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# Serum KL-6 as a novel tumor marker for hepatocellular carcinoma in hepatitis C virus infected patients

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**Abstract**

The up-regulation of MUC1 protein is associated with malignant phenotype of cancer. We investigated the significance of KL-6, one of the MUC1 antigens, as a tumor marker in hepatitis C virus positive hepatocellular carcinoma (HCC). Serum KL-6 was determined in 203 patients with chronic hepatitis (CH), 47 patients with liver cirrhosis (LC) and 78 patients with HCC. KL-6 was higher in HCC compared to non-HCC ($p = 0.0005$) and was higher in patients with multiple HCC nodules compared to a single nodule ($p = 0.02$). There was no correlation between KL-6 and existent tumor markers for HCC such as alpha-fetoprotein, lens culinaris agglutinin-reactive alpha-fetoprotein or des-gamma-carboxyprothrombin. In the prospective analysis, the cumulative incidence of HCC was significantly greater in CH and LC patients with high initial KL-6 (above 400 U/ml) compared to the others ($p = 0.02$). Moreover, in the prospective observation of 25 patients whose HCC was completely cured by radiofrequency ablation therapy, the cumulative incidence of distant recurrences was significantly greater in patients with high initial KL-6 compared to the others ($p = 0.005$). These results suggest that serum KL-6 could be a novel tumor marker in the diagnosis and the prediction of prognosis of HCC that may have additive value to the existent markers.
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*Keywords:* KL-6; Hepatocellular carcinoma; Carcinogenesis; Tumor marker

## 1. Introduction

It has been demonstrated that the expression of MUC1 protein is increased in cancer of various organs such as stomach [1,2], thymus [3], colon [4–8], pancreas [9,10], lung [9,11], breast [9,12], lymphocyte [13] and liver [14]. The impact of the up-regulation of MUC1 is that it is specifically associated with malignant phenotype of cancer such as increased metastasis potential and poor prognosis [2,6–8,12,15–17]. Recently, genome wide profiling using cDNA micro array also depicted the independent prognostic value of MUC1 in papillary thyroid cancer [18].

KL-6 is one of the MUC1 antigens originally identified as a circulating pulmonary adenocarcinoma associated antigen [9]. It is thought to be released into serum upon cell damage [19], and already has been widely used as a marker for the activity of intestinal pneumonitis [20–23]. As well, the serum level of KL-6 is reported to be elevated in cancer of various organs [3,9,24,25]. A recent study in hepatocellular carcinoma suggested that serum level of KL-6 might represent an up-regulation of MUC1 in carcinoma tissue [25]. Thus, measurement of serum KL-6, which is less invasive and more convenient compared to the histological examination of MUC1, may have potential diagnostic and prognostic value in clinical practice.

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Hepatocellular carcinoma (HCC) is one of the major causes of death worldwide. HCC detected at early stage could be cured by percutaneous radiofrequency ablation therapy but a high rate of intrahepatic distant recurrence leads finally to a high mortality rate [26]. It is, therefore, essential to identify a serological tumor marker that is associated with the early diagnosis, the prediction of recurrence and the overall prognosis. Thus, the associations between existent tumor markers and prognosis of HCC have been studied vigorously [27–31].

In the present study, we investigated the potential significance of serum KL-6 as a tumor marker in hepatitis C virus positive HCC.

## 2. Patients and methods

### 2.1. Patients and materials

Serum was obtained from a total of 328 consecutive patients with chronic HCV infection who visited our hospital during September to November of 2003. Chronic HCV infection was diagnosed by the presence of HCV-RNA in serum, determined by the reverse transcription-polymerase chain reaction method. The presence of chronic liver disease was diagnosed on the basis of persistent elevation of ALT levels for more than 6 months and the histological or the radiological finding of chronic liver disease. The stage of hepatic fibrosis on liver biopsy was diagnosed according to the established international classification [32]. The diagnosis of HCC was radiologically made by multi-slice computer tomography with dynamic enhancement by the contrast-medium. Patients comprised of 203 chronic hepatitis patients (CH), 47 liver cirrhosis patients (LC) and 78 HCC patients.

### 2.2. Measurement of serum KL-6

Serum level of KL-6 was measured using a commercially available enzyme-linked immunosorbent assay kit (Eitest KL-6, Eisai Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions.

### 2.3. Associations of serum KL-6 levels with various clinical features

Serum KL-6 was analyzed in terms of the clinical status of liver disease (CH, LC or HCC), the fibrosis stage of the liver and various biochemical blood tests including well established hepatic fibrosis markers such as type III procollagen, type IV collagen and hyaluronic acid [33]. Correlation was also analyzed between serum KL-6 and the platelet count that is reported to decrease in accordance with the progression of hepatic fibrosis [34,35]. After the initial measurement of serum KL-6, patients with CH or LC were prospectively followed thereafter for median duration of 1 year to determine the relation between the initial level of KL-6 and the incidence of HCC.

In HCC patients, correlation of serum levels of KL-6 to the serum level of serological tumor markers such as alpha-fetoprotein (AFP), lens culinaris agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) or des-gamma-carboxyprothrombin (DCP) was analyzed. These tumor markers were regarded as positive according to the following criteria: above 100 ng/ml for AFP, above 15% for AFP-L3 and above 40 mAU/ml for DCP. The level of KL-6 was also compared in terms of diameter and number of HCC nodules to investigate the relation with clinical profile of HCC. In 25 patients whose HCC nodules were cured completely by radiofrequency ablation therapy, the relation between the level of initial KL-6 and the incidence of distant recurrences of HCC was analyzed through the prospective follow up for a median period of 1 year.

### 2.4. Statistical analysis

For statistical analysis the STAT View software package were used. Categorical data were analyzed using the Fisher's exact test. Continuous variables were compared with Student's $t$-test or Mann–Whitney's $U$-test. Spearman's rank correlation test was used to analyze a correlation between ordinal and continuous data. A Kaplan–Meier estimate and the log-rank test were used to calculate the median time and significance for the incidence of HCC. A $p$-value of less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. KL-6 and hepatic fibrosis in chronic hepatitis or liver cirrhosis

The mean serum level of KL-6 was $320 \pm 146$ U/ml in chronic hepatitis and $382 \pm 232$ U/ml in LC ($p = 0.02$) (Fig. 1). When CH and LC patients were categorized according to the fibrosis stage of the liver, the mean serum KL-6 level was $300 \pm 130$ U/ml for F1, $322 \pm 134$ U/ml for F2, $366 \pm 158$ U/ml for F3 and $382 \pm 229$ U/ml for F4. There was a correlation between the fibrosis stage and the serum KL-6 level when analyzed by Spearman's rank correlation test ($p = 0.02$) (Fig. 2). KL-6 was positively correlated with type III procollagen ($r = 0.528$, $p < 0.0001$), type IV collagen ($r = 0.319$, $p = 0.012$), hyaluronic acid ($r = 0.294$, $p = 0.023$) and negatively correlated with platelet counts ($r = -0.376$, $p = 0.002$) (Fig. 3).

### 3.2. Significance of KL-6 as a tumor marker in hepatocellular carcinoma

The mean serum level of KL-6 was $437 \pm 329$ U/ml in HCC patients which was higher compared to non-HCC patients ($p = 0.0005$) (Fig. 1). In addition, KL-6 was elevated disproportional to the fibrosis markers in HCC (type III procollagen ($r = 0.135$, $p = 0.265$), type IV collagen

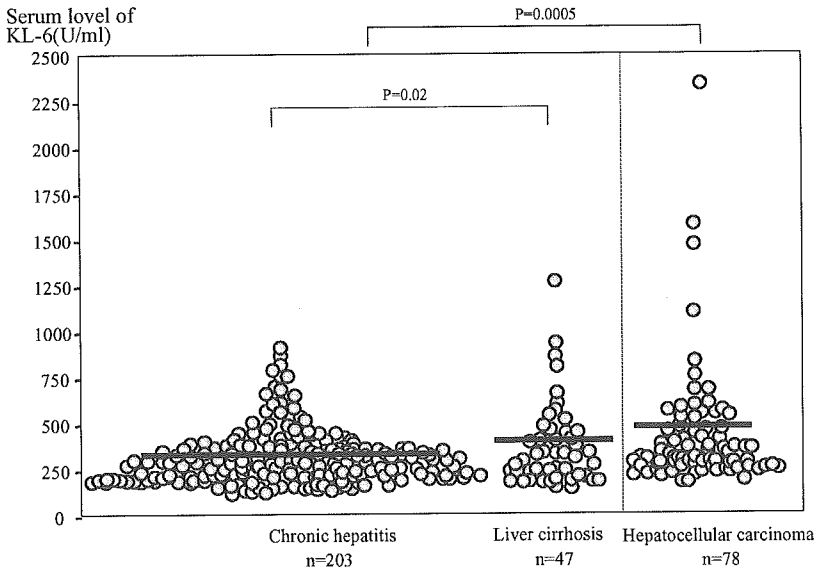


Fig. 1. The associations between serum levels of KL-6 and disease stages. The mean serum level of KL-6 was higher in liver cirrhosis patients compared to chronic hepatitis patients. Furthermore, it was higher in hepatocellular carcinoma patients compared to non-carcinoma patients.

($r=0.105$, $p=0.410$), hyaluronic acid ($r=0.198$, $p=0.091$) and platelet counts ($r=-0.169$, $p=0.151$), data not shown).

Serum level of KL-6 was not significantly correlated with the serum level of AFP, AFP-L3 or DCP (data not shown). There was no significant difference in the level of KL-6 between patients positive and negative for each of these tumor markers (Fig. 4). Out of 78 patients, 24 were positive for one marker, 14 were positive for two markers, 11 were positive for three markers and other 29 ware negative for all three markers. The sensitivity of diagnosing HCC was 63% with existent three markers.

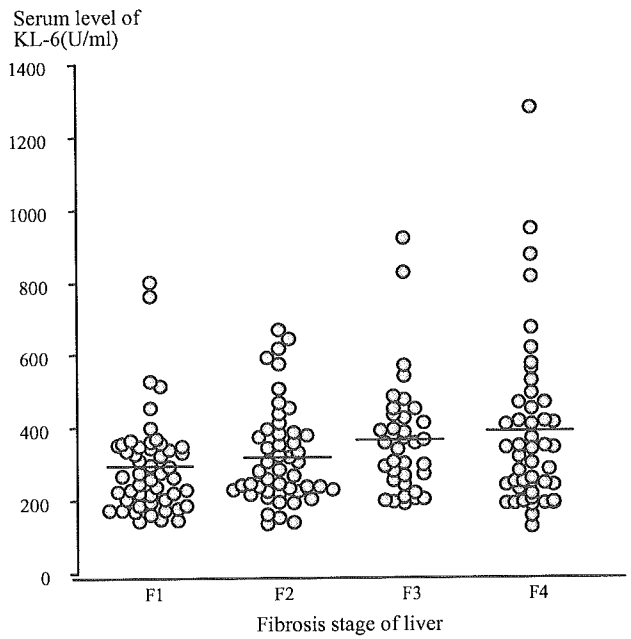


Fig. 2. The association between serum levels of KL-6 and fibrosis stages. The mean serum level of KL-6 increased in parallel with the progression of fibrosis stages in chronic hepatitis and liver cirrhosis patients.

The cut off value of KL-6 in the diagnosis of HCC was determined by receiver operating characteristic curve analysis and was set at 400 U/ml. When KL-6 level above 400 U/ml was regarded as positive, the sensitivity and the specificity in diagnosing HCC was 34 and 77%, respectively. The overall sensitivity of KL-6 was 33% in HCC patients who were positive for at lease one of three markers, and 38% in those negative for all markers, which did not differ significantly (Fig. 5), indicating that KL-6 is independent of other tumor markers. Thus, simultaneous measurement of KL-6 with other markers increased the sensitivity to 50% (in combination with AFP), 59% (in combination with AFP-L3) and 59% (in combination with DCP). The sensitivity of diagnosing HCC improved to 86% when KL-6 was tested in adjunct to existent three markers.

Out of 78 HCC patients, 31 had a single nodule and 47 had multiple nodules. In those with multiple HCC nodules, serum KL-6 was significantly high compared to those with a single HCC nodule ($p=0.02$) (Fig. 6a). In 31 patients with a single HCC nodule, the diameter of the nodule ranged from 5 to 50 mm. No correlation was observed between the size of the tumor and the level of KL-6 ($r=-0.163$, $p=0.39$) (Fig. 6b).

### *3.3. Prospective analysis of the development of HCC from CH and LC in terms of serum KL-6 levels*

Patients with CH or LC were prospectively followed for a median of 1 year to determine the relation between the initial serum level of KL-6 and the cumulative incidence of HCC thereafter. Among a total of 250 patients, HCC developed in 9 patients with the median observation period of 221 days from the initial measurement of KL-6 (range 99–396 days). Initial serum KL-6 level was above 400 U/ml in 58 patients and

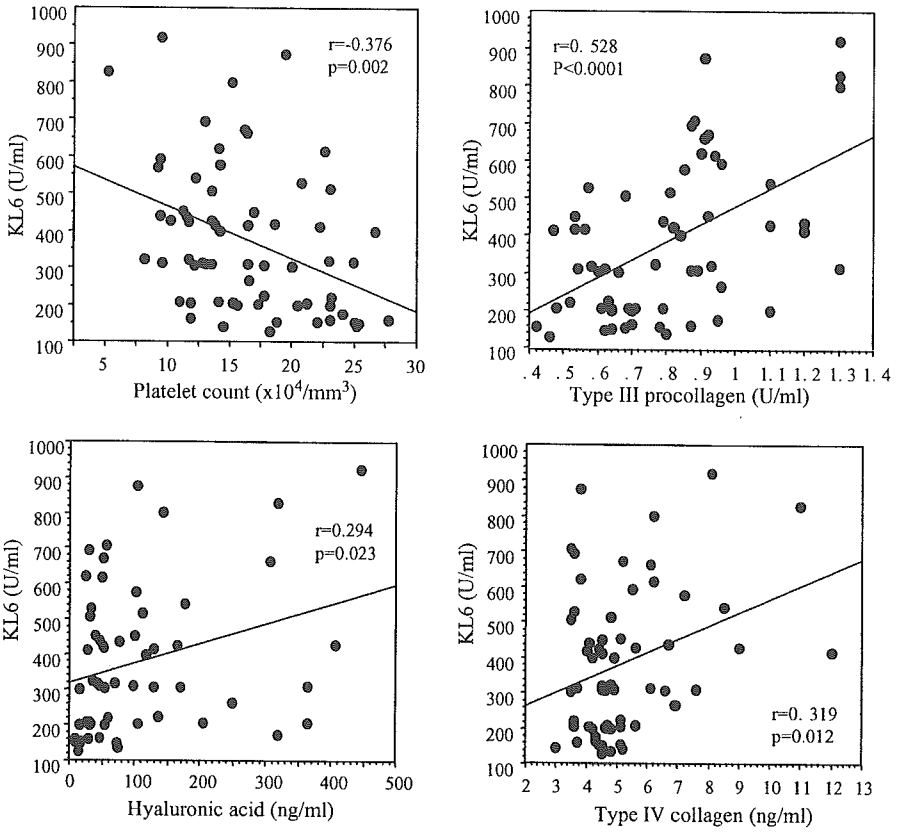


Fig. 3. Correlation between serum levels of KL-6 and fibrosis markers. Serum KL-6 positively correlated with type III procollagen, type IV collagen, hyaluronic acid and negatively correlated with platelet counts in chronic hepatitis and liver cirrhosis patients.

less than 400 U/ml in 192 patients. The cumulative incidence of HCC was greater in patients with initial KL-6 level above 400 U/ml compared to those less than 400 U/ml (6.9% versus 1.6% at 1 year, $p = 0.019$ by Log-rank test) (Fig. 7).

Among nine patients who developed HCC, two patients were in F3 stage of CH and seven were LC (F4), indicating that advanced fibrosis stage was a risk factor associated with the development of HCC, a well established recognition. In comparison of the clinical backgrounds, patients with elevated KL-6 levels were more likely to have LC compared to those without (31% versus 15%, $p = 0.029$). Thus, elevated level of KL-6 and advanced fibrosis stage are confounding variable in the development of HCC, making multivariate analysis inadequate. Thus, the cumulative incidence of HCC from patients with advanced fibrosis stages exclusively (F3 and F4) was analyzed which was greater in patients with ini-

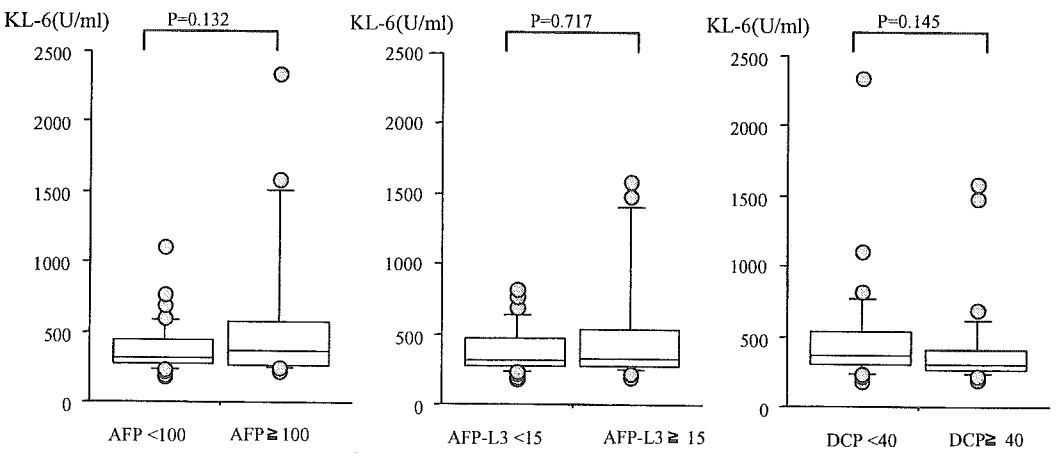


Fig. 4. The association between KL-6 and existent tumor markers. Serum level of KL-6 was compared between patients positive and negative for each of existent tumor markers AFP, AFP-L3 and DCP. These tumor markers were regarded as positive according to the following criteria: above 100 ng/ml for AFP, above 15% for AFP-L3 and above 40 mAU/ml for DCP.

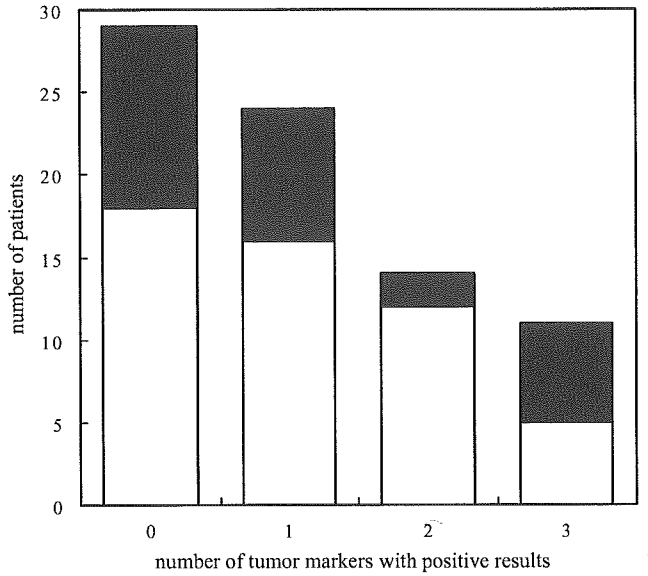


Fig. 5. Positive rate of serum KL-6 in relation to the number of positive tumor markers. Patients were categorized according to the number of positive tumor markers out of AFP, AFP-L3 and DCP (0–3). The white and back portion of the column indicates negative and positive result for KL-6, respectively. The positive rate of KL-6 in each category was 38% for 0, 33% for 1, 14% for 2 and 55% for 3. The overall positive rate was 33% in patients who were positive for at lease one of three markers (average of 1, 2 and 3) which did not differ significantly with those negative for all markers.

tial KL-6 level above 400 U/ml compared to those less than 400 U/ml ($p = 0.042$, Log-rank test).

### 3.4. *Prospective analysis of the distant recurrences of HCC from HCC patients whose HCC nodules were cured completely by radiofrequency ablation therapy*

HCC nodules were treated completely by radiofrequency ablation therapy in 25 patients after the initial measurement of serum of KL-6. These patients were prospectively followed to determine the relation between the initial serum level of KL-6 and the cumulative incidence of distant recurrence of HCC thereafter. Distant recurrence of HCC was observed in 16 patients with the median observation period of 200 days from the initial measurement of KL-6 (range 98–367 days). Initial serum KL-6 level was above 400 U/ml in 6 patients and less than 400 U/ml in 19 patients. The cumulative incidence of distant recurrences of HCC was significantly greater in patients with KL-6 above 400 U/ml compared to those less than 400 U/ml (100% versus 47% at 1 year, $p < 0.005$ by Log-rank test) (Fig. 8).

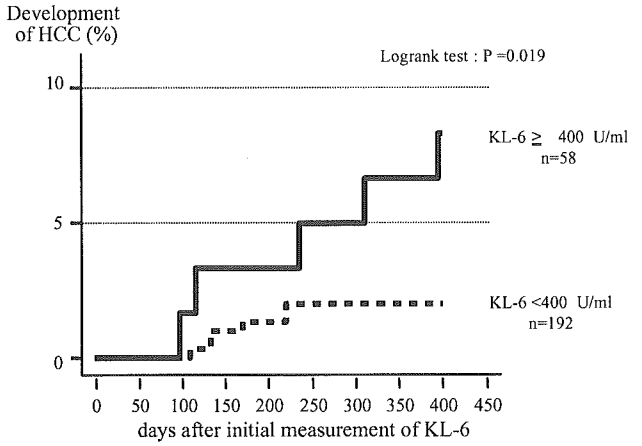


Fig. 7. Prospective analysis of the development of HCC from CH and LC. Patients with CH or LC were divided into two groups according to the initial serum level of KL-6 and the cumulative incidence of HCC was compared prospectively.

## 4. Discussion

In the present study, we found that: (1) serum KL-6 level was higher in HCC compared to CH and LC, (2) high serum

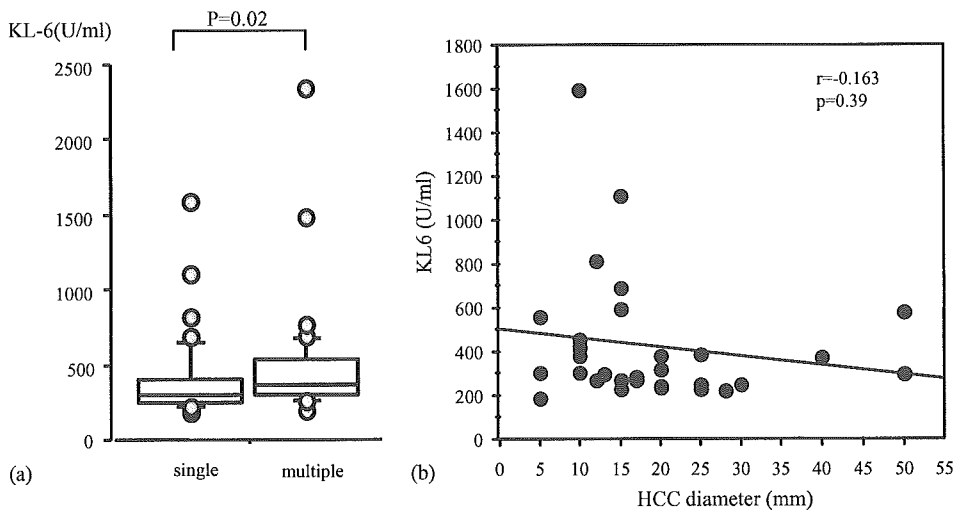


Fig. 6. The association between serum levels of KL-6 and the number and size of HCC nodules. Serum level of KL-6 was compared between patients with a single and multiple HCC nodules (a). In 31 patients with a single HCC nodule, diameter of HCC and the level of KL-6 was compared (b).

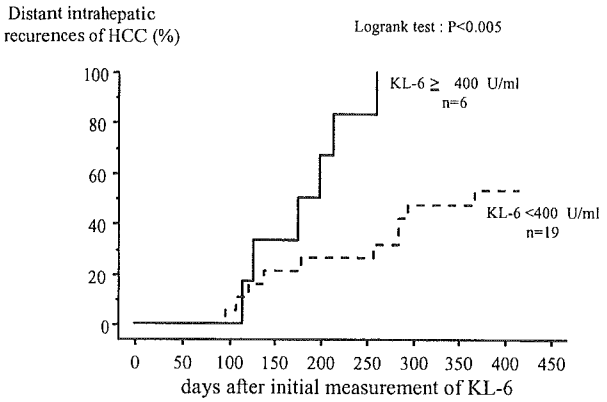


Fig. 8. Prospective analysis of the distant recurrences of HCC among patients treated with radiofrequency ablation therapy. Patients whose HCC nodules were cured by radiofrequency ablation therapy were divided into two groups according to the initial serum level of KL-6 and the cumulative incidence of distant recurrences of HCC was compared prospectively.

level of KL-6 in CH and LC was related to subsequent development of HCC, (3) elevation of KL-6 was independent of existent tumor markers and KL-6 level was high in 38% of patients who were negative for AFP, AFP-L3 and DCP, and (4) high serum level of KL-6 in HCC was related to multiple tumor nodules and subsequent development of intrahepatic recurrence after therapy. These findings suggest the potential diagnostic and prognostic value of serum KL-6 as a novel tumor marker in HCV related HCC.

In patients without HCC, serum KL-6 appeared to be associated with the progression of hepatic fibrosis since it was higher in LC compared to CH and correlated negatively with the platelet count that is reported to decrease in accordance with the progression of hepatic fibrosis. These findings are in accordance with previous two reports [24,36]. In addition we found a positive correlation of serum KL-6 to serum fibrosis markers such as type III procollagen, type IV collagen and hyaluronic acid. The correlation of KL-6 to serum fibrosis markers was an issue of controversy since one report found a correlation to hyarulonic acid [36] while the other found no correlation to hyaluronic acid or procollagen type III [24]. As KL-6 is characterized as a chemotactic factor for human fibroblasts [37], it is tempting to speculate that KL-6 may be related to hepatic fibrogenesis and disease progression. Apparently further study is necessary to confirm the association of KL-6 and hepatic fibrosis.

More importantly, serum KL-6 appeared to be a novel tumor marker with several additive values compared to existent tumor markers for HCV related HCC. Firstly, it may be a potential diagnostic marker since serum KL-6 was high in HCC compared to non-HCC patient. Of interest is that elevation of serum KL-6 was independent of serum level of existent tumor markers such as AFP, AFP-L3 or DCP, which is in agreement with a previous report [25], and high KL-6 was found in 38% of patients who were negative for existent tumor markers. Thus, measurement of KL-6 in combination with existent markers may reinforce the diagnostic power. In fact, sensitivity of diagnosing HCC, which was 63% when existent three markers were tested, improved to 86% when KL-6 was tested in adjunct to existent three markers.

Secondly, elevated serum KL-6 may be useful in identifying patients at high risk for the developing of HCC since CH and LC patients with high initial level of KL-6 had high cumulative incidence of HCC. Previously, Moriyama et al. reported that serum level of KL-6 was correlated to the degree of irregular regeneration of hepatocytes [24] which is a hallmark of a high carcinogenic state of the liver, relating to the development of HCC [38–40]. In this regard, measurement of serum KL-6 may reflect the carcinogenic state of the liver, and thus, the elevated level of KL-6 may be related to a high risk for the development of HCC.

There are several evidences that may explain the functional role of KL-6/MUC1 during the process of carcinogenesis. MUC1 is a member of *trans*-membrane mucins that functions as a signal transducer protein and regulate cell growth [41–43]. The cytoplasmic tail of MUC1 has been shown to be associated with the epidermal growth factor receptor (EGFR), ErbB2, ErbB3, ErbB4 receptor tyrosine kinases [44–47] as well as to Grb2, sos and c-src [48], which suggest that mitogen activated protein kinase (MAPK) signaling system can be triggered by MUC1. Moreover, MUC1 physically interacts with beta catenin [44–46,49,50], which is a key molecule of the Wnt signaling pathway. Beta catenin is normally bound to cadherins which regulate cell to cell adhesion, but binding of MUC1 to beta catenin results in the detachment of beta catenin from cadherin which lead to loss of adhesion and contact inhibition, promoting cellular dissociation and increased proliferation and oncogenic progression [45]. In addition, MUC1 bound beta catenin translocate to the nucleus, which initiate the transcription of growth stimulating genes [44,51]. These findings strongly suggest that KL-6/MUC1 has a functional role in carcinogenesis.

Finally, elevated serum KL-6 may be a prognostic marker. In the present study, high serum level of KL-6 in HCC was not only related to existence of multiple lesions, but also high cumulative incidence of intrahepatic recurrence after radiofrequency ablation therapy. Among 25 patients who were included in the analysis of intrahepatic recurrence, 15 had already repeatedly experienced multiple events of recurrences. Therefore, these patients are in high carcinogenic stage compared to patients with the initial HCC which may explain the high cumulative incidence of intrahepatic recurrence in the present study. The KL-6 level was not associated with the size of HCC, thus we speculate that KL-6 may not simply reflect the volume of the HCC but may be associated with specific biological phenotype of the tumor, possibly representing either highly carcinogenic status of the liver that lead to multicentric de novo carcinogenesis or high potential for intrahepatic distant metastasis.

Previous study has revealed that KL-6 was related to the clinical stage of HCC [25]. Since the clinical stage of HCC, the number of HCC nodules as well as frequent recurrences after therapy are known factors predictive of poor prognosis

[52], KL-6 may be consequently related to poor prognosis. Further follow up study of our cohort will depict the relation between initial KL-6 level and the overall survival period.

The association of KL-6/MUC1 and tumor progression as reflected by invasive growth and metastasis is reported in several cancers. In colorectal cancer, MUC1 and beta-catenin are co-expressed at the invasion front and associated with low grade of differentiation, accelerated course of disease and worse overall survival [7]. In pancreatic cancer, MUC1 is commonly expressed in high grade but not low-grade neoplasia and abundant in almost all conventional adenocarcinoma and associated with aggressive phenotype [10]. In breast cancer KL-6 is related to tumor stage, metastasis and relapse [12]. In papillary thyroid cancer, MUC1 is associated with aggressive course and poor prognosis [18]. These findings are in accordance with our findings in HCC.

In addition to these diagnostic and prognostic values, elevation of KL-6 in HCC may also have clinical implications for the development of novel therapeutic strategies. KL-6/MUC1 is studied as a target for immunotherapy for human adenocarcinomas from various sources, and several MUC1 targeted therapy have been tested in Phase I clinical trials [53,54]. Thus, KL-6/MUC1 could also be a target for therapeutic intervention of HCC in the future.

Our study has limitations as well. Patients with causes of liver injury other than HCV, such as HBV, alcohol, or non-B, non-C, were not included. Further study is necessary to clarify the association between KL-6 and HCC not related to HCV. Another limitation is that the MUC1 producing cell was not defined. MUC1 protein is expressed in various adult normal tissues such as lung, colon, stomach, pancreas and also in liver. However, it is not specified what kind of cells actually produce MUC1 in each tissue. As for the liver, previous paper depicted that HCC cells produce KL-6 [25], demonstrating that abnormally high level of KL-6 may be related to the excess production of MUC1 in HCC cells. It is tempting to speculate that the damaged hepatocytes with altered phenotype produce excess MUC1 which eventually progress to carcinoma. This is obviously beyond the scope of our clinical study but may be elucidated in the future basic investigation.

In conclusion, we found that serum KL-6 may be used as a novel tumor marker of HCV related HCC in adjunct to existent markers to improve the sensitivity of diagnosis, prediction of overall prognosis and identification of high-risk patients for developing HCC. Further study is warranted to delineate the significance of KL-6 in clinical management of HCC and for the future application to therapeutic interventions.

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HEPATOLOGY

# Mutations in the NS5B region of the hepatitis C virus genome correlate with clinical outcomes of interferon-alpha plus ribavirin combination therapy

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**Abstract**

***Background and Aim***: Combination treatments of interferon-alpha (IFN) and ribavirin (RBV) are more effective than those of IFN alone in hepatitis C virus (HCV) infection. However, mechanisms of the action of the combination regimen are not well understood. To elucidate the viral genetic basis of IFN plus RBV combination therapy, genetic variabilities of HCV-1b were analyzed.

***Methods***: We performed pair-wise comparisons of full-length HCV genomic sequences in three patients' sera before and after initiation of IFN plus RBV treatment. Subsequently, we analyzed amino acid sequences of the NS5B region, which codes for the viral RNA-dependent RNA polymerase, and compared these with the outcomes of the therapy in 81 patients.

***Results***: Analysis of the entire HCV sequence in patients who received IFN plus RBV therapy did not show consistent amino acid changes between before and after the initiation of the therapy. NS5B sequence analyses revealed that mutations at positions 300–358 of NS5B, including polymerase motif B to E, occurred more frequently in a group of patients exhibiting a sustained viral response (SVR) or an end-of-treatment response (ETR) compared with a group of patients exhibiting a non-response (NR). Closer examination revealed that mutations at aa 309, 333, 338 and 355 of NS5B occurred significantly more frequently in the SVR plus ETR group than in the NR group ($P = 0.0004$). Multivariate analysis showed that the number of mutations at these four sites was an independent predictor of SVR plus ETR *versus* NR.

***Conclusions***: Particular amino acid changes in the NS5B region of HCV may correlate with outcomes of IFN plus RBV combination therapy.

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**Key words**: amino acid sequence, error catastrophe, RNA-dependent RNA polymerase, transition.

## INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, which can lead to liver cirrhosis and hepatocellular malignancy.[1,2] Interferon (IFN) is the agent of choice for treating HCV infection. However, IFN monotherapy produces sustained virological responses in only 15–20% of patients treated, most of whom relapse after completion of the therapy.[3,4] Several recent studies of combination therapy with IFN alpha 2b and ribavirin (RBV) have shown that the regimen induces higher sustained virological responses than IFN monotherapy. Unfortunately, 50–60% of patients still do not respond to the combination therapy.[5–8]

RBV is a synthetic guanosine analog with broad antiviral actions *in vitro* against various DNA and RNA

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viruses.[9,10] At present, four mechanisms of action have been postulated: (i) immune modulatory effects by a switching of T-cell phenotype from Th2 to Th1 that induces type 1 cytokine responses;[11–13] (ii) inhibition of inosine monophosphate dehydrogenase (IMPDH) leading to depletion of cellular GMP pool;[14] (iii) mutagenic activity against RNA viruses that induces misincorporation of RBV triphosphate into viral RNA leading to error prone replication of viral genome;[15–18] and (iv) inhibition of the activity of HCV NS5B RNA-dependent RNA polymerase (RdRp).[19,20] However, it has not been fully understood which mechanisms of actions of RBV are effective against HCV infection.

Certain genetic structures of viruses may affect the sensitivity to their therapeutic drugs. Nucleoside analogs are widely used against viruses such as human immunodeficiency virus type 1 (HIV) and hepatitis B virus (HBV).[21,22] The antiviral effect of those reagents arises from the inhibition of viral DNA/RNA polymerase activity. However, single or multiple mutation(s) in the viral polymerase confer drug resistance and help the drug resistant strains emerge.[22–30] Also in HCV infection, the INF sensitivity determining region (ISDR) of HCV genome, which we have previously identified, critically determines the virological response to IFN and the treatment outcomes.[31,32] As to RBV, one study of five HCV genotype 1a patients who had undergone RBV monotherapy has reported one mutation in NS5B that may correlate with RBV sensitivity.[33] These findings make us speculate that genetic variability of HCV NS5B region, which codes for RdRp, may correlate with sensitivity to RBV and may influence the outcomes of IFN plus RBV combination therapy.

In the present study, we first analyzed effects of RBV on HCV genomic structure and the viral genetic basis of RBV resistance by performing pair-wise comparisons of full-length HCV genomic sequences in patient sera before and after initiation of IFN plus RBV treatment. Subsequently, we have investigated a hypothesis that genomic variability of HCV RdRp may confer resistance or susceptibility to RBV and may correlate with the outcomes of IFN plus RBV combination therapy. Thus, we analyzed amino acid sequences of the NS5B region and the outcomes of IFN plus RBV combination therapy in 81 patients, and found that certain amino acid variations in the NS5B region may associate with the treatment outcomes.

## METHODS

### Patients of interferon plus ribavirin non-responders

Three patients infected with HCV, genotype 1b, were studied. All patients were non-responders to combination therapy with IFN alfa-2b (Intron A, Schering Plough, Kenilworth, NJ, USA), 6 million units three times per week plus RBV (Rebetoron, Schering Plough), 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. Serum samples were obtained before treatment and at 12 weeks after initiation of the treatment, and pair-wise comparisons of the consensus sequences of full-length HCV genomes were performed. As controls for the IFN plus RBV therapy data, we analyzed our previously published HCV sequence data for three non-responders of IFN monotherapy[32] (deposited with the DDBJ/GenBank/EMBL data libraries under accession number D50483, D50480, D50485, D50481, D50484 and D50482).

### RNA extraction, reverse transcription-polymerase chain reaction and direct sequencing

RNA was extracted from patient sera by the modified acid guanidinium thiocyanate-phenol-chloroform (AGPC) method,[34] using ISOGEN reagent (Wako Pure Chemical Industries, Osaka, Japan), and reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described.[32] Full-length HCV genomes were amplified by nested PCR with 21 partially overlapping sets of primers, as previously reported.[32] M13-forward and M13-reverse sequencing primer sequences were attached to the 5′-termini of sense and antisense nested PCR primers. Each PCR product was purified by a spin filtration column (Suprec-02; Takara). Both strands of the PCR products were cycle sequenced with the PRISM dye termination kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions, and consensus nucleotide sequences were determined by an automated DNA sequencer model 373 A (Applied Biosystems).

### Sequence analyses

Nucleotide sequencing analysis was performed with a software program (MEGA version 2.1) to calculate values for $d_N$ (non-synonymous substitution), $d_S$ (synonymous substitution), $d_N/d_S$ ratios, and the number of point mutations.

### Clinical outcome of combination therapy

Patients were placed into one of three outcome groups.

- Sustained virologic response (SVR): HCV-RNA was not detectable by RT-PCR for 6 months following completion of the therapy.
- End-of-treatment response (ETR): HCV-RNA was not detected at the end of the treatment, but reappeared within 6 months thereafter.
- Non-response (NR): HCV-RNA did not disappear during the treatment.

### Nucleoside sequencing analyses of the NS5b region

Amino acid mutations in the conserved motifs (motif A, B, C, D, E, F)[35–38] in NS5B RdRp were retrospectively analyzed in 81 HCV genotype 1b patients who were

treated with IFN alfa-2b, 6 million units three times per week plus RBV, 800 mg/day (> 12.1 mg/kgBW) for 24 weeks. All patients had biopsy-proven chronic hepatitis with positive serum HCV antibodies and serum HCV-RNA. RNA was extracted from sera of the patients before treatment. NS5B region, including motifs A to F, was amplified by RT-PCR and sequences corresponding to nucleotides 7730–8874 of HCV-J were determined.[32] The deduced amino acid sequences of all patients were aligned and compared with consensus sequences for mutations and analyzed for correlation between amino acid mutations of NS5B and the clinical outcome of the combination therapy.

### Statistical analyses

Comparisons of differences in categorical data between groups were performed using the $\chi^2$ test and Fishers exact test. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test for two groups and by the Kruskal–Wallis test or Scheffé method for three groups. Multivariate analysis was carried out by multiple logistic regression analysis. *P*-values of less than 0.05 were defined as statistically significant.

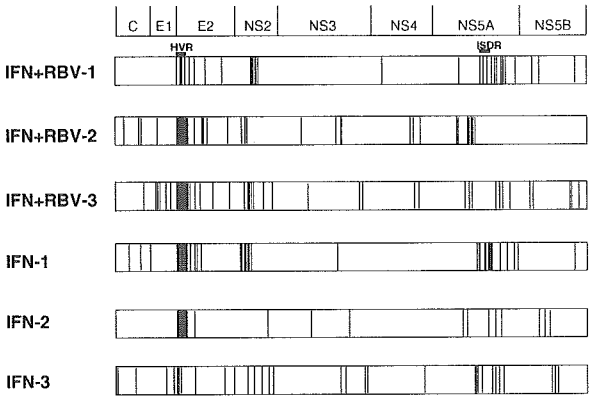


**Figure 1** Schematic representation of the distribution of mutations in amino acid residues during the combination therapy and interferon (IFN) monotherapy. Distributions of amino acid changes in the entire hepatitis C virus (HCV) genome in patient serum before treatment and 12 weeks after initiation of treatment are shown. The upper three data are from patients treated with IFN/ribavirin (RBV) combination therapy (IFN + RBV 1–3), and the lower three data are those treated with IFN monotherapy (IFN 1–3). Vertical lines in each HCV polyproteins show position of amino acid differences during the therapy.

## RESULTS

### Pair-wise comparisons of the full-length HCV genome in three patients before and after initiation of IFN/RBV treatment

HCV genomes from the three study patients comprised 9423 nucleotides and contained an open reading frame of 3010 amino acids. In patient one, 31 amino acid changes were found in the HCV genome. These amino acid changes were clustered in the E2-hypervariable regions (8 of 31) and the NS5A regions (11 of 31). Before treatment, the INF-sensitivity determining lesion (ISDR)[31,32] were 'mutant' type with five amino acid changes compared with consensus sequence, which changed to 'intermediate' type with two amino acid changes after the initiation of treatment. In patient two, 37 amino acid changes were found in the entire HCV genome. The changes were exclusively found in the E2-hypervariable region (16 out of 37 amino acids), while there was no change in the ISDR. In patient three, 56 amino acid changes were found. The changes were exclusively found in the E2 region (24 out of 56 amino acids). Distribution of amino acid changes during the therapy in the three patients treated with combination therapy and three non-responders to IFN monotherapy are illustrated in Figure 1. The numbers of nucleotide changes for the three study patients were 88, 130 and 272, respectively. The $d_N/d_S$ ratios were 0.195, 0.148 and 0.099, respectively. Among the three control subjects who received IFN monotherapy, the numbers of nucleotide changes were 138, 160 and 175, respectively. The $d_N/d_S$ ratios were 0.158, 0.061 and 0.089, respectively. As shown in Figure 2, $d_N/d_S$ ratios tended to be higher in the E2 region than in the other regions during

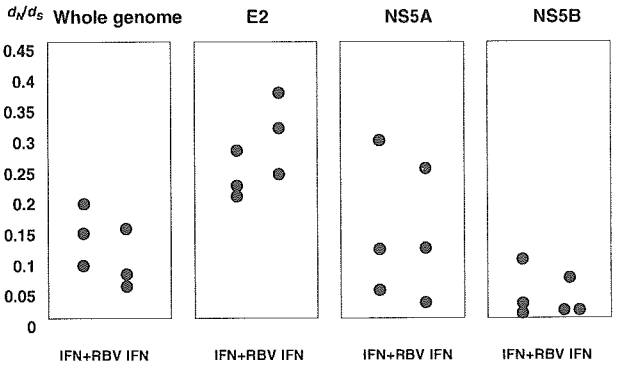


**Figure 2** Ratio of non-synonymous to synonymous distances for the E2, NS5A, NS5B and whole hepatitis C virus (HCV) genome. The $d_N/d_S$ ratio in E2 region tended to be higher than other regions during interferon (IFN) monotherapy and during combination therapy. All pairwise $d_N/d_S$ ratios were calculated using MEGA version 2.1 for each subject.

both IFN monotherapy and combination therapy. The numbers of transitional mutations in patients who received the combination therapy had 71 (80.2% of total mutations), 104 (80.0%) and 218 (80.1%) transitional mutations, respectively, and in patients who received IFN monotherapy these were 108 (78.3%), 131 (81.9%) and 130 (75.4%), respectively. The proportion of transitions among IFN monotherapy patients did not differ from the proportion among combination therapy patients.

Two studies have observed two key transitions, C-to-U and G-to-A, in genomic sequences of RBV-treated RNA viruses.[17,18] In the present study, C-to-U and G-to-A mutations comprised 35.5%, 40.6% and 58% of

total mutations, respectively, in the three patients treated with IFN monotherapy, and 43.2%, 38.3% and 37.8%, respectively, in those treated with combination therapy. These results showed no obvious increase in key mutations of C-to-U and G-to-A associated with the combination therapy (Table 1).

## Sequence analyses of NS5b region in 81 patients treated with IFN and RBV therapy

To study the correlation between the genetic structures of NS5B and the outcome of IFN plus RBV combination therapy, amino acid sequences of HCV NS5B (aa. 61–407), including motif A-F, were analyzed in 81 patients treated with IFN plus RBV combination therapy. The clinical characteristics of the patients are shown in Table 2. Nineteen (23.5%) patients were SVR, 40 (49.4%) were ETR, and 22 (27.2%) were NR. Clinical variables were analyzed according to the results of the combination therapy. Univariate analysis identified fibrosis stage as significantly lower in the SVR patients than in the other patients. No other clinical parameters were significantly correlated with the responses.

The amino acid sequences of the essential motif B to E of NS5B in these 81 patients are aligned with consensus sequences in Figure 3. Comparison of the NS5B sequences between patients with SVR and patients with non-SVR (ETR and NR) showed no obvious differences. Instead, when we compared the sequences of a patient group of SVR plus ETR with those of patients with NR, the mutations at position NS5B 300–358, including motif B to E between, were more frequent in the SVR plus ETR group than in the NR group. When we analyzed mutations of individual amino acid positions, the frequencies of mutations at aa 309, 333, 338 and 355 of NS5B (the four sites) were found to be more frequent in patients with SVR or ETR than those with NR (Fig. 4). The total number of amino acid changes at these four sites was significantly higher in patients with SVR or ETR than those with NR ($0.93 \pm 0.89$ *vs* $0.27 \pm 0.70$, $P = 0.0004$). In 19 SVR patients, five patients had no mutations, 10 patients had one mutation, and four patients had two or more mutations at the four sites. In the 40 ETR patients, 18 patients had no mutations, 13 patients had one mutation, and nine patients had two or more mutations at the four sites. In 22 NR patients, 19 patients had no mutations, two patients had one mutation, and one patient had three mutations at the four sites (Fig. 5a). The SVR rates were 11.9% (5 of 42) and 35.9% (14 of 39) in patients who had none and one or more mutations at the four sites, respectively (Fig. 5b). Patients with increased mutations at the four sites tended to be in the SVR or ETR groups. We subsequently analyzed various clinical factors by multivariate analysis among the three response groups to determine the independent predictors for SVR and NR (Table 3). Among these clinical factors, the NS5B mutation described above was independently associated with NR ($P = 0.0185$).

Mutations of the NS5B region, which codes for the viral RdRp, may alter its enzymatic activities which may influence serum virus load of each patient. In our results, however, there was no obvious correlation between the number of NS5B mutations and serum viral loads in each patient, nor was there a difference in the serum virus loads between the patient groups categorized by the numbers of mutations at aa 309, 333, 338 and 355 of NS5B.

**Table 1** Sequence analysis of full genome of hepatitis C virus (HCV) RNA treated with interferon (IFN) plus ribavirin

| | G-to-A and C-to-U | Other transition (A-to-G and U-to-C) |
|---|---|---|
| IFN plus ribavirin | 58.3 | 72.5 |
| No ribavirin (IFN monotherapy) | 60.8 | 70.4 |

Mutations per 10 000 nucleotides. A total of 56 538 nucleotides were sequenced.

## DISCUSSION

In the present study, we have demonstrated that particular amino acid changes in the NS5B region of HCV

**Table 2** Baseline characteristics of the group of 81 patients, segregated according to the clinical outcome of interferon (IFN) plus ribavirin combination therapy

| | SVR | ETR | NR | *P*-value |
|---|---|---|---|---|
| Number of patients | 19 | 40 | 22 | |
| Age (years) | 49.5 ± 12.2 | 55.9 ± 8.1 | 57.2 ± 10.6 | NS |
| Sex (male/female) | 15/4 | 27/13 | 11/11 | NS |
| Baseline ALT (IU/L) | 122.2 ± 88.0 | 80.2 ± 43.2 | 107.4 ± 73.7 | NS |
| Platelet count ($10^3$/mm$^3$) | 16.0 ± 5.5 | 16.3 ± 5.5 | 14.7 ± 4.5 | NS |
| Fibrosis stage (SD) | 1.41 ± 0.71 | 1.92 ± 0.94 | 2.10 ± 0.72 | 0.012† |
| Serum HCV RNA at baseline (KIU/mL) | 480.5 ± 295.7 | 594.6 ± 239.3 | 599.9 ± 271.3 | NS |
| Number of ISDR mutations | 1.73 ± 2.92 | 0.80 ± 1.22 | 1.00 ± 1.80 | NS |

†Significant differences between SVR and others. Values are expressed as mean ± SD, except where noted. ALT, alanine aminotransferase; ETR, end-of-treatment responder; NR,-non-responder; NS, not significant; SR, sustained responder.

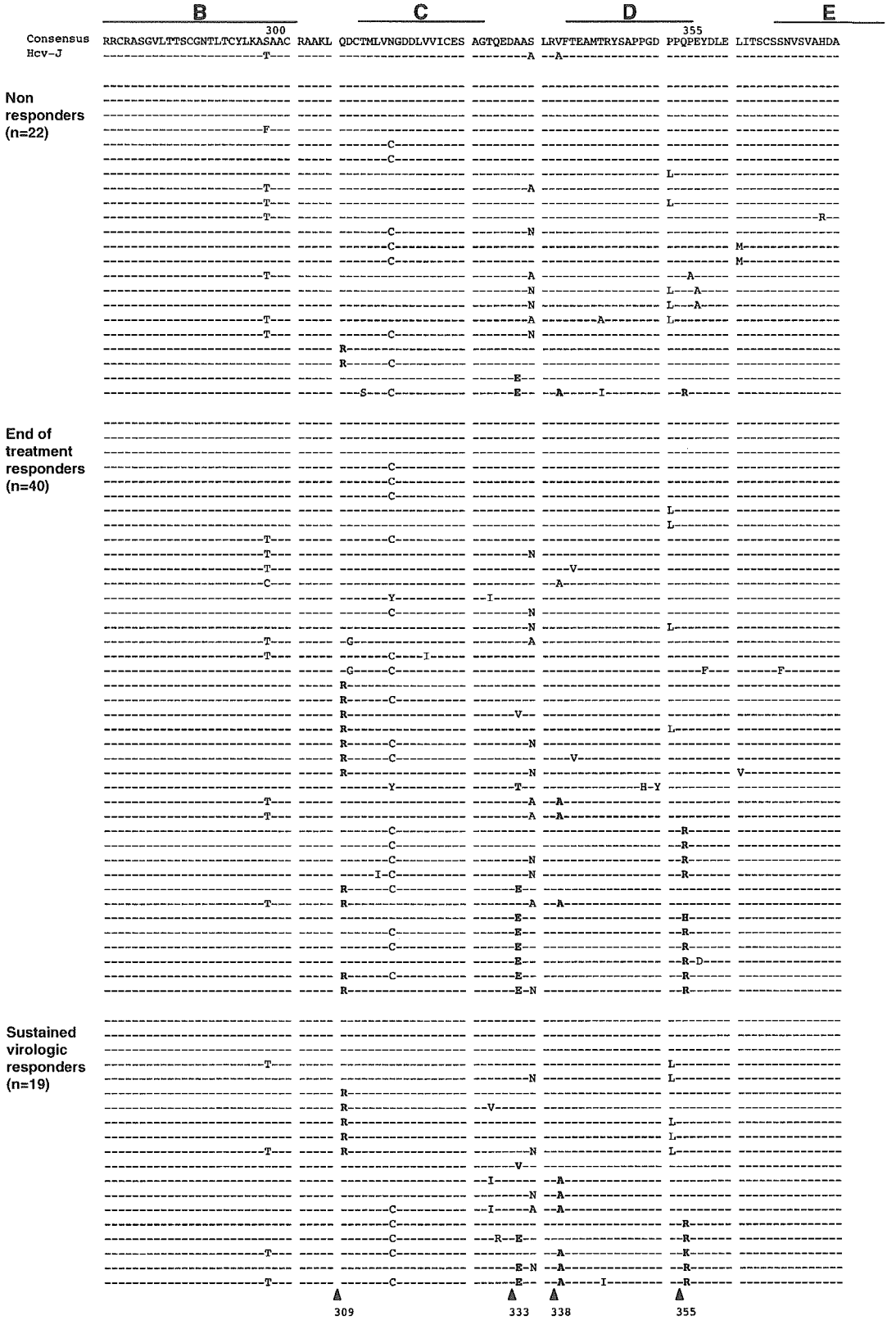


**Figure 3** Amino acid sequence alignments of the NS5B motif B-E in 81 patients with chronic hepatitis C virus (HCV)-1b infection and treated with interferon (IFN) plus ribavirin for 24 weeks. Amino acid residues are indicated by the standard single-letter codes, and dashes indicate the identical amino acid residues with consensus sequence and HCV-J shown at the top. At four amino acid positions (NS5B 309, 333, 338 and 355), usages of amino acid residues differ between non-responders (NR) and others. Changes of this part are indicated by bold letter. Outcome of combination therapy is shown on the left side.

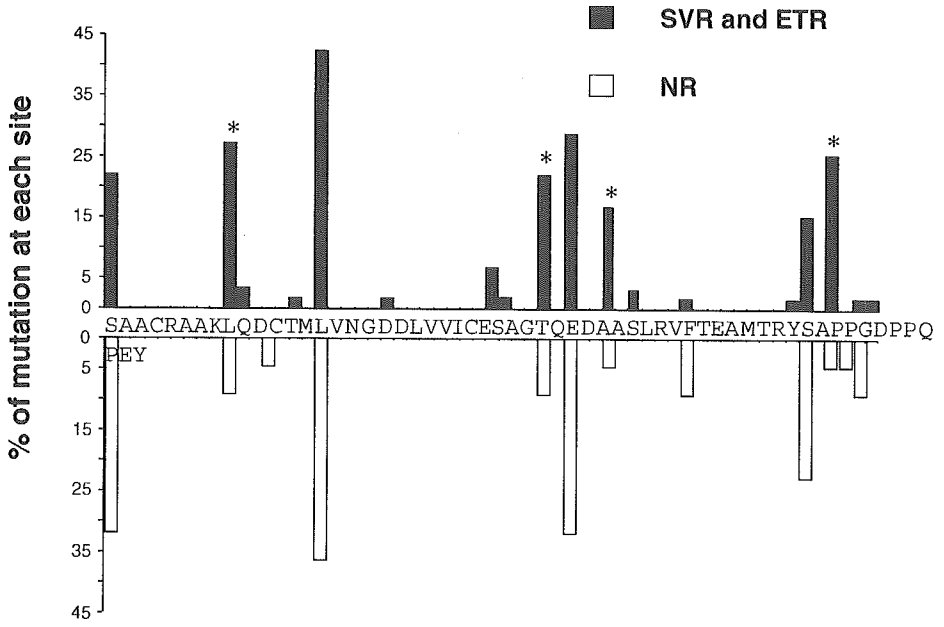


**Figure 4** Relationship between frequency of mutations at each site in NS5B 300–358 and the efficacy of interferon (IFN) plus ribavirin treatment. Amino acid residues are indicated by the standard single-letter codes. Among these 59 sites, mutations of aa NS5B 309, 333, 338 and 355 (identified by *) are frequent in sustained virologic response (SVR) and end-of-treatment response (ETR) patients. NR, non-response.

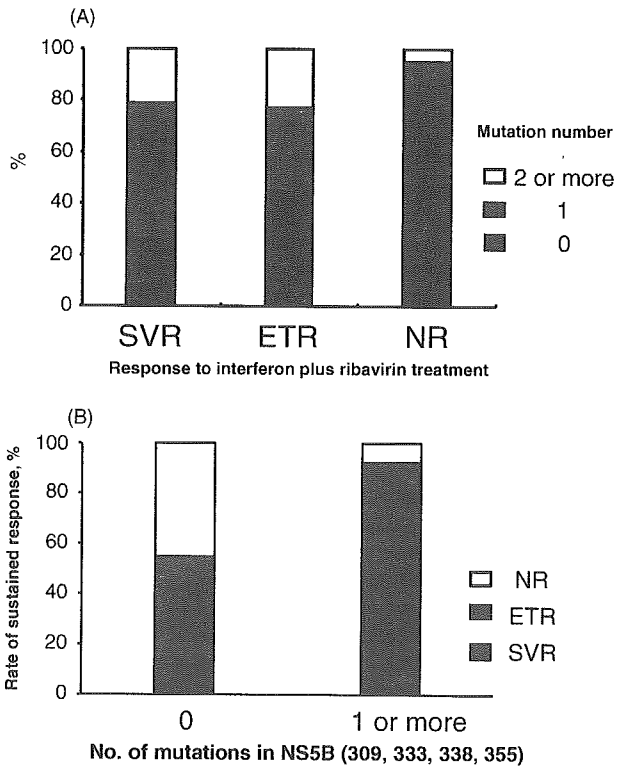


**Figure 5** Relationship between number of mutations in NS5B 309, 333, 338, 355 and the outcome of interferon (IFN) plus ribavirin treatment. (a) Distribution of total numbers of mutations at aa. 309, 333, 338 and 355 of NS5B according to sustained virologic response (SVR), end-of-treatment response (ETR) and non-response (NR) patients. (b) Proportion of SVR, ETR and NR patients between groups with or without mutations at aa. 309, 333, 338 and 355 of NS5B.

**Table 3** Multivariate analysis for the clinical and virological factors affecting virological responses (SVR and NR) to interferon (IFN) plus ribavirin combination therapy in the group of 81 patients

| | Patient with SVR *P*-value | Patient with NR *P*-value |
|---|---|---|
| Age (years) | 0.572 | 0.598 |
| Sex (male/female) | 0.814 | 0.158 |
| Baseline ALT (IU/L) | 0.022 | 0.981 |
| Platelet count ($10^3$/mm$^3$) | 0.749 | 0.627 |
| Mean fibrosis stage (SD) | 0.037 | 0.330 |
| Serum HCV RNA at baseline | 0.227 | 0.890 |
| No. of ISDR mutations | 0.491 | 0.754 |
| No. of NS5B mutations (309,333,338,355) | 0.057 | 0.019 |

ALT, alanine aminotransferase; ETR, end-of-treatment response; ISDR, interferon sensitivity determining region; NR, non-response; SR, sustained response.

correlate with the clinical outcome of combination therapy. Pair-wise comparisons of the full-length HCV genome in three patient sera obtained before and 12 weeks after the start of IFN plus RBV therapy did not show consistent amino acid changes. The results suggest negative evidence against the presence of treatment-resistant viral sub-populations. On the contrary, subsequent analyses of mutation patterns in the NS5B region in 81 patients showed a significant correlation between particular amino acid mutations of NS5B and the outcome of the combination therapy. Mutations of aa. 309, 333, 338 and 355 of the NS5B were significantly more frequent in SVR and ETR patients, in which the virus has been persistently or at least tempo-

rarily eliminated. Total numbers of mutations at the four amino acid positions were significantly more in SVR and ETR patients compared to NR patients ($0.93 \pm 0.89$ *vs* $0.27 \pm 0.70$; $P = 0.0004$). These data suggest that particular amino acid mutations of NS5B-RdRp protein may confer sensitivity to combination therapy.

Recently, several studies on mutational analyses of HCV NS5B have identified several key residues responsible for its RdRp activity. Lohmann *et al.* noted that one single amino acid substitution in NS5B increased the efficacy of colony formation by 500-fold in HCV subgenomic replicon.[39] Cheney *et al.* noted that several amino acid substitutions (K155A, R168A, D225N and R386Q) were detrimental to both *in vitro* polymerase activity and replicon RNA replication in Huh-7 cells.[40] Recently, Young *et al.* suggested that NS5B F415Y mutation in HCV-1a was a key resistant variant for RBV monotherapy.[33] However, Y415 is the consensus residue for all genotypes except for 1a and 6a. In the present study of three non-responders, there was no difference at NS5B Y415 between sera collected before treatment and sera collected 12 weeks after the start of treatment with combination therapy.

The locations of the four mutations within the calculated tertiary structure of NS5B RdRp are illustrated in Figure 6a,b. The mutations in NS5B, which were more frequently found in the SVR and the ETR patients, were clustered in motif B to E of RdRp. The amino acid 309 and 355 are both located on the enzyme surface of the substrate entry site. NS5B 333 and 338 are adjacent to the NTP tunnel (Fig. 6b). Because mutations found in HBV and HIV DNA polymerase/reverse transcriptase are known to be located on the surface of the catalytic domain, the mutations in HCV RdRp that were found in the present study may considerably affect their enzymatic activity. Our preliminary data have shown that the HCV subgenomic replicon carrying point mutations in aa. 141 in NS5B less efficiently than the original sequences. Further studies are needed to clarify the role of these point mutations in NS5B in determining the activity of RdRp.

A recent study by Crotty *et al.* has shown that direct antireplicative effects of RBV on viruses include 'error catastrophe' theory in which misincorporations of RBV triphosphate into the viral genome lead to accumulation of mutations in the viral genome and yield defective virus genome. Characteristic pattern of nucleotide mutations by RBV are an increase of G-to-A and C-to-U transition mutations.[17,18] In our present study, although the majority of the mutations were transitions, there was no significant difference in the ratios of the G-to-A and C-to-U mutations between IFN monotherapy and combination therapy (Table 1). One explanation for the discrepancy is that the concentration of RBV in clinical use is too low to act as a mutagen. The clinically achievable blood concentration of RBV is 10–30 μM.[41] On the contrary, an *in vitro* study of polio virus has shown that RBV concentration of 100 μM is required to increase the mutation frequency by at least 1.2-fold.[17] Highly mutated HCV can be excluded or escape detection by RT-PCR and minor clone of HCV quasi-species are excluded by direct sequence of nested PCR products. Therefore, although it is not clear whether RBV is a mutagen against viral genome, our results suggest other mechanisms of RBV contribute to suppress HCV replication, such as inhibition of enzymatic activities of viral RNA polymerase.

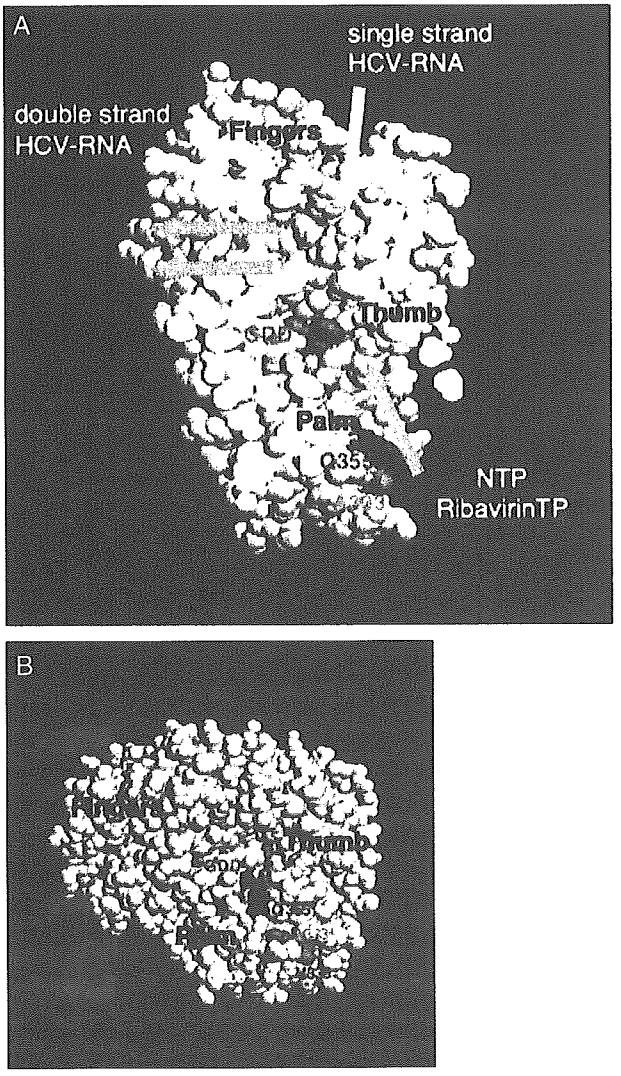


**Figure 6** Crystal structure of the hepatitis C virus (HCV) NS5B-RNA dependent RNA polymerase (RdRp). The molecular model of NS5B was constructed using 1QUV from Protein Data Bank (PDB). A space-filling representation of each atom is shown. Graphics were generated using Rasmol 2.7.2.1. (a) Cross-section of the RdRp at level of nucleotide tunnels. The single stranded HCV RNA enters the enzyme through a groove at the top of the finger domain, and the NTP or ribavirin enters the enzyme through the right lower dNTP tunnel (between β fingers and thumb). The essential GDD motif is shown in pink. NS5B 309, 333, 338 and 355 are shown in yellow, orange, green and red, respectively. (b) View from the dNTP entry site.

Many studies have endeavored to identify factors predictive of the outcome of IFN plus RBV combination therapy. Factors that have been examined include pretreatment clinical parameters such as baseline viral load, degree of fibrosis, and gender.[42] One study has

found early viral response (two-log decline of HCV RNA) to be predictive of SVR.[43] Another study showed that ISDR mutations were correlated with the SVR in chronic HCV 1b infection in Taiwan.[44] In the present study, multivariate analysis identified baseline ALT and the degree of fibrosis as independent factors for SVR. Further multivariate analysis showed that the number of mutations at positions NS5B 309, 333, 338 and 355 were independently associated with NR ($P = 0.0185$). The possible implications of our results are that the number of the above-described NS5B mutations is an independent predictive factor and that the parameter predicts NR patients exclusively from SVR or ETR patients. Our results which may enable prediction of NR before initiation of therapy might be of value when we consider indication for IFN plus RBV antiviral therapy or when making a decision about early cessation of the therapy, which may avoid possible side-effects and therapy costs. Although further studies of a larger population of patients are needed, the mutation number might be used to tailor therapy and is a useful factor for clinicians in making a clinical decision to stop treating HCV infection with combination therapy.

Given the absence of proven anti-HCV agents other than IFN and RBV, these combinations will continue to dominate therapy against HCV. Our present results provide evidence of a significant correlation between the response to IFN plus RBV combination therapy in patients with chronic HCV-1b infection and the amino acid changes that were present before therapy in conserved regions of NS5B. Certain amino acid changes in the HCV NS5B-RdRp domain may correlate with the clinical outcome of combination therapy and could thus be an initial predictor for response to IFN plus RBV combination therapy.

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# Mutagenic effects of ribavirin and response to interferon/ribavirin combination therapy in chronic hepatitis C☆

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See Editorial, pages 553–555

***Background/Aims*: To elucidate whether ribavirin acts as a mutagen in the clinical setting and to clarify the relationship between ribavirin-induced mutations and virological response to combined therapy.**

***Methods*: Thirty-four patients with hepatitis C virus (HCV) genotype 1b received ribavirin monotherapy for 4 weeks, followed by a 24-week course of IFN/ribavirin therapy. HCV mutations during a non-treatment observation period and during subsequent ribavirin monotherapy were determined, and the relationship between mutations and response to subsequent IFN/ribavirin therapy was evaluated.**

***Results*: Serum HCV significantly decreased from 6.90 to 6.56 $\log_{10}$copy/ml in response to ribavirin monotherapy ($P<0.0001$). Nucleotide mutations in the NS5A and NS5B regions occurred during ribavirin monotherapy at a rate of $2.9\times10^{-2}$/site/year and $1.3\times10^{-2}$/site/year, respectively, a significantly higher rate than the mutation rates during the prior non-treatment observation period ($0.60\times10^{-2}$/site/year and $0.24\times10^{-2}$/site/year, $P=0.02$, respectively). Mutation rates in the NS5A region were significantly higher in sustained viral responders (SVRs, $n=10$) than in non-responders ($8.8\times10^{-2}$/site/year vs. $0.38\times10^{-2}$/site/year, $P=0.0005$, respectively). In the NS5A region, non-synonymous mutations only occurred in SVRs.**

***Conclusions*: Ribavirin may act as a mutagen, and mutations occurring during ribavirin therapy correlate with the virological response to subsequent IFN/ribavirin combination therapy.**

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***Keywords*: NS5A; NS5B; ISDR; HCV; HCV dynamics**

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☆ The authors who have taken part in this study declared that they have not a relationship with the manufacturers of the drugs involved either in the past or present and did not receive funding from the manufacturers to carry out their research. The nucleotide sequences reported in this paper will appear in the DDBJ/EMBL/GenBank with accession numbers AB207766 through AB207801.

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*Abbreviations:* HCV, hepatitis C virus; IFN, interferon; RdRp, RNA dependent RNA polymerase; PCR, polymerase chain reaction; SVR, sustained viral responder; NR, non-responder; ISDR, interferon sensitivity determining region.

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## 1. Introduction

Ribavirin, a synthetic guanosine analog, has broad antiviral effects against both DNA and RNA viruses. Although ribavirin monotherapy has minimal efficacy on hepatitis C viral (HCV) eradication [1–3], studies have reported higher sustained response rates following combination therapy with interferon (IFN)-alfa and ribavirin than following IFN-alfa monotherapy [4–7]. Several mechanisms of action of ribavirin have been proposed [8]. In vitro and animal studies, in particular, have demonstrated that the antiviral activity of ribavirin is exerted through its potent mutagenic effects on RNA viruses after being incorporated into newly synthesized genomes by viral RNA-dependent RNA polymerase (RdRp) [9–11]. Still, little information is available regarding the mechanisms responsible for the increased virological efficacy associated with concurrent administration of ribavirin and IFN. No clinical studies to date have determined whether ribavirin induces mutations in the clinical setting nor examined the relationship between the mutagenic effects of ribavirin and viral response to IFN/ribavirin combination therapy.

The present study evaluated a set of patients with chronic hepatitis C. To elucidate whether ribavirin acts as a mutagen in the clinical setting, for each subject the sequential nucleotide mutations occurring during ribavirin monotherapy were compared with mutations occurring in the same patient during the non-treatment observation period immediately preceding the initiation of ribavirin monotherapy as a control. The relationship between mutations observed during ribavirin monotherapy and viral response to subsequent IFN/ribavirin combination therapy was also determined.

## 2. Methods

### 2.1. Patients

Among patients with biopsy-proven chronic hepatitis C hospitalized at the Musashino Red Cross Hospital from December 2001 to June 2002, 34 patients of HCV genotype 1b with a high viral load (>100 kcopies/ml by Amplicor-HCV monitor assay; Roche Molecular Diag. Co., Tokyo, Japan) were included in the present study (Table 1). Patients with liver cirrhosis, autoimmune hepatitis, and alcoholic liver injury were excluded from the study. No patient was positive for hepatitis B virus-associated antigen/antibody or anti-human immunodeficiency virus antibody. No patient received immunomodulatory therapy before enrolment in the study. Written informed consent was obtained from all patients, and this study was approved by the ethical committee of Musashino Red Cross Hospital in accordance with the Helsinki Declaration.

### 2.2. Treatment protocol and study design

Treatment schedule and time points for sequential genetic analysis are described in the upper part of Fig. 1. Following a non-treatment observation period, all patients received oral ribavirin daily for 4 weeks. Ribavirin dosage was 600 mg daily for patients who weighed less than 60 kg and 800 mg daily for patients who weighed between 60 and 80 kg. All patients subsequently received a 24-week course of treatment consisting of intramuscular IFN-alfa 2b (Intron, Schering-Plough, Kenilworth, NJ) at an initial dosage of 6 MU daily in combination plus daily oral ribavirin at the same dosage (600 mg daily or 800 mg daily) as was given during pretreatment monotherapy. After the first 2 weeks of IFN/ribavirin combination therapy, the IFN dosing frequency was reduced to 6 MU three times a week for the remaining 22 weeks.

**Table 1**
**Clinical characteristics of the study patients**

| | |
|---|---|
| No. of patients | 34 |
| Age (years) | 59±8 |
| Gender (M/F) | 14/20 |
| Liver histology | |
| A1/A2/A3 | 14/15/5 |
| F1/F2/F3 | 10/15/9 |
| Number of ISDR mutations (0/1) | 22/12 |
| Baseline data | |
| ALT (IU/L) | 81±56 |
| Platelet count ($\times 10^3$/ml) | 150±52 |
| Viral load (KIU/ml) | 572±204 |
| SVR (%) | 29.4 (10/34) |

Values are expressed as mean±standard deviation.

Nucleotide sequences of the NS5A and NS5B regions of the HCV genome were determined at the following time points: (1) enrolment into the study; (2) end of the non-treatment observation period (immediately before initiation of ribavirin monotherapy); (3) end of the 4-week ribavirin monotherapy (immediately before initiation of IFN/ribavirin combination therapy). For each patient, nucleotide changes between time points 1 and 2 during the non-treatment observation period (mean: 6 months, range: 2–48 months) were used as a control to determine whether mutations observed during the period of ribavirin monotherapy (between time points 2 and 3) represented true effects of ribavirin. Mutation rates for the non-treatment observation period were compared with the mutation rates during the subsequent ribavirin monotherapy period. Moreover, the relationships between observed mutations and the clinical outcome of the subsequent IFN/ribavirin therapy were evaluated.

### 2.3. Nucleotide sequencing

Nucleotide sequences of the NS5A and NS5B regions of the HCV genome were determined by direct sequencing of polymerase chain reaction (PCR)-amplified DNA, as previously described [12,13]. In brief, after RNA was extracted from sera of the study subjects, NS5A and NS5B regions were amplified by RT-PCR using Taq polymerase, and the sequences corresponding to nucleotides 6703–7320 and 7730–8874 of HCV-J were determined [13]. The sequences of the primers for the NS5A region were the same as described previously [12]. Sequences for the NS5B region, which were amplified with two partially overlapping sets of primers, were as follows: NS5B1-5′ outer set, 5′GGTGAATACCTGGAAATCAAAGAAA3′; NS5B1-3′ outer set, 5′AGAAATGAGTCATCAGAATCATCCT3′; NS5B1-5′ inner set, 5′TGTAAAACGACGGCCAGTATGGGCTTCTCATATGACAC3′; NS5B1-3′ inner set, 5′CAGGAAACAGCTATGACCCATGATGATGTTGCCTAGCC3′: NS5B2-5′ outer set, 5′GCAGAAGAAGGTCACCTTTGACAGA3′; NS5B2-3′ outer set, 5′TCGGGGGCCAAGTCACAACATTGGT3′; NS5B2-5′ inner set, 5′TGTAAAACGACGGCCAGTTTTGACAGACTGCAAGTCCT3′; NS5B2-3′ inner set, 5′CAGG AAACAGCTATGACCTTCTCAGTGACCGTTGAGTC3′. M13-forward and M13-reverse sequences were attached to the 5′-termini of sense and anti-sense nested PCR primers. Both strands of the PCR products were cycle sequenced with the PRISM dye termination kit (CN402069, Applied Biosystems, Chiba, Japan), and nucleotide sequences were determined by an automated DNA sequencer Model 373A (Applied Biosystems).

Mutations resulting from ribavirin therapy were defined as the detection of a new nucleotide which was not detected as even a minority population in the prior specimen from that subject. Electropherograms were read by two independent readers without knowledge of the patients' backgrounds and outcomes. A nucleotide detected in the post-ribavirin monotherapy specimen was considered to be a 'new nucleotide' only when both readers could not identify any tracking peak of this nucleotide in a prior specimen.

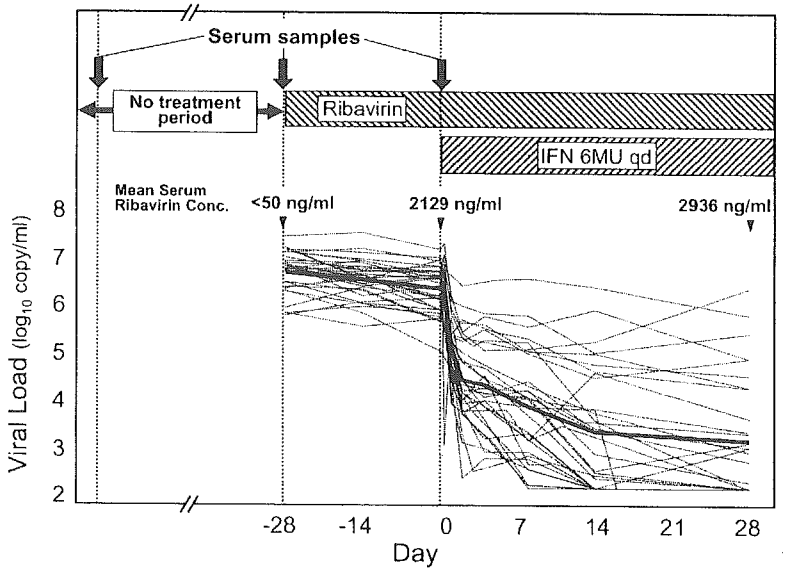


**Fig. 1. Treatment schedule and HCV dynamics during ribavirin monotherapy and subsequent IFN/ribavirin combination therapy. After a non-treatment observation period, all patients received oral ribavirin daily for 4 weeks and subsequently received intramuscular IFN-alpha 2b in combination with daily oral ribavirin. For the first 2 weeks of IFN/ribavirin combination therapy, 6 MU of IFN-alpha 2b was given daily; the IFN dosing frequency was then reduced to 6 MU three times a week for the remaining 22 weeks of combination therapy. Serum HCV dynamics of individual patients are shown in dotted lines, and the solid line represents the mean of these values. The nucleotide sequences were serially analyzed at the time points indicated by the arrows. Closed triangles indicate the time points of serum ribavirin concentration measurements.**

Peaks less than 10% of the dominant peak were considered to be background signals. To calculate values for *dN* (nonsynonymous substitution), *dS* (synonymous substitution), *dN/dS* ratios, and the number of point mutations, analyses of nucleotide sequences were performed using a software program (MEGA version 2.1.). Separate *dN/dS* ratios were determined for each NS5 region in patients who had nucleotide mutations in the corresponding NS5 regions. To determine the locations of amino acid mutations in the tertiary structure of the NS5B molecule, a crystal structure model of HCV NS5B-RdRp was constructed using 1QUV from the Protein Data Bank. A space-filling representation of each atom was generated using Rasmol 2.7.2.1. The deduced amino acid sequences of the NS5A and NS5B regions were also compared with a prototype HCV 1b strain, HCV-J [14].

### 2.4. HCV dynamics in serum

To analyze the effect of ribavirin on viral dynamics, HCV-RNA concentrations were quantified just before and at the end of ribavirin monotherapy, and also at 4, 8, 24, 48, 96, 192, and 336 hours after initiating IFN/ribavirin combination therapy, using real-time detection PCR, as reported previously [15–17]. The detection sensitivity of this assay is approximately 10 copies/ml, and the dynamic range is from 10 copies/ml to more than $1\times10^8$ copies/ml [17]. For each patient, the viral decline curve was plotted on a semilogarithmic scale, and the slopes of the exponential viral declines were calculated for each viral decline phase by a straight-line fit of the data.

### 2.5. Definitions of response to therapy

A patient negative for serum HCV-RNA during the first six months following the completion of IFN/ribavirin combination therapy was defined as a sustained viral responder (SVR), and a patient positive for HCV-RNA during this time period was defined as a non-responder (NR).

### 2.6. Statistical analysis

Categorical data were compared by the chi-square test or Fisher's exact test. Distributions of continuous variables were analyzed by the Student's *t*-test for two groups. All tests of significance were two-tailed, and *P* values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. HCV dynamics

During the 4-week period of ribavirin monotherapy, the mean serum HCV-RNA level significantly decreased from 6.90 to 6.56 $log_{10}$ copy/ml ($P<0.0001$, paired t test) (Fig. 1). Serum HCV dynamics after the start of subsequent IFN/ribavirin combination therapy demonstrated a biphasic kinetic pattern of HCV-RNA decline. The exponential decay slopes for the first phase and the second phase were $2.00\pm0.77$ $log_{10}$/day and $0.15\pm0.14$ $log_{10}$/day, respectively.

### 3.2. The effect of ribavirin on HCV gene mutation and the relationship between mutations and virological response to IFN/ribavirin combination therapy

In a pairwise comparison of the NS5 sequences before and after ribavirin monotherapy, new HCV gene mutations occurring during ribavirin monotherapy were observed in the NS5A region in10 patients and in the NS5B region in 8 patients. Mean gene mutation rates in the NS5A and NS5B regions during ribavirin administration were $2.9\times10^{-2}$/site/year and $1.3\times10^{-2}$/site/year, respectively, rates which were significantly higher compared with the rates observed during the prior non-treatment observation periods in