Table 6. Logistic regression analysis with the highest Δwaist circumference or Δbody mass index quartile as an independent variable and a low eGFR at the second visit as a dependent variable in individuals without a low eGFR at the first visit

Variables	Age adjusted		Multivariate adjusted*	
	OR (95% CI)	p value	OR (95% CI)	p value
Male (n = 1,535)				
ΔWC-Q1, Q2, Q3	1.00	–	1.00	–
ΔWC-Q4	0.84 (0.39–1.83)	0.667	0.88 (0.40–1.91)	0.737
Female (n = 959)				
ΔWC-Q1, Q2, Q3	1.00	–	1.00	–
ΔWC-Q4	1.30 (0.60–2.86)	0.508	1.37 (0.62–3.03)	0.432
Male (n = 1,535)				
ΔBMI-Q1, Q2, Q3	1.00	–	1.00	–
ΔBMI-Q4	1.98 (1.07–3.66)	0.030	1.94 (1.04–3.61)	0.037
Female (n = 959)				
ΔBMI-Q1, Q2, Q3	1.00	–	1.00	–
ΔBMI-Q4	1.22 (0.53–2.83)	0.644	1.23 (0.53–2.89)	0.631

BMI = Body mass index; WC = waist circumference.
* Multivariate adjusted: Adjusted for age, systolic blood pressure, HDL-cholesterol, LDL-cholesterol, fasting plasma glucose, and smoking status.

status of albuminuria or a low eGFR may be altered when men without micro-/macroalbuminuria or a low eGFR, respectively, gain BMI substantially, although such a relationship was not apparent in female subjects. Future studies should be directed toward elucidating whether these observed gender differences were, in part, due to the greater prevalence of other risk factors, such as increased blood pressure, elevated fasting glucose levels, and reduced insulin sensitivity [20, 21], in men than in women.

Several studies have investigated the possible association between the obesity index and CKD. A high BMI has been reported to be associated with CKD [6, 10, 11]. Chou et al. [22] reported that in elder Taiwanese, the waist-hip ratio, body weight and WC, but not BMI, were predictors of a low eGFR, and that among these predictors, the waist-hip ratio may be the best anthropometric index for predicting a low eGFR. Foster et al. [23] showed that the association between obesity with an increased risk of developing stage 3 CKD was not independent, but was confounded by other cardiovascular disease risk factors. These findings suggest that the mode of association between certain obesity index and CKD might differ according to the study design and population studied.

Whether changes in obesity parameters would result in changes in CKD status has also been investigated in several previous studies. Changes in body weight were found to be associated with parallel changes in albuminuria in 6,894 participants of the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study during a 4.2-year follow-up period [12]. In addition, moderate weight loss induced by a hypocaloric and normoproteinic diet in overweight patients with chronic proteinuria resulted in a significant decrease in proteinuria [13]. Furthermore, weight loss induced by an inhibitor of gastrointestinal lipase was associated with the reduction of urinary albumin excretion [14]. Therefore, most, if not all, studies showed that body weight reduction in overweight subjects resulted in a reduction of proteinuria, which was in agreement with the observation in the current study. Compared to the association between changes in obesity parameters and proteinuria, fewer numbers of studies have analyzed the relationship between change in body weight and change in eGFR. In the above-mentioned analysis in the PREVEND study, weight loss or gain did not significantly bring about a change in GFR [12]. Other studies showed that GFR was decreased after weight loss in extremely obese patients, presumably by the mechanisms of amelioration of obesity-associated hyperfiltration [24, 25]. In the current study, BMI gain of ≥0.33 was associated with a significantly higher risk for a low eGFR

at the second visit in men, but not in women, who were free from a low eGFR at the first visit. Taking all these results together, it is suggested that the relationship between weight loss and GFR change may also differ according to the target population. Interestingly, high BMI is known to be associated with better survival in dialysis patients [26] designated as a risk factor paradox [27].

The current study has several limitations. First, we retrospectively analyzed data on individuals who underwent general health screening at our institute in two consecutive years; therefore, individuals who did not visit our institute the following year for unknown reasons were not enrolled in the current study, which may cause some biases. Second, we excluded subjects those who were taking anti-hypertensive agents during either visit. This may have excluded from the study population some hypertensive subjects with proteinuria. Whether or not a body weight change results in a change in CKD status in such hypertensive individuals is nonetheless an important question. However, we do not have data on which class of anti-hypertensive agents had been used, which might affect the development, amelioration or elimination of CKD. Third, we used the MDRD equation for the estimation of GFR, which may result in a certain degree of inaccuracy. In addition, changes in weight will be affected not only by the changes in fat mass, but also those in muscle mass, and eGFR determined by MDRD formula is also highly dependent on muscle mass, as this formula takes only serum creatinine into account. We have to be careful in interpreting the results of the current study, as changes in muscle mass will lead to bias when assessing the association between obesity parameters and eGFR. Fourth, our findings may not be immediately applicable to non-Japanese populations, as the GFR estimated using serum creatinine is again more than slightly affected by muscle mass.

In conclusion, a BMI reduction of ≥ 0.42 or a WC reduction of ≥ 3.0 cm over a 1-year period in men with micro-/macroalbuminuria at the first visit significantly reduced the risk for micro-/macroalbuminuria at the second visit, and a BMI gain of ≥ 0.33 over a period of a year in men without micro-/macroalbuminuria or a low eGFR at the first visit significantly increased the risk for micro-/macroalbuminuria or a low eGFR during the 1-year follow-up. Such associations were not statistically significant in female subjects. Our data indicated that reducing body weight in overweight/obese men with micro-/macroalbuminuria and that maintaining an ideal body weight in non-overweight men without micro-/macroalbuminuria or a low eGFR are both important targets of lifestyle in terms of renoprotection.

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Functional phenotypes and gene expression profiles of peripheral blood mononuclear cells in chronic hepatitis C patients who developed non-Hodgkin's B-cell lymphoma

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ABSTRACT

Epidemiological data have indicated a close relationship between chronic HCV infection and non-Hodgkin's B-cell lymphoma (B-NHL). In this study, functional phenotypes and gene expression profiles of PBMCs were analyzed in chronic hepatitis C (CHC) patients who developed B-NHL. The frequencies of effector $CD8^+$ T cells and cytotoxic natural killer cells increased in CHC patients with B-NHL compared to those in CHC patients without B-NHL. These phenotypic changes may reflect the host's immune response to neoplasia. The mRNA expression levels of several oncogenes increased in CHC patients without B-NHL, but were much higher in CHC patients with B-NHL, while mRNA levels of type I IFNs were decreased in CHC patients without B-NHL and were nearly negligible in CHC patients with B-NHL. Interestingly, the mRNA expression levels of activation-induced cytidine deaminase and caspase recruitment domain-containing proteins markedly increased in CHC patients without B-NHL but decreased in CHC patients with B-NHL. These results are discussed in view of the possible involvement of HCV infection in B-cell lymphomagenesis.

Introduction

Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus belonging to the *Flaviviridae* family. It has infected nearly 200 million people worldwide and is therefore an important public health problem [1]. The liver is considered to be the primary target of HCV infection, which is also associated with a number of extrahepatic manifestations, such as mixed cryoglobulinemia, rheumatoid factor production, Sjogren-like syndrome, and B-cell lymphoproliferative disorders that may develop into overt non-Hodgkin's B-cell lymphoma (B-NHL) [2]. Recent epidemiological data have suggested a close relationship between chronic HCV infection and B-NHL [3]; however, experimental data concerning these conditions remain elusive. The aim of the present study was to compare the functional phenotypes and gene expression profiles of PBMCs between CHC patients without B-NHL and those with B-NHL. Results of this study suggest the possible involvement of HCV infection in the progression of B-NHL.

Materials and methods

Patients and PBMCs. Thirteen healthy subjects, 19 CHC patients without B-NHL, and 13 CHC patients with B-NHL were enrolled in this study. Demographic and clinical data were as follows: (1) CHC patients without B-NHL ($n = 19$): gender (M:F = 10:9); mean age (range) = 59.6 years (38–79 years); mean alanine aminotransferase (ALT) (IU/L) (SD) = 64.4 (23.5); mean aspartate aminotransferase (AST) (IU/L) (SD) = 61.3 (19.3); HCV genotype (No.) = 1b (18) and 2b (1); and mean HCV RNA titer = 2077 KIU/mL. (2) CHC patients with B-NHL ($n = 13$): gender (M:F = 7:6); mean age (range) = 73.1 years (52–85 years); ALT (IU/L) (SD) = 28.5 (12.4); AST (IU/L) (SD) = 32.4 (11.2); HCV genotype (No.) = 1b (13); and mean HCV RNA titer = 1288 KIU/mL. All CHC patients were confirmed to be negative for other viral infections, including hepatitis B virus and human immunodeficiency virus infections. Study protocols were approved by the review board at the National Institute of Infectious Diseases and Jikei University School of Medicine. All donors gave written informed consent. PBMCs were isolated by Fi-

Abbreviations: AID, activation-induced cytidine deaminase; CARD, caspase recruitment domain; CHC, chronic hepatitis C; HCV, hepatitis C virus; B-NHL, B-cell non-Hodgkin's lymphoma; NK, natural killer; PBMCs, peripheral blood mononuclear cells.

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coll-HyPaque (Pharmacia Biotech, Quebec, Canada) gradient centrifugation.

Flow cytometry. The following fluorescence-conjugated Abs were used for flow cytometry: Peridinin-chlorophyll-protein complex-anti-CD3 (Cat. 347344, BD Biosciences, San Jose, CA), APC-anti-CD8 (Cat. IM2469, Beckman Coulter, Fullerton, CA), PE-anti-CCR7 (Cat. FAB197P, R&D Systems, Minneapolis, MN), FITC-anti-CD45R (Cat. A07786, Beckman Coulter), and APC-anti-CD56 (Cat. IM2474, Beckman Coulter). Cells were washed twice with cold PBS containing 0.2% BSA and incubated with an appropriate combination of conjugated Abs for 30 min on ice. Stained cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data were collected using CellQuest software (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Semiquantitative real-time PCR. Total RNA was extracted from lymphoid cells using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan). cDNA was synthesized using SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo(dT) 12–18 primers (Invitrogen). PCR amplification was performed using SYBR Premix Ex Taq™ II (TAKARA Shuzo, Kyoto, Japan) with gene-specific primers (Bex Co., Ltd., Tokyo, Japan) available in the public database RTPrimerDB under the following entry codes (in parentheses): CCND2 (803), AID (2285), IFNA (3541), IFNB (3542), FN1 (3523), LMO2 (693), and GAPDH (3539); real-time PCR primer sets (http://www.realtimeprimers.org/): CCND1 (JS-209); and the Universal ProbeLibrary Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp; Roche Applied Science): MYC (#34, 04687671001), TP53 (#58, 04688554001), MLLT3 (#25, 04686993001), serine/threonine kinase 15 (STK15; #79, 04689020001), FHIT (#33, 04687663001), CASP1 (#17, 04686900001), and CASP4 (#29, 04687612001). The sequences of primers for RIG-I were 5′-GTG CAA AGC CTT GGC ATG T-3′ (forward) and 5′-TGG CTT GGG ATG TGG TCT ACT C-3′ (reverse).

Real-time PCR was performed for 45 cycles at 94 °C for 1 min and 60 °C for 25 s (two-step) using a Light Cycler (Roche Diagnostics, Basel, Switzerland). Amplification of predicted fragments was confirmed by melt curve analysis and gel electrophoresis. Standard curves were obtained with 10-fold serial dilutions of the amplified products. Measured amounts of mRNA were normalized against

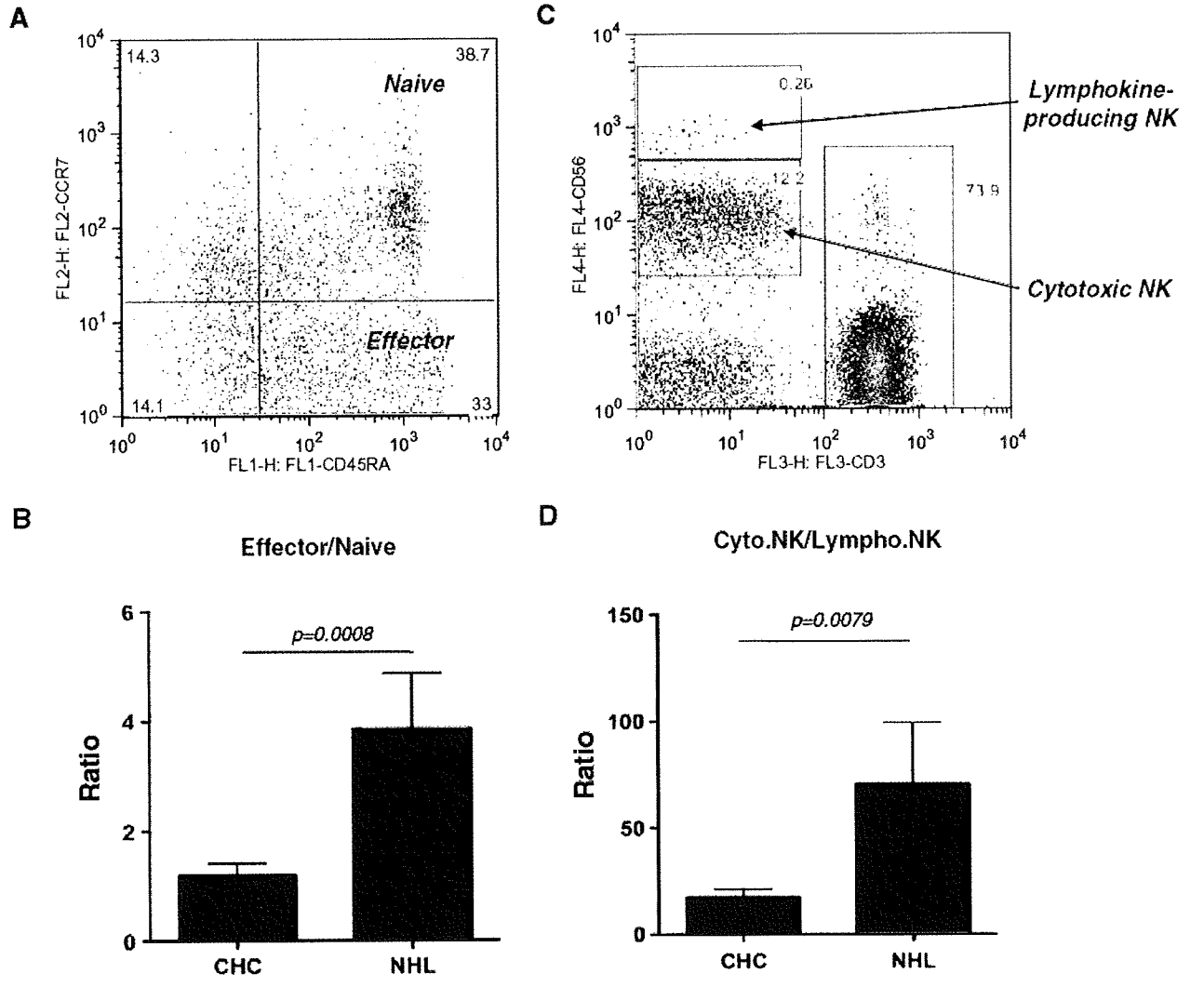

Fig. 1. Phenotypic analysis of PBMCs. (A) CD3'CD8'cells (CD8' T cells) were analyzed by 2-color flow cytometry (CD45RA vs. CCR7). Fractions of naïve CD8' T cells (CD45RA'CCR7') and effector CD8' T cells (CD45RA'CCR7) are shown. (B) Ratios of effector CD8' T cells to naïve CD8' T cells in CHC patients without B-NHL (n = 19) and CHC patients with B-NHL (referred to as NHL; n = 7) are shown. Error bars indicate SEM. (C) Lymphocytes were analyzed by 2-color flow cytometry (CD3 vs. CD56). The $CD56^{dim}$ fraction (cytotoxic NK cells) and $CD56^{bright}$ fraction (lymphokine-producing NK cells) are shown. (D) Ratios of cytotoxic NK cells ($CD56^{dim}$) to lymphokine-producing NK cells ($CD56^{bright}$) in CHC patients without B-NHL (n = 19) and CHC patients with B-NHL (referred to as NHL; n = 7) are shown. Error bars indicate SEM.

the amounts of GAPDH mRNA. The mRNA expression levels of normal PBMCs were arbitrarily defined as 1.0.

Statistics. Unpaired two-tailed Student's *t* tests at the 95% confidence level ($p < 0.05$) were applied in all cases using Prism ver.4 (GraphPad Software, Inc., San Diego, CA).

Results and discussion

Functional phenotypes of PBMCs in CHC patients with or without B-NHL

Based on CD45RA and CCR7 expressions, $CD8^+$ T cells are classified into four subsets: naïve cells ($CD45RA^+CCR7^+$), central memory cells ($CD45RA^-CCR7^+$), effector memory cells ($CD45RA^-CCR7^-$), and effector cells ($CD45RA^+CCR7^-$), as shown in Fig. 1A [4]. The ratio of effector $CD8^+$ T cells to naïve $CD8^+$ T cells markedly increased in CHC patients with B-NHL compared to that in CHC patients without B-NHL, as shown in Fig. 1B. These results suggest that phenotypic changes, i.e., naïve to effector, occurred in PBMCs during the course of B-NHL development.

Human natural killer (NK) cells constitute approximately 15% of PBMCs and are divided into two subsets based on the cell surface density of CD56, i.e., $CD56^{bright}$ and $CD56^{dim}$ [5], as shown in Fig. 1C. There is ample evidence to suggest that these NK cell subsets have unique functional attributes and distinct roles in human immune responses [6]. The $CD56^{dim}$ NK subset has higher cytotoxicity than the $CD56^{bright}$ subset, which is capable of producing abundant lymphokines [5]. The ratio of cytotoxic NK cells ($CD56^{dim}$) to lymphokine-producing NK cells ($CD56^{bright}$) markedly increased in CHC patients with B-NHL compared to that in CHC patients without B-NHL, as shown in Fig. 1D.

Taken together, these phenotypic changes in PBMCs of CHC patients with or without B-NHL may be attributed to the development of B-NHL and may thus reflect the host's immune response to neoplasia.

Gene expression profiles of PBMCs in CHC patients with or without B-NHL

Our preliminary analyses of the dynamics of innate immune-related gene expressions in PBMCs demonstrated that the mRNA expression levels of type I IFN, i.e., IFN-α and IFN-β, decreased significantly, while those of caspase recruitment domain (CARD)-containing proteins, such as RIG-I, caspase 1, and caspase 4, markedly increased in CHC patients without B-NHL compared to normal subjects. The mRNA expression levels of various lymphomagenesis-related genes were also analyzed, and the significant increase in the mRNA expression levels of AID in CHC patients was of particular interest. These levels were thought to be closely associated with the occurrence of B-NHL [7]. In this study, the mRNA expression levels of interest were those of the above-mentioned genes in PBMCs of CHC patients with or without B-NHL. Relative mRNA expression levels of 15 genes in PBMCs were analyzed (Fig. 2), and significant differences were observed between the three groups: normal, CHC patients without B-NHL, and CHC patients with B-NHL. Interestingly, there seemed to be three distinct categories in terms of mRNA expression level dynamics: (A) upregulated in CHC patients without B-NHL and further more in CHC

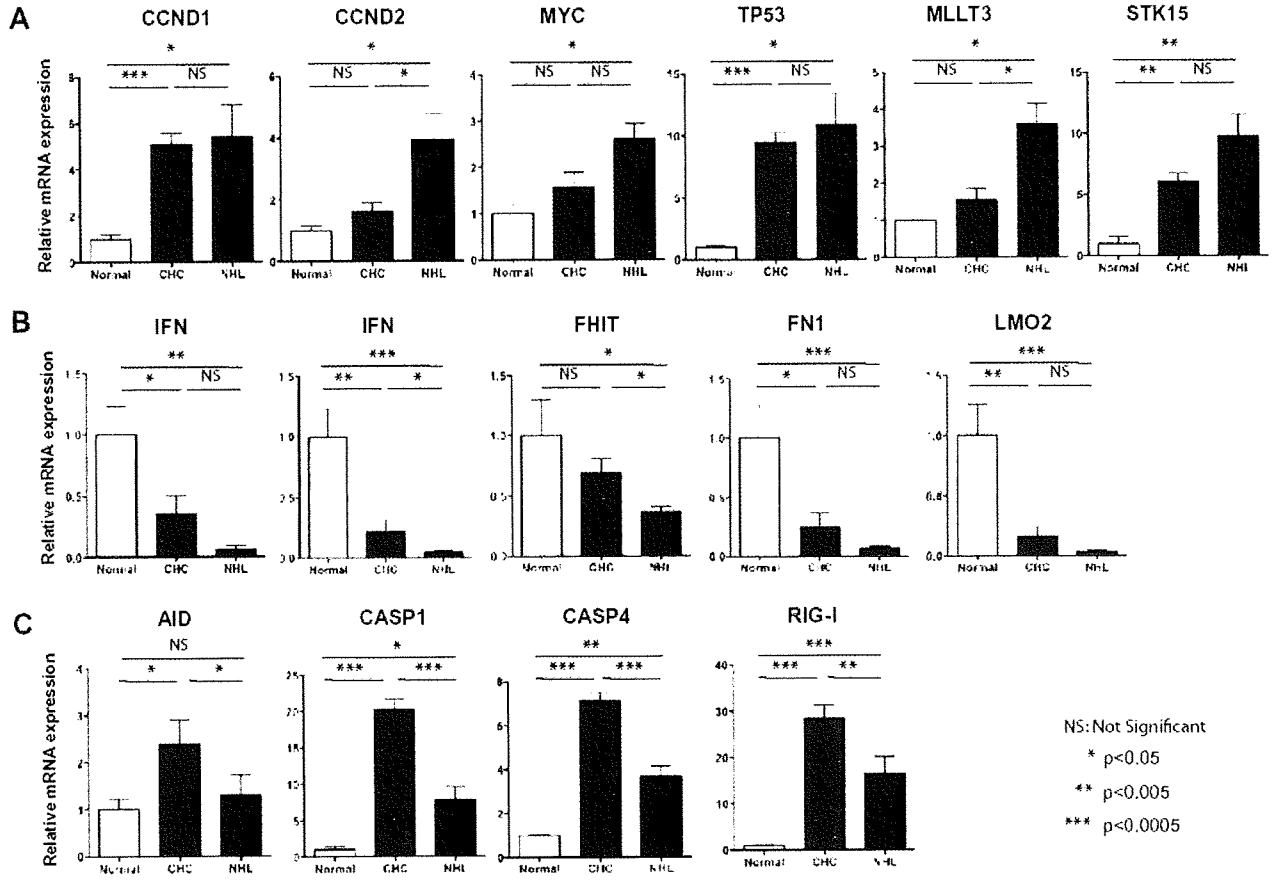

Fig. 2. Gene expression profiles of PBMCs. mRNA expression levels of 15 genes in normal subjects ($n = 7$), CHC patients without B-NHL ($n = 7$), and CHC patients with B-NHL (referred to as NHL; $n = 7$) are shown. mRNA expression dynamics are categorized into A, B, and C (see text). Error bars indicate SEM.

patients with B-NHL, (B) downregulated in CHC patients without B-NHL and further more in CHC patients with B-NHL, and (C) upregulated in CHC patients without B-NHL and downregulated to normal levels in CHC patients with B-NHL.

Oncogenes and proto-oncogenes are included in category A in which gene expression levels are highly upregulated in CHC patients with B-NHL. Overexpression of CCND1, which alters cell cycle progression, is frequently observed in various tumors and may contribute to tumorigenesis [8]. Furthermore, CCND2 is known to be expressed at constitutively high levels in B-NHL [9] and may thus be correlated with B-cell lymphomagenesis. It is reported that STK15 is strongly expressed in histologically aggressive NHL [10]. Taken together, these results suggest that upregulation of these genes is responsible for the development of B-NHL.

In contrast, the mRNA expression levels of type I IFN, i.e., IFN-α and IFN-β, markedly decreased in CHC patients without B-NHL and were almost negligible in CHC patients with B-NHL (category B). This downregulated type I IFNs gene expression may be due to strategies employed by HCV to evade antiviral innate immune responses in the host [11]. Expression of fragile histidine triad (FHIT), fibronectin 1 (FN1) and LIM domain only 2 (LMO2) mRNAs was also downregulated in CHC without B-NHL and further downregulated in CHC with B-NHL. Decreased expression of FHIT is correlated with worse prognosis in CHC patients with B-NHL [12]. Lossos et al. reported that augmented expression of FN1 and LMO2 is correlated with prolonged survival in patients with DLBCL [13]. Thus, downregulation of these genes in CHC patients may be correlated with B-NHL malignancy.

Up- and downregulation of mRNA expression was noted in AID and CARD-containing proteins, including CASP1, CASP4, and RIG-I (category C). AID is essential for both somatic hypermutation (SHM) and class switch recombination of immunoglobulin genes in B cells [14]. It has recently been proposed that AID is instrumental in initiation and progression of B-NHL because a malfunction in either of the above processes is apparently responsible for generating chromosomal translocations and aberrant SHM, which are the two main causes of genetic lesions associated with B-NHL [15].

Interestingly, expression of AID mRNA, which was significantly enhanced in CHC patients without B-NHL, was downregulated to normal levels in CHC patients with B-NHL. We speculate that HCV infection triggers abnormal expression of AID mRNA and that after B-NHL develops, AID expression is not necessarily upregulated further. Downregulation of AID mRNA expression in CHC patients with B-NHL may reflect lower HCV RNA titers when compared to those of CHC patients without B-NHL (2077 KIU/mL in CHC patients without B-NHL vs. 1288 KIU/mL in CHC patients with B-NHL).

CARDs are interaction motifs found in a wide array of proteins, particularly those involved in inflammatory and apoptotic processes [16]. Caspases 1 and 4, which are CARD-containing proteins, are classified as inflammatory caspases [17]. HCV infection is a major cause of liver disease characterized by inflammation, cell damage, and fibrotic reactions of hepatocytes; apoptosis has also been implicated in the pathogenesis. Enhanced expression of both caspase mRNA may be a consequence of HCV infection in CHC patients without B-NHL. On the other hand, both caspases are also involved in apoptosis and thought to suppress tumor progression [18]. Therefore, reduced mRNA expression of CASP1 and CASP4 in CHC patients with B-NHL may be correlated with B-NHL progression.

RIG-I, another CARD-containing protein, is a cytoplasmic sensor molecule for dsRNA, including HCV RNA, and plays a critical role in antiviral innate immune responses [19]. RIG-I mRNA expression increased in CHC patients without B-NHL ($p < 0.0005$) and decreased in CHC patients with B-NHL ($p < 0.005$). This may account for the fact that enhanced RIG-I expression results in augmentation of antitumor activity by producing inflammatory cytokines and inducing apoptosis [20].

In conclusion, the present study indicates noticeable alterations in both functional phenotypes and gene expression profiles between PBMCs of CHC patients without B-NHL and those of CHC patients with B-NHL. Our results support the notion that HCV infection is at least partly responsible for the development of B-NHL in CHC patients.

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Differential susceptibility of peripheral blood CD5$^+$ and CD5$^-$ B cells to apoptosis in chronic hepatitis C patients

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ABSTRACT

A body of evidence has suggested a close link between chronic hepatitis C virus (HCV) infection and B cell abnormalities, including mixed cryoglobulinemia, rheumatoid factor (RF) production, and lymphoproliferative disorders that may develop into non-Hodgkin's lymphoma. Recent studies have demonstrated the expansion of CD5$^+$ B cells in the peripheral blood of chronic hepatitis C patients (CHC). As CD5$^+$ B cells, which are capable of producing autoantibodies and RF, are apparently crucial for the development of HCV-associated pathogenesis, the fate of both the CD5$^+$ and CD5$^-$ B cell subsets upon chronic HCV infection is of interest. In this study, the degree to which chronic HCV infection induces apoptosis in each B cell subset was investigated. Our results demonstrated that peripheral CD5$^-$ B cells were more susceptible to apoptosis than CD5$^+$ B cells in CHC. Furthermore, plasma levels of IL-4, IL-10, and IL-12 were significantly elevated in CHC, thus suggesting that these interleukins protect CD5$^+$ B cells from apoptosis. The rationale for the differential susceptibility of distinct B cell subsets in CHC is also discussed with regard to extrahepatic manifestations associated with chronic HCV infection.

Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease, affecting nearly 200 million people worldwide [1]. Although the liver is considered to be the primary target, HCV infection is also associated with extrahepatic manifestations such as mixed cryoglobulinemia (MC), rheumatoid factor (RF) production and B cell lymphoproliferative disorders that may develop into overt non-Hodgkin's B-lymphoma (B-NHL) [2]. However, the pathogenic relationship between HCV infection and these immunological disorders remains uncertain.

The CD5$^+$ B cell subset identified in early 1980s [3] has attracted considerable interest because of its association with autoimmune pathology [4]. CD5$^+$ B cells are the predominant B cell population in the fetus but are rare in adults, and seem to constitute a primitive but effective first line of defense against foreign pathogens [5]. The expansion of CD5$^+$ B cells in rheumatoid arthritis [6], Sjoegren syndrome [7] and MC [8] has also implicated them in the development of autoimmune disorders. Interestingly, these disorders seem to be closely correlated with HCV infection [9]. Curry et al. recently demonstrated the expansion of CD5$^+$ B cells in peripheral blood from chronic hepatitis C patients (CHC) [10] and within the liver of non-cirrhotic CHC [11]. These results strongly suggest a correlation between HCV pathogenesis and the expansion of peripheral CD5$^+$ cells; however, the mechanisms underlying this CD5$^+$ B cell subset-restricted expansion upon HCV infection remain unknown.

In this study, we compared the susceptibility of the peripheral CD5$^+$ and CD5$^-$ B cell subsets to apoptosis in both normal subjects and CHC. Our results demonstrated the differential susceptibility to apoptosis between the two B cell subsets in CHC but not in normal subjects. Enhanced levels of anti-apoptotic cytokines in CHC plasma may be responsible for the apoptosis resistance of CD5$^+$ B cell in CHC.

Materials and methods

Patients and samples. A total of 25 CHC were enrolled in this study. Demographic and clinical data were as follows: gender (M:F) = 15:10; mean age (range) = 60.8 years (44–80 years); HCV genotype (No.) = 1b (15) and 2a (10); mean HCV RNA (KIU/mL) (SD) = 1836 (1506); mean alanine aminotransferase (ALT) (U/L) (SD) = 57.3 (18.1); and mean aspartate aminotransferase (AST) (U/L) (SD) = 47.3 (17.7). The study protocols were approved by the Review Board at the National Institute of Infectious Disease. All donors gave written informed consent before phlebotomy. A

Abbreviations: B-NHL, non-Hodgkin's B-lymphoma; CHC, chronic hepatitis C patients; HCV, hepatitis C virus; MC, mixed cryoglobulinemia; RF, rheumatoid factor.

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total of 15 normal control subjects included anonymous volunteer blood donors visiting the Japanese Red Cross Blood Center (Tokyo, Japan), who were confirmed to be negative for HCV, HBV, and HIV.

Clinical tests. HCV genotype was determined by PCR of the core region with genotype-specific primers [12]. HCV RNA was quantified by the Roche Amplicor assay (Roche Diagnostics, Branchberg, NJ), and results were standardized to international units (IU). Determination of serum levels of ALT and AST was performed using standard methods.

Flow cytometry. The following fluorescence-conjugated antibodies (Abs) were used for flow cytometry: allophycocyanin-anti-CD19 (MHCD1905; Invitrogen, Carlsbad, CA); and PerCP-Cy5.5-anti-CD5 (Cat. 341089; BD Biosciences, San Jose, CA). Cells were washed twice with cold PBS containing 0.2% BSA, followed by incubation with an appropriate combination of directly conjugated Abs for 30 min on ice. Stained cells were analyzed by FACSCallibur (Becton Dickinson, San Jose, CA). Data were collected using CellQuest software (Becton Dickinson, San Jose, CA) and were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Assessment of apoptotic cell death. Levels of Annexin V binding to both $CD5^+$ and $CD5^-$ B cells were assessed with a commercially available Annexin V apoptosis detection kit Annexin V-FITC (PN IM3546, Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions.

Plasma cytokine assay. Plasma samples were analyzed for IL-2, IL-4, IL-5, IL-10, IL-12p40/p70, IL-13, IFN-γ, and TNFα using a Human Cytokine Thirty-plex antibody bead kit (BioSource Int. Inc., Camarillo, CA) according to the manufacturer's instructions. Assay results were obtained using a Luminex 100™ reader (Luminex Co., Austin, TX).

Statistics. Unpaired (two-tailed) Student's *t*-test was applied at the 95% confidence level ($p < 0.05$) using Prism ver.4 (GraphPad Software, Inc., San Diego, CA) in all cases.

Results and discussion

Increased frequency of peripheral blood $CD5^+$ B cells in CHC

The frequencies of peripheral blood $CD19^+$ cells (B cells) and $CD5^+$ cells (mostly T cells) were comparable between normal subjects and CHC, as shown in Fig. 1A and B, respectively. Although CD5 is primarily a pan-T cell marker, a particular B cell subset, termed B-1 or B-1a cells, expresses the CD5 molecule [13,14]. When the percentages of peripheral $CD5^+$ cells in $CD19^+$ cells was analyzed, a significant increase was noticed in CHC when compared to normal subjects (Fig. 1C). It was also verified that the frequencies of peripheral $CD5^+CD19^+$ cells were significantly elevated in CHC (Fig. 1D). These results were concordant with a previous study by Curry et al., in which they concluded that immune complex formation by expanded $CD5^+$ B cells may limit the development of progressive liver disease [10].

$CD5^+$ B cells, which are characterized by the production of low-affinity IgM with RF activity [5], have been shown to expand in patients with MC [8] and in those with Sjoegren's syndrome [7]. Interestingly, the correlation between chronic HCV infection and the above-mentioned manifestations has been widely appreciated [15]. Therefore, we aimed to investigate the fate of both the $CD5^+$ and $CD5^-$ B cell subsets upon chronic HCV infection.

Differential susceptibility of peripheral blood $CD5^+$ and $CD5^-$ B cells to apoptosis in CHC

The levels of spontaneous apoptosis among peripheral blood $CD5^+$ and $CD5^-$ B cells in both normal subjects and CHC were analyzed using three-color flow cytometry by staining with allophycocyanin-anti-CD19, PerCP-Cy5.5-anti-CD5 and Annexin V-FITC. Representative staining data from experiments analyzing eight normal subjects and ten CHC with similar results are shown in Fig. 2A and B, respectively. The patterns of Annexin V binding were almost identical between $CD5^+$ and $CD5^-$ B cells in normal subjects (Fig. 2A). In contrast, as shown in Fig. 2B, $CD5^-$ B cells bound to much larger amounts of Annexin V than $CD5^+$ B cells in CHC. The percentages of each cell subset bound to large amounts of Annexin V are shown in Fig. 2C (the cut-off point was tentatively set at a fluorescence intensity of 2000). It was concluded that, $CD5^-$ B cells were more vulnerable to apoptosis than $CD5^+$ B cells upon HCV infection; in other words, $CD5^+$ B cells were apparently resistant to apoptosis.

Elevation of anti-apoptotic cytokine levels in CHC plasma

A number of cytokines, including IL-2, IL-4, IL-10, IL-12p40/p70, IL-13, IFN-γ, and TNFα, are known to suppress apoptosis of leukemic $CD5^+$ B cells, and may be closely involved in the pathogenesis

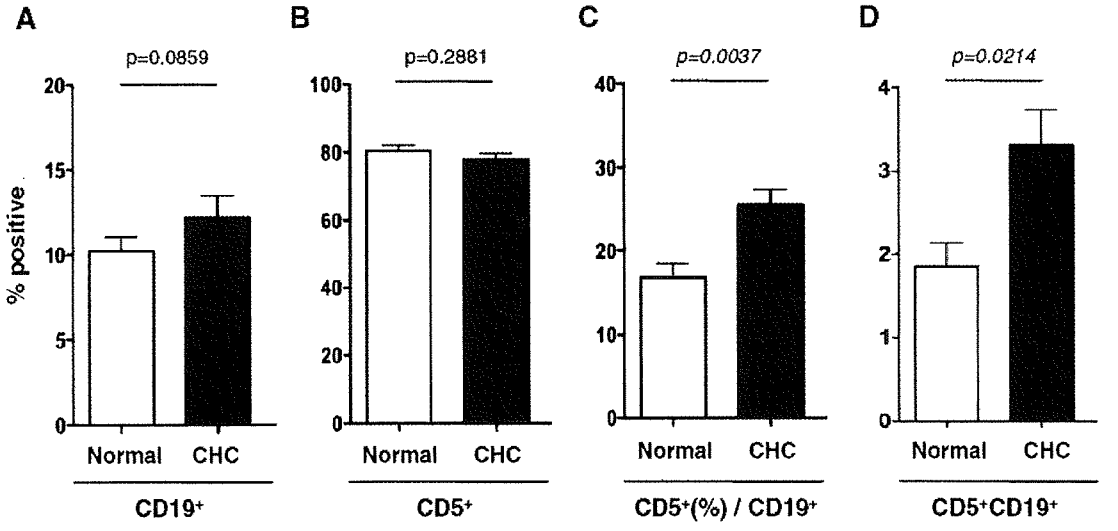

Fig. 1. Flow cytometric analysis of PBMC from normal subjects and CHC. Lymphocytes were identified by forward and orthogonal light scatter characteristics. Percentages of $CD19^+$ cells (A), $CD5^+$ cells (B), $CD5^+$ (%) in $CD19^+$ cells (C), and $CD5^+CD19^+$ cells (D) in normal ($n = 10$) and CHC ($n = 25$) are shown with SEM bars and *p*-values.

of chronic lymphocytic leukemia (B-CLL) [16,17]. Some anti-apoptotic cytokines, i.e., IL-4 and IL-10, have been shown to protect cord blood CD5$^+$ B cells from apoptosis [18,19]. These findings prompted us to examine the cytokine levels in CHC plasma. Our assumption was that anti-apoptotic cytokine levels are elevated in CHC, thereby preventing apoptosis of CD5$^+$ B cells. As shown in Fig. 3A, B, and C, plasma levels of IL-4, IL-10, and IL-12 were significantly elevated in CHC when compared with normal subjects, which supported our prediction. In addition, plasma levels of other anti-apoptotic cytokines for leukemic CD5$^+$ B cells, i.e., IL-2, IL-13, IFN-γ, and TNFα,

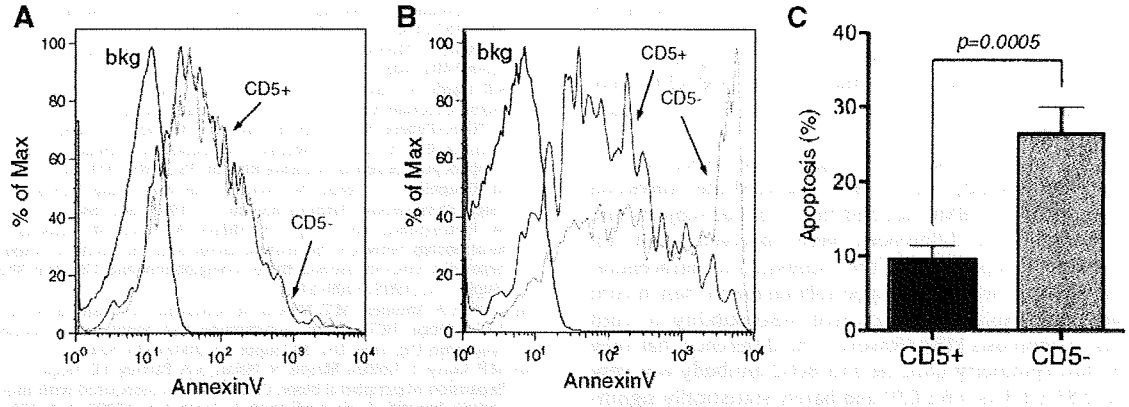

Fig. 2. Annexin V binding to CD5$^+$ and CD5$^-$ B cells. Representative staining patterns for Annexin V binding to CD5$^+$ (red line) and CD5$^-$ (green line) B cells are shown in normal subjects (A) and in CHC (B). Blue lines indicate background (bkg) staining in negative controls. (C) Summary of data on Annexin V binding to CD5$^+$ (red bar) and CD5$^-$ (green bar) B cells in CHC ($n = 10$) are shown with SEM and p-values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

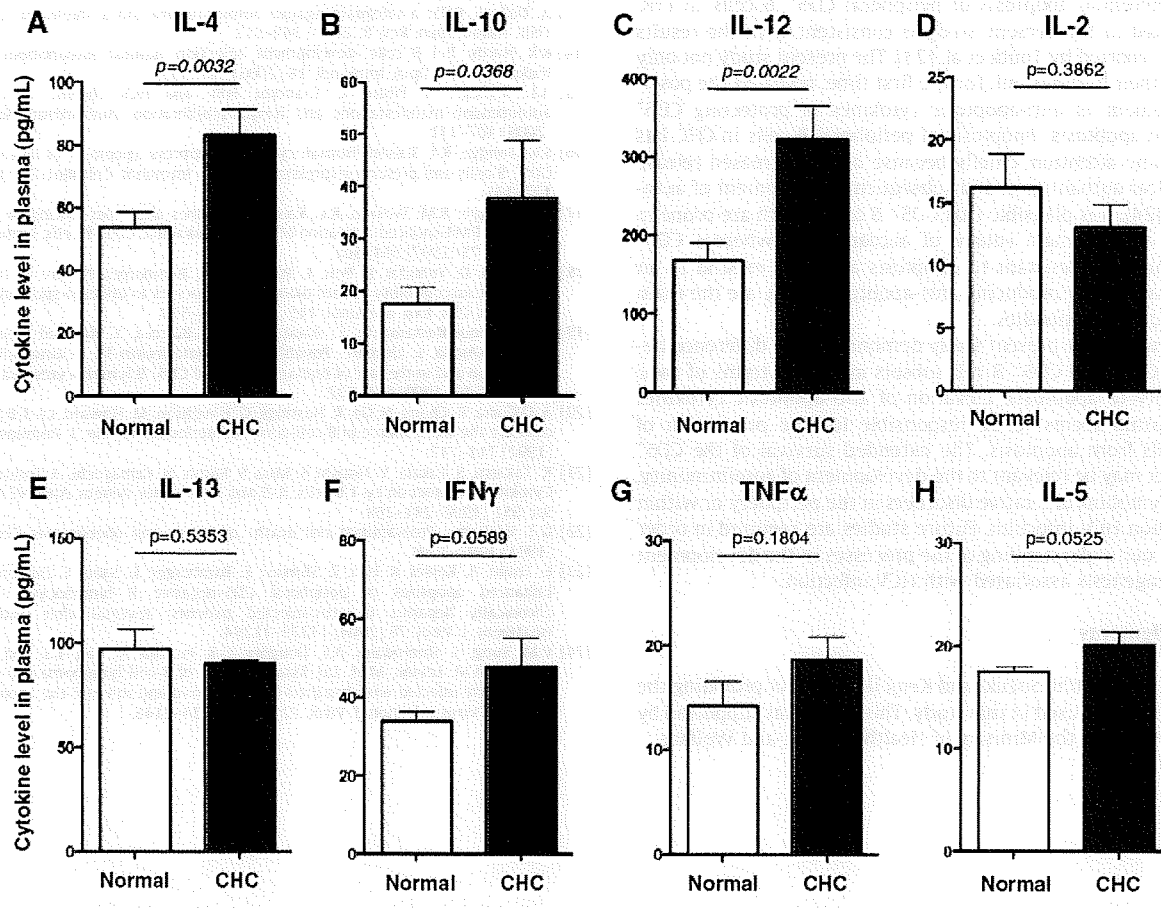

Fig. 3. Plasma levels of cytokines. Amounts of IL-4 (A), IL-10 (B), IL-12 (C), IL-2 (D), IL-13 (E), IFN-γ (F), TNFα (G), and IL-5 (H) were simultaneously determined in plasma samples from both normal ($n = 14$) and CHC ($n = 11$), and are shown with SEM and p-values.

were not significantly elevated (Fig. 3D–G). These results reflect the differences between leukemic CD5$^+$ B cells and CHC CD5$^+$ B cells in terms of the functional expression of cytokine receptors.

The elevation of IL-10 levels in CHC plasma is of particular interest because CD5$^+$ B cells themselves are known to produce IL-10 [20], and this autocrine loop may result in further expansion of the CD5$^+$ B cell subset. As IL-5 plays a critical role in the development of CD5$^+$ B cells and may be responsible for prolonging the lifespan of immature CD5$^+$ B cells [21], we were interested in the plasma levels of IL-5 in CHC. As shown in Fig. 3H, no significant differences in IL-5 levels between normal subjects and CHC were found, suggesting the minimum involvement of IL-5 in the expansion of CD5$^+$ B cells in CHC.

Enhanced expression of Bcl-2 is thought to be responsible for resistance to apoptosis [22]. We therefore analyzed the expression levels of Bcl-2 mRNA in PBMC purified from normal subjects and CHC, but no significant differences were observed (data not shown). Kessel et al. reported that the expression of intracellular Bcl-2 protein in B cells was higher in peripheral blood than in cord blood, which may explain the increased susceptibility of cord blood B cells to apoptosis [18]. However, the difference that they observed in flow cytometry utilizing anti-Bcl-2 antibody was very subtle (MFI: 2.85 ± 1.3 vs 1.6 ± 0.9) and barely statistically significant ($p = 0.05$). Toubi et al. analyzed the Bcl-2 expression levels in B cells from healthy individuals and HCV-positive patients, but did not detect any differences between them [23]. Thus, the underlying mechanism, other than augmented expression of Bcl-2, responsible for the resistance to apoptosis recognized in the CD5$^+$ B cell subset remains uncertain and needs to be clarified.

The preferential apoptosis of peripheral CD5$^-$ B cells in CHC demonstrated in the present study is consistent with the results previously reported by Toubi et al. [23]. The present study not only confirmed their findings but, for the first time, revealed the possible involvement of anti-apoptotic cytokines in protecting CD5$^+$ B cells from apoptosis. Apoptosis of peripheral B cells in CHC has received some attention, chiefly because of the increased release of cell nuclear autoantigens and subsequent development of autoimmunity [24]. It is plausible that CD5$^-$ B cells, which are prone to apoptosis, are the main source of autoantigens, whereas CD5$^+$ B cells, which are resistant to apoptosis and may expand in an autocrine fashion by producing anti-apoptotic IL-10, are the main producers of autoantibodies.

In conclusion, the present study demonstrated a difference between the CD5$^+$ and CD5$^-$ B cell subsets in CHC in terms of their susceptibility to apoptosis. Elevation of anti-apoptotic cytokines in CHC plasma seems to be responsible for the prevention of CD5$^+$ B cells from apoptosis. The extended survival of the CD5$^+$ B cell subset may be relevant to the development of autoimmunity, as well as lymphoproliferative disorders in the periphery or within the liver upon HCV infection. Future studies are required in order to enhance our understanding of the processes in the development of the pathogenesis associated with HCV infection.

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Double-Filtration Plasmapheresis plus IFN for HCV-1b Patients with Non-Sustained Virological Response to Previous Combination Therapy: Early Viral Dynamics

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Key Words
Chronic hepatitis C · Double-filtration plasmapheresis · Early viral dynamics · Genotype 1b · High viral load · Interferon β · Non-sustained virological responder · Peginterferon plus ribavirin combination therapy

Abstract
Double-filtration plasmapheresis (DFPP) was approved in Japan in April 2008 for the retreatment of chronic hepatitis C patients with genotype 1b and high viral loads, whose hepatitis C virus was not eradicated by earlier IFN therapy or by pegylated IFN plus ribavirin (PEG-IFN/RBV) combination therapy. In this study, we assessed the early viral dynamics of 9 patients with non-sustained virological response to the combination therapy. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks showed a reduction of ≥1 log in the viral load in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9) and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment. By contrast, DFPP plus consecutive intravenous IFN-β for 4 weeks reduced the viral load by ≥1 log in 33% (2/6), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks. The viral load declined by ≥2 log in 50% (3/6) at 4 weeks after the start of treatment. DFPP plus consecutive intravenous IFN-β for 4 weeks is a promising treatment for non-sustained virolgical response patients.

Introduction

Hepatitis C virus (HCV) infection is the major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) in industrialized countries. HCV infection is manageable, however, and its complications can be prevented by antiviral therapy [1, 2]. Currently, the most effective treatment for chronic HCV infection is based on pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy [3]. Nonetheless, sustained

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virological response (SVR) rates for those infected with the most resistant genotypes (HCV-1a and HCV-1b) still hover around 50% [3, 4].

To surmount this SVR rate with combination therapy, several trials have been undertaken, two of which are: (1) retreatment with combination therapy and (2) double-filtration plasmapheresis (DFPP). By the protocol-defined primary analysis of the former, the SVR rate has been 16% at most, even for a 72-week induction group [5].

The use of DFPP [approved in Japan in April 2008 for the retreatment of chronic hepatitis C (CHC) patients with genotype 1b and high viral loads] together with IFN administration has produced a substantial reduction in the viral load during the early stages of treatment and has effected a high SVR [6], suggesting that this treatment is a new modality for CHC patients in difficult-to-treat states. In this study, we used DFPP plus IFN to enhance the efficacy of the treatment of CHC patients whose HCV was not eradicated by earlier PEG-IFN/RBV combination therapy, and we assessed early viral dynamics associated with SVR.

Patients and Methods

Patients

Nine patients (aged 43–66 years) whose HCV had not been eradicated by earlier PEG-IFNα-2b plus RBV combination therapy carried out between 2008 and 2009 were enrolled in this study. The patients were divided into 2 groups: partial responders (PR; relapse after the end of therapy) and non-responders (NR; no disappearance of HCV RNA during therapy). All the patients were confirmed to be HCV RNA positive with high transaminase levels persisting for 6 months or longer, and with HCV RNA genotype 1b at levels exceeding 10^5 log IU/ml in blood (as determined before the start of therapy by real-time PCR). Also, the patients were negative for hepatitis B surface antigen. Patients with platelet counts of $\leq 10 \times 10^4/\mu l$, leukocyte counts of $\leq 3{,}000/\mu l$, or hemoglobin levels of ≤ 12 g/dl were excluded from the study.

Each patient gave written informed consent and agreed to receive concomitant DFPP, and the study was approved by the review board of the Kobe Asahi Hospital.

DFPP and Blood Collection

Blood collected from the peripheral vein for DFPP by a Plasmaflo™ OP-18W filter (Asahi Kasei Medical, Tokyo, Japan) was separated into plasma and cell components. The virus was then removed from the plasma by a second filter (Cascadeflo™ EC-50W; Asahi Kasei Medical) of an average pore size of 30 nm. For each session, the final volume of treated plasma was 50 ml/kg; the number of sessions was 5 over 2 weeks, and the time of DFPP, based on the reduced plasma fibrinogen levels during DFPP, was decided by the physicians and as required by the patients.

Types of IFN for 4 Weeks with DFPP

During DFPP, the patients were treated with different kinds of IFN: patient 1 with PEG-IFNα-2b plus RBV for 4 weeks; patients 2 and 3 with IFN-β 3 MU twice daily for 2 weeks and PEG-IFNα-2a plus RBV for 2 weeks; patients 4 and 9 with IFN-β 3 MU twice daily for 2 weeks and IFN-β 6 MU daily for 2 weeks; patient 5 with IFN-β 3 MU twice daily for 10 days and IFN-β 6 MU daily for 18 days, and patients 6, 7 and 8 with IFN-β 3 MU twice daily for 4 weeks. The dose of PEG-IFNα-2b was 1.5 μg/kg and 180 μg of α-2a per week. The RBV dose was 800 mg/day with α-2b and 600–800 mg/day with α-2a. After DFPP plus IFN treatment for 4 weeks, all patients were scheduled to receive PEG-IFN/RBV combination therapy (patient 1: PEG-IFNα-2b 1.5 μg/kg per week plus RBV 800 mg/day; patients 2–9: PEG-IFNα-2a 180 μg per week plus RBV 600–800 mg/day).

Amino Acid Substitutions in the Core Region (aa 30 and aa 91) and Number of IFN Sensitivity-Determining Region Mutations

We measured pre-treatment factors such as prediction of clinical outcome of therapy, amino acid sequence variation in the NS5A region (referred to as IFN sensitivity-determining regions) and in the core protein regions (aa 70 and aa 91) of HCV with a given genotype, and the viral load.

HCV RNA Measurement

The quantity of HCV RNA was measured by real-time PCR (detection limit 1.2 log IU/ml), by HCV core antigen (detection limit 20 fmol/l), and by RT-PCR (Amplicor HCV monitor v 2.0; Roche; detection limit 50 IU/ml).

Virus Removal at Second Filter Inlet and Outlet

Plasma was collected twice from the inlet and outlet of the second filter during 1 session of DFPP: once when the treated plasma volume reached half of the target quantity, and once when DFPP was completed. The change in the quantity of HCV RNA was evaluated through the plasma samples collected.

Viral Reduction and Viral Response Rate

The quantity of HCV RNA was converted to a log value at the beginning of the treatment (A) and at each of the virus measurement points (B). Δlog was then calculated: $\Delta\log = \log A - \log B = \log (A/B)$.

Evaluation of DFPP Safety

The subjective and objective adverse events of DFPP were observed, and five clinical factors were measured (platelet and lymphocyte counts, and hemoglobin, albumin and fibrinogen levels) before the first session of DFPP, before successive sessions on the second, third, fourth, fifth and sixth days, and 2 weeks after the last session.

Statistical Analysis

Statistical analysis consisted of analysis of variance for patient background factors, and the paired t test for quantities of HCV RNA at the second filter inlet during DFPP. The t test was used for viral load reductions and Fisher's exact test for viral response rates among the groups. The t test was 2-tailed, and differences of $p < 0.05$ were considered significant.

Table 1. Early viral dynamics with DFPP plus IFN treatment

Case	Age/ sex	Type of IFN for 4 weeks with DFPP	Viral dynamics after DFPP+IFN						Viral dynamics of previous treatment (PEG-IFN/RBV)				Viral mutation		
			before treatment	log drop			unit		before treatment	log drop	unit	outcome	aa 70	aa 91	ISDR
				24 h	1 wk	2 wks	4 wks			4 wks					
1	66/M	PEG-IFNα-2b/RBV 4 wks	6,510	0.5	0.6	0.6	1.1	fmol/l	452	0.7	KIU/ml	NR	wild	wild	0
2	65/F	IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks	7.5	0.4	1.3	2.6	1.0	log IU/ml	2,800	ND	KIU/ml	PR	wild	wild	0
3	52/F	IFN-β (3 MU 2/day) 2 wks → PEG-IFNα-2a/RBV 2 wks	5.8	0.4	1.0	1.6	+0.2	log IU/ml	6.3	0.2	log IU/ml	NR	wild	wild	1
4	47/F	IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks	6.8	0.6	0.3	0.4	0.4	log IU/ml	2,900	0.3	KIU/ml	NR	mutant	mutant	1
5	52/F	IFN-β (3 MU 2/day) 10 days → IFN-β (6 MU 1/day) 18 days	5.5	1.4	1.5	1.2	1.9	log IU/ml	782	0.6	fmol/l	NR	wild	mutant	1
6	61/F	IFN-β (3 MU 2/day) 4 wks	6.5	1.2	3.4	5.0	4.8	log IU/ml	8,450	2.6	fmol/l	NR	wild	wild	0
7	66/F	IFN-β (3 MU 2/day) 4 wks	5.3	0.0	0.8	1.2	1.3	log IU/ml	11,500	0.8	fmol/l	NR	mutant	wild	1
8	43/F	IFN-β (3 MU 2/day) 4 wks	3,460	0.5	0.2	1.3	2.2	fmol/l	745	0.1	fmol/l	NR	wild	mutant	1
9	43/M	IFN-β (3 MU 2/day) 2 wks → IFN-β (6 MU 1/day) 2 wks	7.2	0.6	1.4	2.5	2.9	log IU/ml	426	0.1	KIU/ml	NR	wild	wild	0

PEG-IFN/RBV: PEG-IFNα-2a (180 μg per week) plus RBV (600–800 mg/day) or PEG-IFNα-2b (1.5 μg/kg per week) plus RBV (800 mg/day). IFN-β: 3 MU twice daily or 6 MU daily. ND = Not done; aa = amino acid; ISDR = interferon sensitivity-determining region.

Results

Of the 9 patients, 1 was PR and 8 were NR. Virus mutation in the core region was as follows: wild type (7 patients) and mutant type (2 patients) at aa 70; wild type (6 patients) and mutant type (3 patients) at aa 91. IFN sensitivity-determining regions demonstrated mutation 1 (5 patients) and mutation 0 (4 patients), while mutation 2 was not seen in any patient. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks showed a reduction in the viral load of ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9) and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The early viral dynamics after DFPP plus consecutive intravenous IFN-β treatment for 4 weeks showed a reduction in the viral load of ≥1 log in 33% (2 of 6 patients), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. The reduction of the viral load by ≥2 log was observed in 50% (3 of 6 patients) at 4 weeks after the start of treatment (table 1).

Discussion

New drugs to replace IFN as well as drugs that can be used in combination with IFN are being actively developed. Also, attempts are being made to find ways to physically remove HCV particles from the blood. Granulocyte apheresis, plasma exchange and hemofiltration have been applied to HCV-infected patients for the treatment of cryoglobulinemia and vasculitis, modalities which have been shown to reduce HCV RNA in the blood during treatment [6–11]. The mechanisms of the clinical results of plasmapheresis have been described, whereby HCV in the blood is related to the effects of IFN therapy that could be enhanced by removing the virus from blood [12–14]. Low-density lipoprotein-cholesterol apheresis and plasma exchange in hypercholesteremic patients with HCV infection reduces the quantity of HCV RNA in the blood of some patients [15]. Hemodialysis, hemofiltration and peritoneal dialysis in chronic dialysis patients infected with HCV significantly lower HCV RNA levels in the blood [16]. Combined granulocyte apheresis with IFN therapy for CHC [17–19] and the prerequisite for early reduction of the virus in the treatment of CHC [20, 21] are essential. Thus, the potential effectiveness of IFN therapy combined with early physical removal of the virus is of particular interest.

Asahina et al. [22] studied HCV dynamics in both serum and peripheral blood mononuclear cells in 44 patients, with HCV genotype 1b and high viral loads, randomly assigned to 4 treatment groups: (1) combination therapy with 6 MU daily of IFNα-2b plus 800 mg of RBV; (2) monotherapy with 6 MU daily of IFNα-2b; (3) monotherapy with twice-daily intravenous administration of 3 MU of IFN-β, and (4) monotherapy with daily intravenous administration of 6 MU of IFN-β. HCV RNA levels measured serially by highly sensitive real-time PCR and HCV dynamics in both serum and peripheral blood mononuclear cells have demonstrated a 'biphasic' pattern. The exponential decay slopes of the second phase have been significantly higher in the combination or the twice-daily dose regimen groups than in group 2 or 4 (0.10 ± 0.08 vs. 0.02 ± 0.09 or 0.16 ± 0.09 vs. 0.02 ± 0.04 day^{-1}; $p < 0.05$ and $p < 0.0005$, respectively) [22]. Kim et al. [23] observed that a daily dose of IFN-β 6 MU for 4 weeks effects a 2 log decrease in the HCV RNA load in 7 patients with genotype 1b and high viral loads.

In this study, early viral dynamics were assessed in the 9 patients non-SVR to the combination therapy. The overall viral dynamics of DFPP plus IFN treatment with or without RBV for 4 weeks reduced the viral load by ≥1 log in 22% (2 of 9 patients), 55.6% (5/9), 77.8% (7/9), and 77.8% (7/9) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively. DFPP plus consecutive intravenous IFN-β treatment for 4 weeks reduced the viral load by ≥1 log in 33% (2/6), 50% (3/6), 83.3% (5/6) and 83.3% (5/6) at 24 h, 1, 2 and 4 weeks after the start of treatment, respectively.

The prerequisite for early virological response (EVR; indicating negative HCV RNA at 12 weeks) has been emphasized in predicting SVR and non-SVR in CHC patients undergoing IFN treatment; those who do not reach EVR fail to respond to further therapy. Treatment discontinued in patients not reaching EVR would reduce drug costs by more than 20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for CHC is highly desirable [24].

To be able to predict SVR with PEG-IFN/RBV treatment, reduction of the HCV RNA viral load by week 4 is considered essential. A 2 log reduction in the HCV RNA viral load by week 4 is a prerequisite to achieving SVR with PEG-IFN/RBV treatment [25]. In our study of DFPP plus consecutive intravenous IFN-β treatment for 4 weeks, a reduction in the viral load of ≥2 log was achieved in 50% (3 of 6 patients) at 4 weeks after the start of treatment.

From the above considerations, DFPP plus consecutive intravenous IFN-β treatment for 4 weeks is a promising regimen for non-SVR patients with genotype 1b and high viral loads, previously treated with PEG-IFN/RBV therapy. Further study is needed to elucidate the SVR rate in a larger number of patients given DFPP plus IFN treatment, especially with consecutive intravenous IFN-β.

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Disclosure Statement

No conflict of interest exists.

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Outcome and Early Viral Dynamics with Viral Mutation in PEG-IFN/RBV Therapy for Chronic Hepatitis in Patients with High Viral Loads of Serum HCV RNA Genotype 1b

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Key Words

Chronic hepatitis · Early viral dynamics · IFN/RBV resistance-determining region · HCV RNA genotype 1b · High viral load · PEG-IFN/RBV combination therapy · Virological response, prediction

Abstract

We investigated whether sustained virological response (SVR) and non-SVR by chronic hepatitis C patients to pegylated interferon plus ribavirin (PEG-IFN/RBV) combination therapy are distinguishable by viral factors such as the IFN/RBV resistance-determining region (IRRDR) and by on-treatment factors through new indices such as the rebound index (RI). The first RI (RI-1st; the viral load at week 1 divided by the viral load at 24 h) and the second RI (RI-2nd; the viral load at week 2 divided by the viral load at 24 h) were calculated. The subject patients were divided into 3 groups based on RI-1st and RI-2nd: an RI-A group (RI-1st $\leq$1.0), an RI-B group (RI-1st >1.0 and RI-2nd <0.7) and an RI-C group (RI-1st >1.0 and RI-2nd $\geq$0.7). The SVR rate was 71.4% (10/14) in the RI-A group, 46.2% (6/13) in the RI-B group and 20.0% (3/15) in the RI-C group (p = 0.005 between the RI-A group and the RI-C group). In IRRDR $\geq$6 and IRRDR $\leq$5 the SVR rate was 81.3% (13/16) and 23.1% (6/26) (p = 0.0002), respectively. By combining RI and IRRDR as a predicting factor, the SVR rate was 87.5% (7/8) in the RI-A group ($\geq$6 mutations in the IRRDR) and 7.7% (1/13) in the RI-C group ($\leq$5 IRRDR mutations) (p = 0.0003).

Introduction

Recently, global consensus has obtained that a combination of IFN or pegylated IFN plus ribavirin (PEG-IFN/RBV) is the treatment of choice for chronic hepatitis C (CHC). Notwithstanding this treatment regimen, sustained virological response (SVR) rates of those infected with the most resistant genotypes [hepatitis C virus (HCV)-1a and -1b] still hover at ~50% [1, 2]. It is therefore worthwhile to identify the predictive factors that allow the selection of patients who would achieve eradication

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of HCV RNA either before or during therapy, especially since IFN/RBV combination therapy is costly and has several side effects [3].

Predictors of the effectiveness of IFN-based therapy can be classified into pretreatment and on-treatment factors. Pretreatment factors comprise: (1) host factors such as age, gender, obesity, alcohol consumption, hepatic iron overload, fibrosis, immune responses and co-infection with other viruses, and (2) viral factors that mainly include viral genotypes and loads, particular amino acid sequence variations in the NS5A region [4, 5] and in the core protein region of HCV [6] within a given genotype. Moreover, the mean number of mutations in variable region 3 (V3) plus its upstream flanking region of NS5A [amino acid 2334–2379, referred to as IFN/RBV resistance-determining region (IRRDR)] is significantly higher in HCV isolates obtained from patients who later achieve SVR by PEG-IFN/RBV than in those from non-SVR patients. On-treatment factors are mainly related to viral kinetics within the first few weeks of treatment [7].

In the current study, with the aim of investigating whether SVR and non-SVR can be distinguished by viral factors such as IRRDR and by on-treatment factors through new indices such as the rebound index (RI), we calculated the first RI (RI-1st; the viral load at week 1 divided by the viral load at 24 h) and the second RI (RI-2nd; the viral load at week 2 divided by the viral load at 24 h), as proposed by Nomura et al. [8].

Patients and Methods

The 42 patients included in this study, who all demonstrated high viral loads (>100 KIU/ml) of serum HCV RNA of genotype 1b, had been diagnosed with CHC on the basis of abnormal serum alanine aminotransferase persisting for at least 6 months, and of positive HCV RNA assessed by RT-PCR. None of the patients was positive for hepatitis B surface antigen or other liver diseases (autoimmune hepatitis, alcoholic liver disease). All the patients received a regimen of PEG-IFNα-2b (peginterferon alpha-2b; PegIntron; Schering-Plough, Kenilworth, N.J., USA) (1.5 μg/kg/week, subcutaneously) in combination with RBV (ribavirin; Rebetol; Schering-Plough) 600–1,000 mg/day for 48 weeks. RBV was administered at a dose of 600 mg/day (3 capsules) to patients weighing <60 kg, 800 mg/day (4 capsules) to those weighing <80 kg and 1,000 mg/day (5 capsules) to those weighing ≥80 kg.

The efficacy of the combination therapy was evaluated by HCV RNA negativity determined by qualitative RT-PCR analysis at the end of therapy (end of therapy response) and 6 months after the completion of therapy (SVR). The amount of HCV RNA was also measured quantitatively by RT-PCR (Amplicor HCV monitor v. 2.0; Roche) before therapy. The lower detection limit of the assay was 5 KIU/ml. Samples collected during and after therapy were also determined by qualitative RT-PCR (Amplicor; Roche), which has a higher sensitivity than quantitative analysis, and the results were labeled as positive or negative. The lower limit of the assay was 50 IU/ml.

SVR was defined as undetectable serum HCV RNA at 24 weeks after the cessation of treatment, and non-SVR as detectable HCV RNA at 24 weeks after the discontinuation of treatment. Informed consent was obtained from all patients enrolled in the study after thoroughly explaining the aims, risks and benefits of the therapy.

The amount of HCV core antigen was assessed by the IRM assay (Ortho Clinical Diagnostics, Tokyo, Japan), which provides a good correlation between the amount of HCV core antigen and the amount of HCV RNA, as shown in our previous study [9]. The HCV core antigen was measured on days 0, 1 (24 h), 7 (1 week) and 14 (2 weeks) according to the detection limit of 20 fmol/l established by the manufacturer.

RI-1st was defined as the coefficient derived by dividing the viral load of HCV core antigen at week 1 by that at 24 h, and RI-2nd was defined as the coefficient derived by dividing the viral load at week 2 by that at 24 h [8].

The patients were divided into 3 groups based on RI-1st and RI-2nd: group A (RI-1st ≤1.0), group B (RI-1st >1.0 and RI-2nd <0.7) and group C (RI-1st >1.0 and RI-2nd ≥0.7).

NS5A sequence analysis (IRRDR) was performed as described [4]. Briefly, the sequences of the amplified fragments were determined by direct sequencing without subcloning with the use of a Big Dye Deoxy Terminator cycle sequencing kit and an ABI 337 DNA sequencer (Applied Biosystems, Japan). The aa sequences were deduced and aligned with Genetyx Win software v. 7.0 (Genetyx Corp., Tokyo, Japan). Numbering of aa throughout the manuscript is according to the polyprotein of HCV genotype 1b prototype HCV-J.

Statistical Analysis

Differences between the groups were assessed by the χ^2 test, Fisher's exact test or Student's t test, the Mann-Whitney test and the Kruskal-Wallis test. $p < 0.05$ was considered statistically significant.

Results

Of the 42 patients treated with combination therapy, 19 (45.2%) achieved SVR and 23 (54.8%) were still HCV RNA positive (non-SVR) 6 months after therapy. No significant differences were observed in patient characteristics between SVR and non-SVR, except in platelet counts and the degree of fibrosis (table 1), or among the RI-A, -B and -C groups (table 2).

The SVR rate was 71.4% (10/14), 46.2% (6/13) and 20.0% (3/15) in the RI-A, -B and -C groups, respectively, with a significant difference between the RI-A and -C groups ($p = 0.005$), but not significant between the RI-A and -B groups and the RI-B and -C groups (fig. 1). In the 14 patients of the RI-A group, HCV RNA turned negative

Table 1. Host-dependent, virus-related profile by response (SVR and non-SVR)

	SVR	Non-SVR	p value
Gender (M/F), n	11/8	13/10	NS
Age, years	56.7 ± 8.8	59.3 ± 10.5	NS
HCV RNA level, KIU/ml	1,685 ± 1,477	1,660 ± 1,363	NS
HCV core antigen, fmol/l	7,044 ± 6,763	9,343 ± 12,563	NS
Body weight, kg	59.9 ± 11.5	59.8 ± 13.6	NS
Treatment history (retreatment/naïve)	6/13	13/10	NS
Platelet count ($\times 10^4$/mm^3)	18.7 ± 4.4	14.8 ± 5.4	NS
F0, 1/F2, 3	12/2	5/10	0.004

Table 2. Host-dependent, virus-related profile by response (RI-A, -B and -C groups)

	RI-A	RI-B	RI-C	p value
Gender (M/F), n	7/7	9/4	8/7	NS
Age, years	60.0 ± 5.9	58.5 ± 9.4	56.1 ± 12.8	NS
HCV RNA level, KIU/ml	1,401 ± 1,014	2,053 ± 1,286	1,593 ± 1,772	NS
HCV core antigen, fmol/l	6,084 ± 5,106	7,674 ± 5,038	11,000 ± 15,837	NS
Body weight, kg	62.1 ± 16.6	59.5 ± 10.4	58.2 ± 10.1	NS
Treatment history (retreatment/naïve)	3/11	7/6	9/6	NS
Platelet count ($\times 10^4$/mm^3)	15.3 ± 3.5	18.3 ± 5.9	16.3 ± 6.0	NS
F0, 1/F2, 3	7/3	5/4	5/5	0.004

Table 3. SVR rate between IRRDR ≤5 and IRRDR ≤6 in RI-A, -B and -C groups

	RI-A		RI-B		RI-C	
	IRRDR ≤5	IRRDR ≥6	IRRDR ≤5	IRRDR ≥6	IRRDR ≤5	IRRDR ≥6
SVR	3	7	2	4	1	2
Non-SVR	3	1	5	2	12	0
SVR rate, %	50.0	87.5	28.6	66.7	7.7	100
p value	NS		NS		0.0024	
			0.0003			

by week 4 in 3 patients, week 8 in 5 patients, week 12 in 5 patients and was positive in 1 patient throughout the treatment. In the 13 patients of the RI-B group, HCV RNA was negative by week 4 in 1 patient, week 8 in 2 patients, week 12 in 4 patients, at and after week 16 in 5 patients and remained positive throughout the treatment in 1 patient. In the 15 patients of the RI-C group, HCV RNA was negative by week 12 in 1 patient, on and after week 16 in 6 patients and remained positive throughout the treatment in 8 patients (fig. 2).

The SVR rate was 81.3% (13/16) in the group with ≥6 mutations in IRRDR, and 23.1% (6/26) in those with ≤5 (fig. 3), with a significant difference between the 2 groups (p = 0.0002).

By combining RI and IRRDR, the SVR rate was 87.5% (7/8) in the RI-A group (IRRDR ≥6) and 7.7% (1/13) in the RI-C group (IRRDR ≤5) (table 3), with a significant difference between the 2 groups (p = 0.0003).

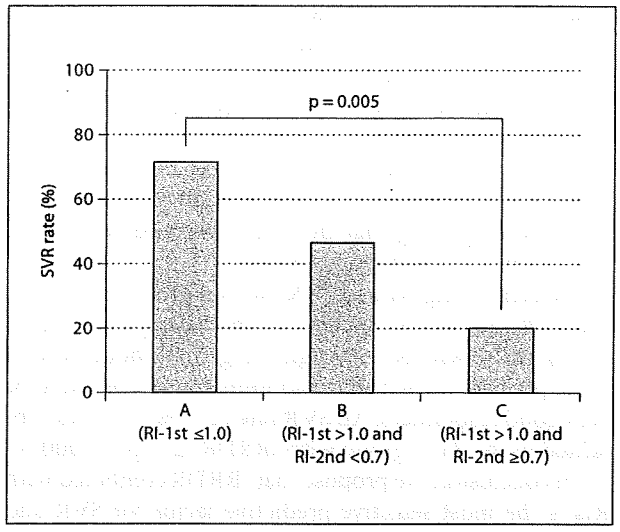

Fig. 1. SVR rate in RI-A, -B and -C groups. The overall SVR rate was 71.4, 46.2 and 20.0%, respectively. Significant difference in SVR rate is indicated.

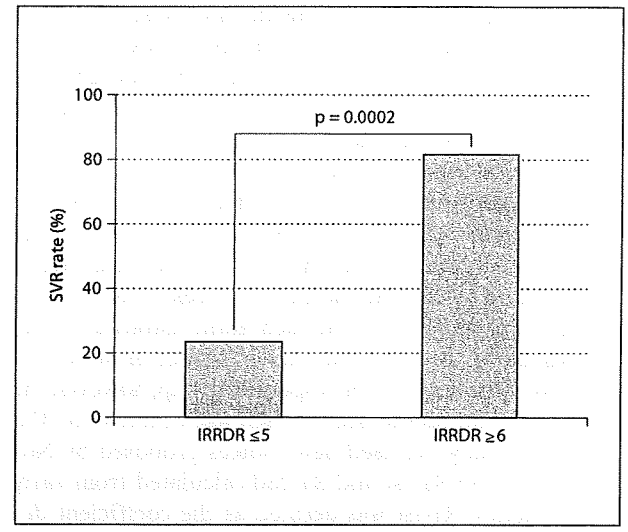

Fig. 3. SVR rate and IRRDR number. The SVR rate was 23.1% in IRRDR ≤5 and 81.3% in IRRDR ≥6, which was significantly different.

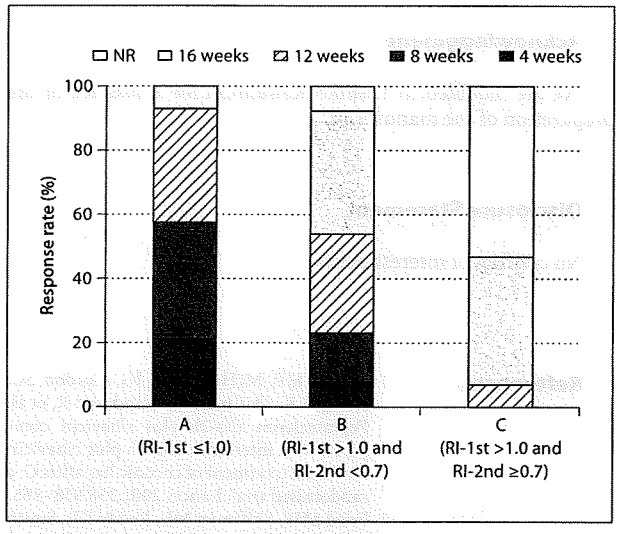

Fig. 2. Relation between response time and virus dynamics. In the 14 patients of the RI-A group, HCV RNA turned negative by week 4 in 3 patients, week 8 in 5 patients, week 12 in 5 patients and remained positive throughout the treatment in 1 patient. In the 13 patients of the RI-B group, HCV RNA was negative by week 4 in 1 patient, week 8 in 2 patients, week 12 in 4 patients, at and after week 16 in 5 patients and remained positive throughout the treatment in 1 patient. In the 15 patients of the RI-C group, HCV RNA was negative by week 12 in 1 patient, at and after week 16 in 6 patients and remained positive throughout the treatment in 8 patients.

Discussion

The importance of early virological response (EVR; signifying HCV RNA negative at 12 weeks) has been emphasized in predicting SVR and non-SVR in CHC patients undergoing IFN treatment; those not reaching EVR do not respond to further therapy. Discontinuation of treatment in patients not reaching EVR would reduce drug costs by more than 20%; consequently, early confirmation of viral reduction after initiating antiviral therapy for CHC is worth investigating [10].

Treatment with IFN induces a decline in HCV RNA levels that can be mathematically measured in 2 phases. The decline in the first phase, usually measured at 24 or 48 h, probably reflects direct inhibition of intracellular production and release of HCV [11], with IFN efficacy ranging from about 70% (approx. 0.7 log units) for standard IFN (given 3 times a week) to more than 90% (1 log unit) for high daily doses of standard IFN or PEG-IFN (given once a week) [12, 13]. The decline in the second phase, beginning after 24–48 h, is slower and more variable than that in the first phase, and is thought to reflect continued inhibition of replication and the gradual elimination of virus-infected cells [11]. The decay in the first phase has little correlation with the IFN dose, but is more rapid with PEG-IFN than with standard IFN preparations [10].