

For instance, in this study, only 20% of patients with Q70 and wild-type ISDR achieved SVR, compared with 70.6% of those with non-Q70 and mutant-type ISDR. However, the majority of patients were classified into those with non-Q70 and wild-type (50.3%), and another factor for improving SVR prediction was considered necessary. Three genome-wide association studies of SVR to pegylated-IFN- α and ribavirin combination therapy for chronic hepatitis C patients with genotype 1 from Japan, the USA and Australia identified SNPs of IL28B associated with IFN responsiveness (17–19). SVR was achieved in 13.8% of patients with IL28B minor allele (TG and GG) and in 58% with IL28B major allele (TT) in this study, and the SNP of IL28B was associated with the response to IFN in patients with HCV genotype 1b, as in previous reports. The effects of both host and viral factors on IFN responsiveness would affect the IFN treatment outcome. Thus, the SNP of IL28B was considered in the analysis of aa substitutions in the HCV core and the NS5A region for improving the prediction of SVR. The strain with the worst SVR outcome was Q70 and wild-type ISDR, with an SVR of 20%. When IL28B was considered in the analysis of patients with Q70 and wild-type ISDR, 8.1% of patients with TG/GG for IL28B achieved SVR compared with 35.7% of those with TT for IL28B. These results indicate the effects of both host and viral factors on IFN responsiveness. The best responders were 47 patients who simultaneously had non-Q70, mutant-type ISDR and TT allele; 35 (74.5%) achieved SVR. The clear suggestion of a correlation between the combination of the SNP of IL28B and aa substitutions in the core region 70 and ISDR with IFN responsiveness would not be supported in the non-Q/mutant/G allele and the Q/mutant/G allele groups because of the small number of patients. Both mutations in core region 70 and ISDR were strongly associated with IL28B genotype. Thus, the prevalence of patients with core 70 non-Q, ISDR mutant, and IL28B genotype G was rare, and it was difficult to find these combinations. Patients infected with IFN-resistant strains Q70 and wild-type ISDR could be clearly identified as non-responders to IFN therapy (8.1%) by the IL28B genotype; the positive predictive value for non-SVR was 91.9%. Meanwhile, patients infected with IFN-sensitive strains non-Q70 and mutant-type ISDR showed that the positive predictive value for SVR was 74.5%. Montes-Cano *et al.* (38) reported that the influence of IL28B would be stronger among patients infected with an IFN-resistant genotype (HCV genotype 1) than in those infected with an IFN-sensitive genotype (HCV genotype non-1). The SNP of IL28B would be strongly associated with the response to IFN, especially for poor responders.

Interleukin28B genotype was associated with spontaneous viral clearance, as well as IFN responsiveness (17, 20). A Spanish study found that the prevalence of HCV genotype depends on IL28B genotype and speculated that IL28B would be a candidate to explain HCV genotype differences in the IFN response (38). The

IFN-resistant strain (Q70) was detected more frequently in patients with the IL28B minor allele (TG and GG) (56.3%) than in those with the IL28B major allele (TT) (18.3%). The present study showed similar results: patients with IL28B G allele, which is associated with poor response to IFN, seemed to more frequently have the IFN-resistant strain (Q70). Further study is needed to clarify the effect of the IL28B gene on differences in IFN response between each HCV genotype and subgenotype. The IL28B polymorphism might regulate the expression of hepatic interferon-stimulated genes and cause the difference in IFN responsiveness (39). The association between IL28B genotypes and IL28B gene expression is controversial (18, 19, 39). The effects of the SNP of IL28B on gene expression and mechanisms against HCV infection are still under debate. Although the effect of the SNP of IL28B was unclear, as were the aa substitutions of the core region and ISDR, these factors could be used to predict SVR in patients infected with genotype 1b. The SNP of IL28B plays an important role in choosing optimal therapy and avoiding unnecessary treatment.

In conclusion, the SNP of IL28B and aa substitutions in the core region and ISDR were associated with response to IFN in patients with HCV genotype 1b. Combined use of both host and viral factors could improve prediction of the IFN response.

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Antiviral Combination Therapy With Peginterferon and Ribavirin Does not Induce a Therapeutically Resistant Mutation in the HCV Core Region Regardless of Genetic Polymorphism Near the *IL28B* Gene

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An association has been reported between genetic polymorphism near *IL28B* gene and the prevalence of mutation of hepatitis C virus (HCV) core region residue 70, both of which have been associated with a lack of virologic response to antiviral combination therapy with peginterferon (PEG-IFN) and ribavirin. This study investigated whether PEG-IFN/ribavirin combination therapy induces amino acid (AA) mutation at residue 70 of HCV and whether genetic polymorphism near *IL28B* gene affects it. AA substitutions at residue 70 of the HCV core region were measured and compared before and after combination therapy in 65 non-responders and 88 relapsers to the combination therapy. In three patients in whom both wild-type AA (arginine) and mutant-type AA (glutamine or histidine) were detected at residue 70 before treatment, only mutant-type AA was identified after treatment. In two patients who had wild-type AA solely before treatment, both wild-type and mutant-type AAs were identified at residue 70 after treatment. In five patients, in whom the AA had changed at residue 70 between before and after treatment, four patients carried the TT genotype at a polymorphic locus (rs8099917) near the *IL28B* gene and one carried the TG/GG genotype. No difference was found in the prevalence of this change of AA at residue 70 between the TT and the TG/GG genotype. Antiviral combination therapy with PEG-IFN and ribavirin does not appear to induce mutation of HCV core region residue 70 regardless of genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. **J. Med. Virol.** 83:1559–1564, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis C; peginterferon and ribavirin; amino acid substitution of HCV core region residue 70; genetic polymorphisms near the *IL28B* gene; mutation; non-sustained virologic responder

INTRODUCTION

Hepatitis C virus (HCV) causes chronic infection that can result in chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [Niederau et al., 1998; Kenny-Walsh, 1999]. The current standard therapy for patients with chronic HCV infection is the combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although the current treatment regimen has markedly increased the rate of patients with sustained virologic response, which indicates the eradication of HCV, only approximately 50% of patients infected with HCV genotype 1 achieve a sustained virologic response.

Many studies have investigated the potential baseline host- or virus-related factors that are associated

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with the lack of virologic response to IFN-based antiviral therapy. As a host-related factor, recent studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 are strongly associated with a resistance to the combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. Patients having the TT genotype at a polymorphic locus (rs8099917) near the *IL28B* gene show a favorable response to the combination therapy with PEG-IFN and ribavirin, whereas patients having the GG genotype or those who are TG heterozygote show a resistance to the therapy. As for virus-related factors, amino acid (AA) mutations at residue 70 in the HCV core region have been reported to be associated strongly with a resistance to PEG-IFN/ribavirin combination therapy in patients infected with HCV genotype 1b [Akuta et al., 2005, 2007a; Donlin et al., 2007]. Patients with the mutant-type AA (glutamine or histidine) at residue 70 in the HCV core region show a resistance to the combination therapy in comparison to those with the wild-type AA (arginine) at this residue. These host- and virus-related factors are both associated with the outcome of the combination therapy with PEG-IFN and ribavirin independently in a previous report [Hayes et al., 2011].

A previous study reported that the percentage of patients with the mutant-type AA at residue 70 of the HCV core region increases with the progression of chronic hepatitis, suggesting that the mutation of AA at residue 70 (from arginine to glutamine or histidine) occurs in the natural course of chronic HCV infection [Kobayashi et al., 2010a]. Several recent studies have reported a higher prevalence of the mutant-type AA at residue 70 in patients who have the TG/GG genotype of genetic polymorphism of rs8099917 near the *IL28B* gene, which is associated with an unfavorable response to the combination therapy with PEG-IFN and ribavirin, than in patients who have the TT genotype [Abe et al., 2010; Kobayashi et al., 2010b]. These reports suggest that the mutation of AA residue 70 of the HCV core region may occur more frequently in patients with the TG/GG genotype. Especially, the induction of this mutation may occur easily in patients who underwent PEG-IFN/ribavirin combination therapy and failed to clear HCV (non-sustained virologic response), wherein HCV obtained a resistance to combination therapy.

Mutation at HCV core region residue 70 has reportedly been associated with a hepatocarcinogenesis and an insulin resistance [Akuta et al., 2007b, 2009; Nakamoto et al., 2010]. In addition, a recent study reported that patients who have both the TG/GG genotype of rs8099917 near the *IL28B* gene and the mutant-type AA at residue 70 of the HCV core region have shown further resistance even to the triple therapy with telaprevir, PEG-IFN, and ribavirin [Akuta et al., 2010]. It is, therefore, important to clarify whether PEG-IFN/ribavirin combination

therapy induces the mutation of the HCV core region residue 70 in patients who failed to eradicate HCV, and whether genetic polymorphism near the *IL28B* gene are correlated with this mutation. If so, some patients should not undergo the current standard combination therapy in order to prevent the acquisition of the resistance (i.e., mutation at residue 70).

The present study investigated the effects of the combination therapy with PEG-IFN and ribavirin and genetic polymorphisms near the *IL28B* gene on the mutation of HCV core region residue 70 in patients who failed to achieve a sustained virologic response.

PATIENTS AND METHODS

Patients and Treatment

Three hundred and forty six patients with chronic hepatitis C who had been infected with HCV genotype 1b (as assessed by amplification of core-gene sequences with polymerase chain reaction (PCR) using genotype-specific primers [Ohno et al., 1997]) and pretreatment HCV-RNA level of $>100 \times 10^3$ IU/ml [as assessed by a quantitative PCR assay (Amplicor GT-HCV Monitor, Version 2.0; Roche Molecular Systems, Pleasanton, CA)] underwent antiviral combination therapy with PEG-IFN and ribavirin between January, 2007 and December, 2009 at the Ogaki Municipal Hospital or the Nagoya University Hospital. Of these patients, 19 patients dropped out and their outcome could not be defined. Among the remaining 327 patients, 274 patients who gave written informed consent for genetic analyses were enrolled to the study (Fig. 1). No patients were coinfecting with hepatitis B virus or human immunodeficiency virus.

All patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough) daily. The initial doses of PEG-IFN and ribavirin and the dose reductions were according to the manufacturer's recommendations. All patients were scheduled to undergo 48 weeks of the treatment. Some patients had an extended treatment duration of up to 72 weeks. In some patients, the treatment was discontinued before 48 weeks because they had a low likelihood of achieving a sustained virologic response, when serum HCV-RNA was positive 24 weeks after starting the therapy. The outcomes of the combination therapy were classified as a sustained virologic response when serum HCV-RNA became undetectable during the treatment and remained undetectable for 6 months after the treatment ended (i.e., eradication of HCV), a relapse when the serum HCV-RNA became undetectable during the treatment period but returned detectable after the treatment, and no-response when the serum HCV-RNA remained detectable during and after the treatment period.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of the Ogaki Municipal Hospital and the Nagoya University School of Medicine. Written

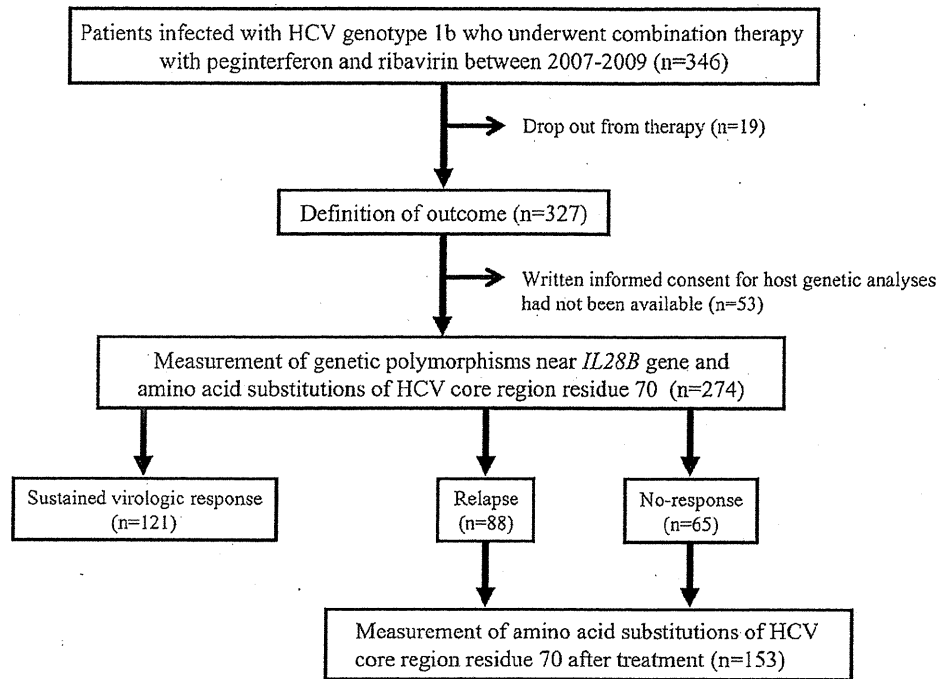


Fig. 1. Schematic representation of the study design.

informed consent was obtained from all patients prior to the study for the measurement of genetic polymorphism of rs8099917 near *IL28B* gene and AA substitution of HCV core region residue 70, and for the use of the laboratory data.

Measurements of Genetic Polymorphism Near the *IL28B* Gene and Amino Acid Substitution of the HCV Core Region Residue 70

Genotyping of polymorphisms of the rs8099917 locus near the *IL28B* gene was carried out in all 274 patients using a Taqman SNP assay (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C_11710096_10, Applied Biosystems).

The AA at residue 70 of the core region of HCV was measured before the treatment in all patients. In patients who failed to achieve a sustained virologic response, that is, patients who showed a relapse or no-response, the AA identity was measured at residue 70 after the treatment and compared pre- to post-treatment AA identity at this residue (Fig. 1). The AA at residue 70 after the treatment was measured in serum samples obtained at the end of treatment in patients who showed no-response. In patients with a relapse, it was measured in serum samples obtained upon the reappearance of HCV-RNA after the completion of the therapy. The AA identity was analyzed by direct nucleotide sequencing according to

a previous report [Akuta et al., 2007c]. The primer pairs used for PCR for direct sequencing the HCV core region were 5'-GCCATAGTGGTCTGCGGAAC-3' (outer, sense primer), 5'-GGAGCAGTCCTTCGTGACATG-3' (outer, antisense primer), 5'-GCTAGCCGAGTAGTGTT-3' (inner, sense primer), and 5'-GGAGCAGTCCTTCGTGACATG-3' (inner, antisense primer).

Statistical Analysis

The chi-square test was used to analyze the differences in percentages between groups.

RESULTS

Patient Characteristics and the Outcome of the Combination Therapy

The characteristics of study patients are shown in Table I. The study patients comprised 139 males (50.7%) and 135 females (49.3%), with a mean age of 58.0 ± 10.4 years. The grade of liver fibrosis according to the METAVIR score [The French METAVIR Cooperative Study Group, 1994] was F0 in 31 patients (11.6%), F1 in 122 patients (45.9%), F2 in 75 patients (28.2%), and F3 in 38 patients (14.3%). Analysis of the genetic polymorphism of the rs8099917 near the *IL28B* gene indicated 202 patients (73.7%) had the TT genotype, three patients (1.1%) had the GG genotype, and the remaining 69 patients (25.2%) were TG heterozygous. Before the treatment, 204 patients (74.4%)

TABLE I. Baseline Characteristics of the Study Patients (n = 274)

Age (years)	55.9 ± 11.2
Sex (female/male)	135 (49.3)/139 (50.7)
Body weight (kg)	58.0 ± 10.4
Alanine aminotransferase (IU/L)	64.5 ± 56.3
Aspartate aminotransferase (IU/L)	53.7 ± 42.2
Gamma-glutamyl transpeptidase (IU)	49.7 ± 48.5
Alkaline phosphatase (IU/L)	267.9 ± 100.6
Albumin (g/dl)	4.07 ± 0.38
Total bilirubin (mg/dl)	0.79 ± 0.30
White blood cell count (/μl)	4933 ± 1331
Hemoglobin (g/dl)	14.0 ± 1.4
Platelet count (×10 ³ /μl)	164 ± 50
Liver histology-activity (A0/A1/A2/A3) ^a	2 (0.7)/147 (55.3)/99 (37.2)/18 (6.8)
Liver histology-fibrosis (F0/F1/F2/F3) ^a	31 (11.6)/122 (45.9)/75 (28.2)/38 (14.3)
HCV-RNA concentration (log ₁₀ IU/ml) ^b	6.34 ± 0.54
Genetic polymorphisms near the <i>IL28B</i> gene (TT/TG/GG) ^b	202 (73.7)/69 (25.2)/6 (2.2)
Amino acid at HCV core 70 (wild type/mutant type/both) ^c	204 (74.4)/64 (23.4)/6 (2.2)
Response (SVR/relapse/NR)	121 (44.2)/88 (32.1)/65 (23.7)

HCV, hepatitis C virus; SVR, sustained virologic response; NR, no-response.

Percentages are shown in parentheses.

^aLiver biopsy was not performed in eight patients.

^brs8099917 genetic polymorphism

^cBefore the treatment.

carried HCV with the wild-type AA at residue 70 of the HCV core region, 64 patients (23.4%) carried the mutant-type AA at residue 70, and both the wild-type AA and the mutant-type AA were identified at residue 70 in the remaining six patients (3.5%).

As a final outcome, 121 patients (44.2%) achieved a sustained virologic response, 88 patients (32.1%) relapsed, and the remaining 65 patients (23.7%) showed no-response (Fig. 1). Treatment was discontinued before 48 weeks in 11 of 65 patients who showed no-response because HCV-RNA remained detectable in serum 24 weeks after starting the therapy. The identity of the AA 70 of the core region of HCV was determined after the treatment in serum obtained at the discontinuation of the therapy in these 11 patients. Table II shows the association between the genetic polymorphisms of the rs8099917 near the *IL28B* gene, the AA substitutions of the HCV core region residue 70, and the outcome of the combination therapy. The wild-type AA was more frequently identified at residue 70 in patients with the TT genotype in comparison to those with the TG/GG genotype (82.2% vs.

52.8%, $P < 0.0001$). The rate of a sustained virologic response was significantly higher in patients with the TT genotype than those with the TG/GG genotype (107 of 202 patients, 53.0% vs. 14 of 72 patients, 19.4%, $P < 0.0001$), as well as being higher in patients carrying HCV with the wild-type AA at residue 70 of the core region than those with the mutant-type AA at this residue (101 of 204 patients, 49.5% vs. 19 of 64 patients, 29.7%, $P = 0.0083$, one patient had both the wild-type and the mutant-type AAs).

Comparison of the Amino Acid at Residue 70 of the HCV Core Region Before and After the Combination Therapy in Patients Who Showed a Relapse or No-Response

Table III shows the comparison of the AA at residue 70 of the HCV core region before and after the combination therapy in patients who showed a relapse or no-response, according to the genetic polymorphisms of the rs8099917 near the *IL28B* gene. In three of five

TABLE II. Association Between the Genetic Polymorphisms Near the *IL28B* Gene, the Amino Acid at the HCV Core Region Residue 70, and the Final Outcome of Peginterferon/Ribavirin Combination Therapy

Genetic polymorphism of rs8099917 near <i>IL28B</i> gene	Amino acid at residue 70 of the HCV core region		
	Wild type (n = 204)	Mutant type (n = 64)	Wild type + mutant type (n = 6)
TT (n = 202)	166 (92/60/14)	31 (14/9/8)	5 (1/2/2)
TG/GG (n = 72)	38 (9/9/20)	33 (5/7/21)	1 (0/1/0)

Outcomes of the combination therapy with peginterferon and ribavirin are shown in parentheses as sustained virologic response/relapse/no-response.

TABLE III. Amino Acid Substitutions of HCV Core Region Residue 70 Before and After the Combination Therapy With Peginterferon and Ribavirin in No-Responders or Relapsers

Amino acid at HCV core region residue 70	After treatment		
	Wild type	Wild + Mutant	Mutant type
Before treatment			
(A) Genetic polymorphisms near the <i>IL28B</i> gene (rs8099917): TT (n = 91)			
No-responders (n = 24)			
Wild type (n = 14)	13	1	0
Wild + mutant (n = 2)	0	0	2
Mutant type (n = 8)	0	0	8
Relapsers (n = 71)			
Wild type (n = 60)	60	0	0
Wild + mutant (n = 2)	0	1	1
Mutant type (n = 9)	0	0	9
(B) Genetic polymorphisms near the <i>IL28B</i> gene (rs8099917): TG/GG (n = 57)			
No-responders (n = 41)			
Wild type (n = 20)	19	1	0
Wild + mutant (n = 0)	0	0	0
Mutant type (n = 21)	0	0	21
Relapsers (n = 17)			
Wild type (n = 9)	9	0	0
Wild + mutant (n = 1)	0	1	0
Mutant type (n = 7)	0	0	7

HCV, hepatitis C virus.

patients in whom both the wild-type and mutant-type AAs had been identified at residue 70 of the HCV core region before treatment, only the mutant-type AA was identified at this residue after the treatment. All three of these patients (two no-responders and one relapser) had the TT genotype of the rs8099917. Both the wild-type and mutant-type AAs were identified at residue 70 after the treatment in two no-responders in whom only the wild-type AA had been identified before the treatment. One of them had the TT genotype at the rs 8099917 and the other patient was TG heterozygous. No change in the HCV core region residue 70 was found after the treatment in patients with the mutant-type AA at this residue before the treatment.

DISCUSSION

The present study investigated whether the combination therapy with PEG-IFN and ribavirin causes the mutation of residue 70 of the HCV core region, and whether the genetic polymorphisms of the rs8099917 locus near the *IL28B* gene influence this mutation. It is thought to be important to verify this issue, because it may be advisable to avoid the treatment of patients who have the TG/GG genotypes by the combination therapy with PEG-IFN and ribavirin so as to avoid an acquisition of the further resistance to emerging new therapies against HCV, as well as to avoid a potential enhancement of hepatocarcinogenesis.

The mutation of the AA at residue 70 was not observed before and after the treatment in all patients who had failed to achieve a sustained virologic response. The mutant-type AA was identified solely at

residue 70 after the treatment in three patients who had both the wild-type and the mutant-type AAs at residue 70 before the treatment. This could be due to the selection of HCV strains with the mutant-type AA at residue 70 by the combination therapy with PEG-IFN and ribavirin, as reported previously [Kurbanov et al., 2010]. In two patients who carried only the wild-type AA before the treatment, the HCV with the mutant-type AA at residue 70 was also detected with the persistence of the wild-type AA at this residue after the treatment. The very minor HCV strain with the mutant-type AA at residue 70 which were not detected before the treatment may have been detected after the treatment due to the reduction of HCV with the wild-type AA at residue 70 by the combination therapy. Indeed, HCV with the mutant-type AA at core region residue 70 was not detectable in serum 6 months after the end of the combination therapy, suggesting that it returned to being a very minor population (data not shown). These two phenomena were observed in patients with both the TT genotype of the rs8099917, that is associated with a favorable response to the combination therapy and those with the TG/GG genotypes that is associated with an unfavorable response, without difference in the prevalence according to the genetic polymorphisms at the rs8099917 near the *IL28B* gene.

In conclusion, PEG-IFN/ribavirin combination therapy does not appear to induce the mutation of the AA at the HCV core region residue 70 regardless of the genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. The combination therapy can be attempted regardless of the genetic polymorphisms near the *IL28B* gene in

treatment-naïve patients without the anxiety for the acquisition of the further resistance to the antiviral therapy. However, future studies should be undertaken to confirm the absence of the mutation at residue 70 of the HCV core region induced by the combination therapy with PEG-IFN and ribavirin. In addition, the effect of the genetic polymorphisms near the *IL28B* gene on the mutation of the AA at the HCV core region residue 70 should be investigated in the long-term observation of the natural course of chronic hepatitis C.

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Mutations in the core and NS5A region of hepatitis C virus genotype 1b and correlation with response to pegylated-interferon-alpha 2b and ribavirin combination therapy

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SUMMARY. Mutations in two regions of hepatitis C virus (HCV) have been implicated in influencing response to interferon (IFN) therapy. Substitutions in the NS5A region of HCV have been associated with response to IFN therapy, and this region has been known as the IFN sensitivity-determining region (ISDR). The mutations in the core region of HCV have also been reported to predict IFN response. 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 IFN therapy. A total of 213 patients who completed IFN treatment were randomly selected. All patients received pegylated-IFN-alpha 2b once each week, plus oral ribavirin daily for 48 weeks. Of the 213 patients, 117 (54.9%) showed early virologic response (EVR), with HCV-negativity, at 12 weeks. Factors related to EVR on multivariate analysis were non-Gln70 and Leu91 in the core

region, and ISDR mutant-type. One hundred and two (47.9%) showed a sustained virologic response (SVR). SVR occurred more frequently in patients without Gln70 (55.4%) than in those with Gln70 (21.3%) ($P < 0.0001$). SVR was achieved in 43.6% of patients with wild-type ISDR and 62.5% of patients with mutant-type ($P = 0.0227$). Of the 34 patients who simultaneously had non-Gln70 and mutant-type ISDR, 26 (76.5%) achieved SVR. Factors related to SVR on multivariate analysis were non-Gln70 and ISDR mutant-type. In conclusion, amino acid substitutions in the core region and ISDR were useful for predicting the response to IFN in patients with HCV genotype 1b.

Keywords: core region, genotype 1b, hepatitis C virus, interferon sensitivity-determining region, interferon therapy, NS5A.

INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and causes chronic hepatitis that can develop into potentially fatal cirrhosis and hepatocellular carcinoma [1]. It has been estimated that 170 million people are infected with HCV worldwide. Therefore, HCV infection is a major global health problem. HCV consists of four structural proteins (core,

envelope 1, envelope 2 and p7) and six nonstructural proteins (NS2–NS5) [2]. HCV core protein was thought to inhibit the antiviral action of interferon (IFN) through down-regulation of transcription of IFN-induced antiviral genes [3,4]. The NS5A region includes the PKR-binding domain, which is associated with viral replication that is affected by IFN [5]. Thus, the core and NS5A regions of HCV appear to be important factors that may affect the response to IFN therapy, and mutations in the core and NS5A regions of HCV have been reported to affect response to IFN therapy [6–10]. The core region of HCV is well conserved, but substitutions of amino acid (aa) 70 and aa 91 are frequently found. Several studies reported a relation between these substitutions in the core region and IFN responsiveness [8,10]. The substitutions in the NS5A region of HCV have been closely associated with response to IFN therapy, and this region is known as the IFN sensitivity-determining region (ISDR) [6]. However, these

Abbreviations: Aa, amino acid; ALT, alanine aminotransferase; EVR, early virologic response; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity-determining region; SVR, sustained virologic response.

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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'-GGGAGGTCTCGTAGACCGTG-CACCATG-3' and antisense 5'-GAGMGGKATRTACCCCA-TGAGRTCGGC-3' and primers for the NS5A-ISDR were sense 5'-TGGATGGAGTGGGTTGCACAGTA-3' and antisense 5'-TCTTCTCCGTGGAGGTGGTATTG-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'-TACGCCGGGGTCAKTRGGGCCCA-3'; and for the NS5A-ISDR, sense 5'-CAGGTACGCTCCGGCGTGCA-3' and antisense 5'-GGGCCTTGGTAGGTGGCAA-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 = 213
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

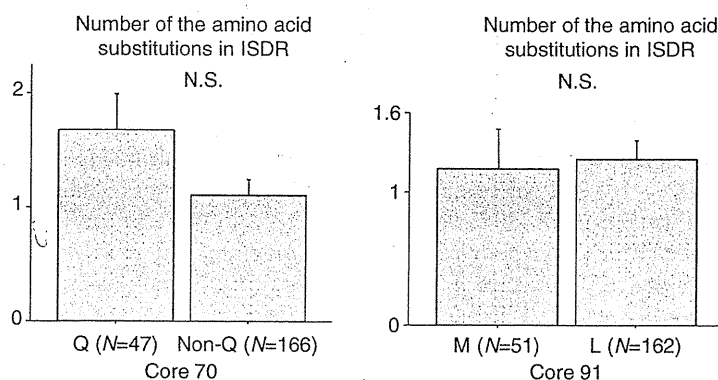


Fig. 1 The association between amino acid substitutions in core region and ISDR. ISDR, interferon sensitivity-determining region; Q, glutamine; L, leucine; M, methionine; NS, not significant.

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 (×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 × 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

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 (×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

Table 6 Univariate analysis: factors predictive of SVR

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 × 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.

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

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.

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.

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

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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

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 ($P = 0.046$, 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

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 pyogenes*, 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-1 β , IL-6 and prostaglandin E₂ (PGE₂)] [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 ≥ 20 years, informed consent and the following normal baseline haematological parameters (within 1 week before DC administration): haemoglobin ≥ 8.5 g/dl; white cell count $\geq 2000/\mu\text{l}$; platelet count $\geq 50\,000/\mu\text{l}$; 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 1.4×10^7 cells in 2 ml per well and allowed to adhere 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 ⁵¹Cr 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 ⁵¹Cr. In a 96-well plate, 2.5×10^3 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) \times 100. Spontaneous release was always < 20% of the total.

NK cell activity

NK cell cytotoxicity against K562 erythroleukemia target cells was measured by using ⁵¹Cr-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]} \times 100.

Intracellular cytokine expression

Freshly isolated PBMCs were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1 μ g/ml ionomycin (Sigma-Aldrich) at 37°C in humidified 7% CO₂ for 4 h. To block cytokine secretion, brefeldin A (Sigma) [27] was added to a final concentration of 10 μ g/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 ($\approx 10\,000$ 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), SART3₁₀₉ (VYDYNCHVDL), multi-drug resistance protein 3 (MRP3)₇₆₅ (VYSDADIFL), MRP3₅₀₃ (LYAWEPSFL), MRP3₆₉₂ (AYVPQQAWI), alpha-fetoprotein (AFP)₄₀₃ (KYIQESQAL), AFP₄₃₄ (AYTKKAPQL), AFP₃₅₇ (EYSRRHPQL), human telomerase reverse transcriptase (hTERT)₁₆₇ (AYQVCGPPL) (unpublished), hTERT₄₆₁ (VYGFVRACL) and hTERT₃₂₄ (VYAETKHFL) were used in this study. Negative controls consisted of an HIV envelope-derived peptide (HIVenv₅₈₄). Positive controls consisted of 10 ng/ml PMA (Sigma) or a CMV pp65-derived peptide (CMVpp65₃₂₈). 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 μ l 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-1 β , 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-1 α , MIP-1 β , 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 \pm 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 \pm 9.5% (mean \pm 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 \pm 14.2% were CD11c-positive (myeloid DC subset) and 14.8 \pm 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-