumour promotion. A role for TNF-alpha in keratinocyte migration? *Oncogene* 2004; **23**: 6954–66.
30 Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* 2005; **330**: 1304–5.