Figure 4. Real-time quantitative RT-PCR for IL-12 and IL-18 mRNA expression in the liver on day 1. IL-12 gene expression was significantly higher in T/M^{Low} mice than in the other groups ($p < 0.05$)
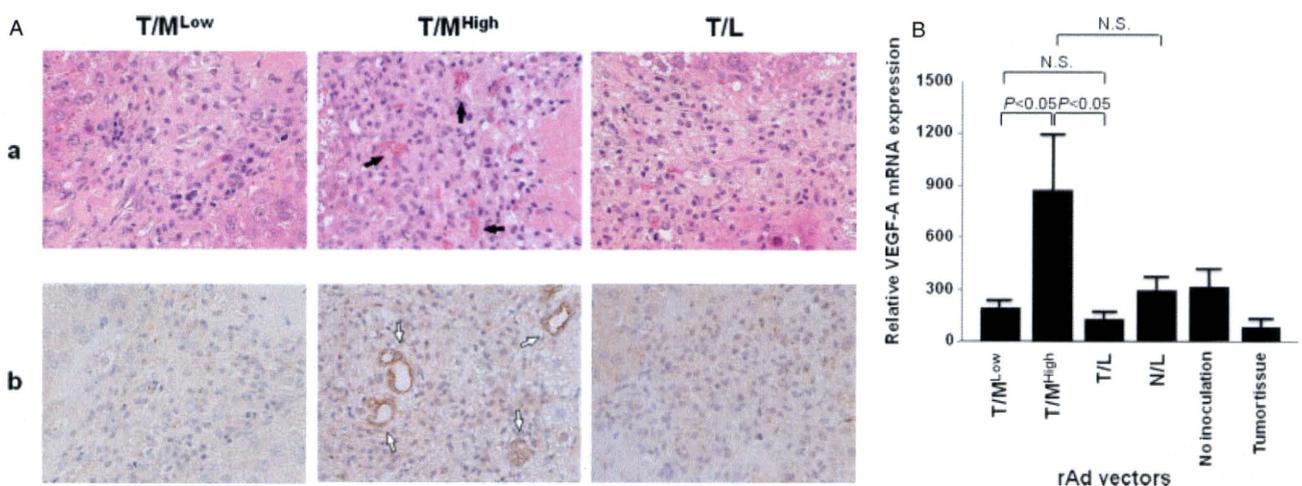

Figure 5. Evaluation of tumor angiogenesis. (A) Morphometric analysis of microvessels in tumor tissues using H&E staining and CD31 immunohistochemical analysis. (a) Representative H&E stained histological sections of day 7 tumor tissues showing intratumoral microvessels containing red blood cells (closed arrow); endothelial cells were not identified. (b) Representative CD31 immunohistochemical staining showing endothelial cell proliferation in tumor tissues (open arrow). Original magnification ×400. (B) Real-time quantitative RT-PCR for VEGF-A mRNA expression in liver on day 3

monocytes/macrophages and IL-12 production on day 1, and the tumor foci showed heavy infiltration by CD4$^+$ and CD8$^+$ T cells on day 14. CTLs specific for BNL cells were induced in mice treated with CCL2/MCP-1. By contrast, the expression of the angiogenic factor VEGF-A was significantly increased in mice treated with a large amount of CCL2/MCP-1. Collectively, these results suggest that the delivery of an adequate amount of CCL2/MCP-1, in conjunction with the HSV-tk/GCV system, may display beneficial antitumor effects, preventing the intrahepatic metastasis of HCC cells.

In the development of this model, we injected 1×10^6 tumor cells infected with recombinant adenoviruses into the portal vein because the injection of fewer cells (e.g. 10^5) resulted in greatly diminished frequencies of metastasis in the mice. The injection of large numbers of cells, however, may have caused the embolization of cell aggregates in the portal vein, which may have contributed to the induction of ischemic necrosis in the liver tissues. The resultant ischemic death of liver cells may be

recognized by immune cells including macrophages, and may result in macrophage activation and the local release of cytokines and chemokines. However, when the mice were injected with control tumor cells (N/L), we observed little infiltration of immune cells, including macrophages and CD4$^+$ and CD8$^+$ T cells, and these mice developed the largest amounts of tumor tissues. These results indicate that any unfavorable effects as a result of ischemic cell death were minimal for the development of intrahepatic metastasis in this model.

This model would be more relevant if ganciclovir treatment was delayed to allow the establishment of tumors. Therefore, we performed the additional experiments with ganciclovir treatment at delayed time point, day 3. Although there was a trend for small amount of MCP-1 to enhance the antitumor effects of the HSV-tk/GCV system, as seen in the experiments on day 1, these differences did not reach statistical significance [T/M^{Low}: 7.64 ± 1.25 ($n = 10$); T/M^{Mod}: 9.24 ± 0.77 ($n = 5$); T/M^{High}: 9.65 ± 1.06 ($n = 8$); T/L: 10.51 ± 1.79
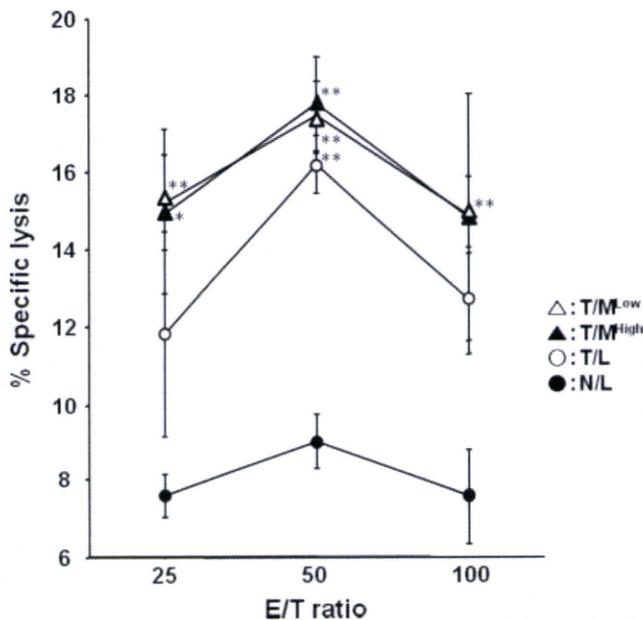
Figure 6. Cytotoxic activities of splenocytes. Splenocytes harvested on day 14 from individual mice stimulated with MMC-treated BNL cells for 7 days were tested in a standard 4-h cytotoxicity assay with ^{51}Cr-labeled target (BNL) or control (CT26) cells. $^*p < 0.05$ and $^{**}p < 0.01$ compared to N/L mice

$(n = 7)$; and N/L: 13.94 ± 1.16 $(n = 5)$]. Consequently, the experiment in which ganciclovir was added 3 days after tumor inoculation failed to show a significant antitumor effect. The weakness of this approach may be still the low relevance of the tumor model. The reason is that MCP-1 gene expression by rAds may not be sufficient to enhance antitumor effect at day 3 because the transgene expression gradually diminished with the tumor growth. In our previous studies, MCP-1 production reached peak level on day 2 and decreased after day 3 [6].

Mice treated with small amounts of CCL2/MCP-1 showed enhancement of antitumor effects. The amount of CCL2/MCP-1 delivered, however, was not correlated with monocyte/macrophage accumulation. Although activated monocytes/macrophages are indicative of the potential to eliminate tumor cells [24–26], infiltrating macrophages may enhance tumor growth by secreting growth and angiogenic factors, including VEGF [26–28]. Immunohistochemical analysis of CD31 revealed that microvessels in HCCs were increased in the mice treated with large amounts of CCL2/MCP-1. We also observed a close correlation between the amounts of CCL2/MCP-1 delivered and the levels of VEGF expression. These findings suggest that large amounts of CCL2/MCP-1 may recruit macrophages to induce tumor cell killing and, simultaneously, to facilitate tumor growth, probably by promoting angiogenesis, thus resulting in a reduction of antitumor effects.

CCL2/MCP-1 is a member of the CC chemokine superfamily that promotes the migration of macrophages/monocytes, T lymphocytes, natural killer cells and natural killer T cells not only to sites of inflammation, but also to tumor tissues, which may contribute to the

inhibition of tumor growth [29–31]. In addition, the production of CCL2/MCP-1 by tumor tissues has been reported to be associated with favorable prognoses in human pancreatic cancer [31] and neuroblastoma [30]. By contrast, CCL2/MCP-1 may promote tumor growth by chemoattracting tumor-associated macrophages for tumor angiogenesis, or by acting on tumor cells as an autocrine growth factor [29,32,33]. Consistent with this notion, a Japanese study of 135 breast cancer patients found that the women with high levels of tumor-associated CCL2/MCP-1 showed a significantly shorter relapse-free survival [34]. Taken together, the biological and immunological effects of CCL2/MCP-1 appear to vary greatly depending on the diverse microenvironments of cancer tissues.

Two major types of activated macrophages have been described: M1 (classical) and M2 (alternative) [35–38]. M1 macrophages, which play a critical role in the development of antitumor immunity, are characterized by high IL-12 and low IL-10 production. By contrast, M2 macrophages produce reduced amounts of IL-12 but higher levels of Il–10. We found that IL-12 expression was significantly increased in mice treated with a small amount of CCL2/MCP-1 but not in mice treated with a large amount of CCL2/MCP-1, despite the marked infiltration of monocytes/macrophages in the latter. In addition, members of the MCP family have been reported to dose-dependently inhibit IL-12 production by antigen-presenting cells (APCs) [39,40]. Because of the different local concentrations of CCL/MCP-1, we hypothesized that the M1/M2 ratio of recruited monocytes/macrophages may differ in T/M^{Low} and T/M^{High} mice. Indeed, we found that the proportions of M1 cells among infiltrating cells were significantly higher in T/M^{Low} than in T/M^{High} mice. Therefore, M1 monocyte/macrophage polarization may be suppressed in mice treated with large amounts of CCL2/MCP-1, resulting in the reduction of antitumor immunity and the promotion of tumor growth.

Significant tumor infiltration of CD4$^+$ and CD8$^+$ T cells 14 days after transfer was observed in mice treated with the HSV-tk/GCV system plus CCL2/MCP-1. Local secretion of CCL2/MCP-1 by tumor cells may lead to the recruitment and activation of antigen-presenting monocytes/macrophages [41,42]. Once attracted to the tumor tissues, these APCs may ingest pathogenic antigens and transport them to local lymphoid organs, where the antigens are presented to naive T cells, thus establishing a T cell-mediated antitumor response [43]. Tumor growth may thus be impeded by tumor antigen-specific CD4$^+$ and CD8$^+$ T cells.

Although the data obtained in the present study appear to be promising, several problems remain to be solved before clinical application. Our liver metastasis model using a mouse HCC cell line may not be comparable to intrahepatic metastasis of HCC in human patients. However, HCC patients treated by nonsurgical procedures, including percutaneous radiofrequency ablation therapy and transcatheter arterial chemotherapy [44,45], could also be administered rAds to reduce the incidence of

intrahepatic recurrence and metastasis. The present study demonstrated that, in a mouse model, there is a negative impact on tumor development in the presence of a low level of CCL2/MCP-1, whereas high levels of the protein complicate the situation by having a positive impact on tumor growth (i.e. a balance is required). The therapeutic effects may vary with different tumors. The direct correlation between the overexpression of VEGF in tumor cells and tumor angiogenesis has been demonstrated previously [46], and large amount of CCL2/MCP-1 might be less effective in the treatment of hypervascular tumors such as HCC. However, other cancers resistant to anti-angiogenic drug (e.g. pancreatic cancer) [47,48], probably do not need a good blood supply for tumor growth. In the treatment of hypovascular tumors that are resistant to anti-angiogenic drug, CCL2/MCP-1 may enhance the antitumor effects via activation of M1 macrophages.

Additionally, in the present study, we did not perform *in vivo* delivery experiments of the vectors to existing tumors. There would be many complicated factors affecting the delivery of HSV-tk and CCL2/MCP-1 genes in therapeutic approaches [49,50]. Intra-arterial administration of rAds may result in the induction of immunogenicity or cytotoxicity, especially when spread via blood flow. Extremely high-dose rAds have been found to cause severe unexpected side-effects [51]. To overcome these problems, highly tumor-specific promoters may be needed. In our previous studies, human alpha-fetoprotein (AFP) promoters specific for liver cancer cells were used in an immunodeficient nude mouse models [6,52]. A reporter gene was specifically expressed in AFP producing tumors that were xenografted subcutaneously and disseminated in the liver and lung. However, HSV-tk gene expression was not enhanced sufficiently to kill established tumor cells [53] because the transcriptional activity of AFP promoter was relatively low. Furthermore, neither promoters, nor delivery systems were found to be specific for the BNL mouse tumor cell line. Although better methods of tumor-specific gene delivery and expression are needed, the use of *ex vivo* infection techniques has been found to reproduce tumor specific gene expression *in vivo*.

Conclusions

Although problems with rAds remain to be resolved before clinical application, the results obtained in the present study suggest that a new strategy, consisting of immune gene therapy accompanied by a suicide gene system, can be used to treat HCC and tumors of other lineages.

Acknowledgements

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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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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

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; Peg-Intron; 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	0.02
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	NS

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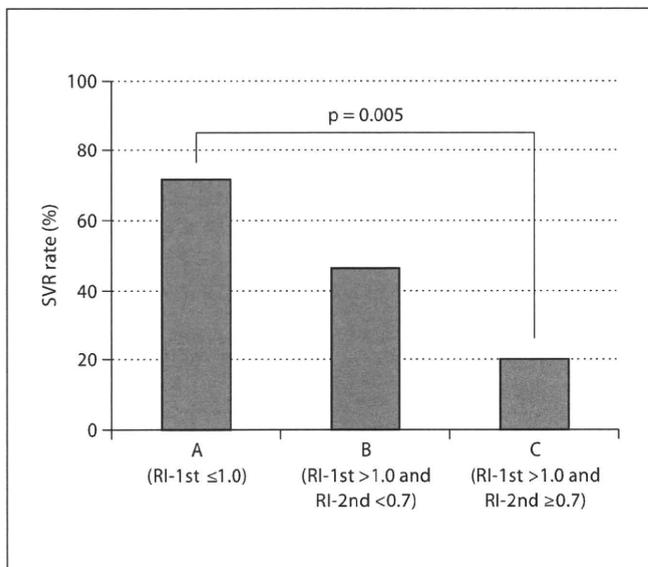
Fig. 1. SVR rate in RI-A, -B and -C groups. The overall SVR rate was 71.4, 46.2 and 20.0%, respectively. Significant difference in SVR rate is indicated.
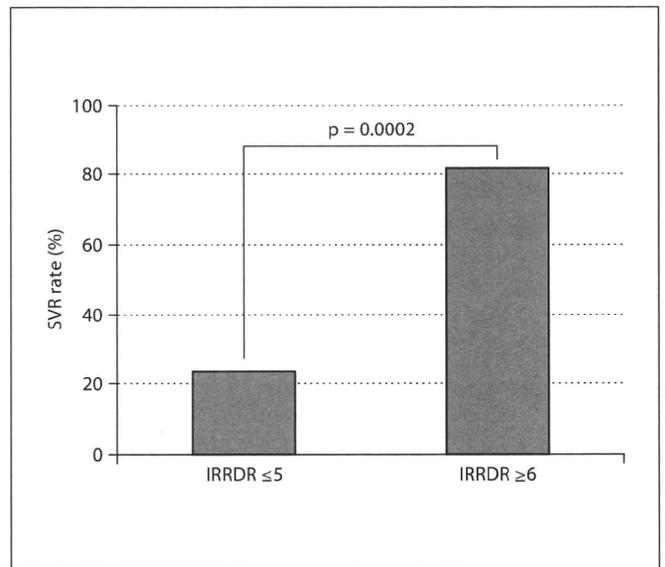

Fig. 3. SVR rate and IRRDR number. The SVR rate was 23.1% in IRRDR ≤ 5 and 81.3% in IRRDR ≥ 6, which was significantly different.
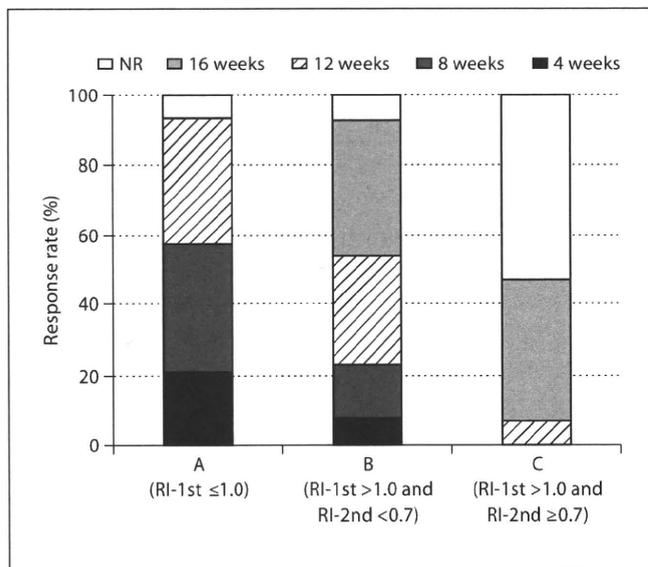

Fig. 2. Relation between response time and virus dynamics. In the 14 patients of the RI-A group, HCV RNA turned negative by week 4 in 3 patients, week 8 in 5 patients, week 12 in 5 patients and remained positive throughout the treatment in 1 patient. In the 13 patients of the RI-B group, HCV RNA was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

Secondary Structure of the Amino-Terminal Region of HCV NS3 and Virological Response to Pegylated Interferon Plus Ribavirin Therapy for Chronic Hepatitis C

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The aim of the study was to identify a predictive marker for the virological response in hepatitis C virus 1b (HCV-1b)-infected patients treated with pegylated interferon plus ribavirin therapy. A total of 139 patients with chronic hepatitis C who received therapy for 48 weeks were enrolled. The secondary structure of the 120 residues of the amino-terminal HCV-1b non-structural region 3 (NS3) deduced from the amino acid sequence was classified into two major groups: A and B. The association between HCV NS3 protein polymorphism and virological response was analyzed in patients infected with group A (n = 28) and B (n = 40) isolates who had good adherence to both pegylated interferon and ribavirin administration (>95% of the scheduled dosage) for 48 weeks. A sustained virological response (SVR) representing successful HCV eradication occurred in 33 (49%) in the 68 patients. Of the 28 patients infected with the group A isolate, 18 (64%) were SVR, whereas of the 40 patients infected with the group B isolate only 15 (38%) were SVR. The proportion of virological responses differed significantly between the two groups (P < 0.05). These results suggest that polymorphism in the secondary structure of the HCV-1b NS3 amino-terminal region influences the virological response to pegylated interferon plus ribavirin therapy, and that virus grouping based on this polymorphism can contribute to prediction of the outcome of this therapy. *J. Med. Virol. 82:1364–1370, 2010.* © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis C; interferon; ribavirin; interaction; polymorphism

INTRODUCTION

Hepatitis C virus (HCV) is the major pathogen that causes chronic liver diseases with a risk of progression to cirrhosis and hepatocellular carcinoma. Currently, the standard treatment for chronic hepatitis C is antiviral therapy using pegylated interferon (Peg-IFN) plus ribavirin (RBV), and this approach is most effective for eradication of HCV viremia. However, even with the widely used treatment regimen of 48 weeks, the rate of sustained virological response (SVR), which indicates eradication of viremia, is still approximately 50% for patients infected with the therapy-resistant HCV genotype 1b (HCV-1b) with a high viral load [Manns et al., 2001; Bruno et al., 2004; Hadziyannis et al., 2004]. It would be useful to predict the virological response to this therapy and to identify patients who would obtain beneficial therapeutic effects before treatment, in order to avoid any serious side effect and to eliminate those who would not be helped by the treatment. In the future it will be important to establish a protocol of tailor-made medicine for chronic hepatitis C.

Both the HCV genotype and pre-treatment viral load are major viral factors that influence the response to IFN-based antiviral therapy, but IFN resistance is also partly due to variation of the amino acid sequence encoded by HCV itself. Enomoto et al. [1996] proposed that variation of 40 amino acids within the NS5A region (aa 2,209–2,248), which is referred to as the IFN sensitivity-determining region (ISDR), is well correlated with IFN responsiveness. ISDR and its adjacent sequence bind and inhibit the enzymatic activity of a double-stranded RNA-activated protein kinase (PKR), which can have an antiviral effect, and therefore the combined region is referred to as the PKR-binding domain (PKR-BD) [Gale et al., 1997, 1998]. A correlation between sequence variation in the PKR-BD and IFN responsiveness has been reported [Nousbaum et al., 2000], and some reports show a correlation between IFN responsiveness and the sequence diversity of variable region 3 (V3) (aa 2,356–2,379) or surrounding regions near the carboxy terminus of NS5A [Murphy et al., 2002; Sarrazin et al., 2002; Puig-Basagoiti et al., 2005]. A high degree of amino acid substitution in the V3 and pre-V3 regions (aa 2,334–2,355) of NS5A, which is referred to as the IFN/RBV resistance-determining region (IRRDR) (aa 2,334–2,379), has been associated with SVR in Peg-IFN/RBV combination therapy for patients infected with HCV-1b [El-Shamy et al., 2007, 2008]. In addition to these findings in non-structural proteins of the virus, amino acid substitution in a structural region of HCV has been reported to be a predictive viral marker for the virological response to PegIFN/RBV therapy. Amino acid polymorphisms in the HCV core region (Arg70 vs. Gln70 and Leu91 vs. Met91) correlate with virological outcome and on-treatment viral kinetics in Peg-IFN/RBV therapy [Akuta et al., 2006, 2007], and a double wild-type HCV core (Arg70 and Leu91) may be a significant predictor of SVR in Peg-IFN/RBV therapy [Akuta et al., 2007].

Interactions between viral and host proteins in infected cells may influence therapeutic effects and the natural history of infection, since the HCV NS3 region has a significant effect on immunity. The amino-terminal part of this region encodes a serine protease, for which the minimum activity has been mapped to a region between aa 1,059 and 1,204 [Yamada et al., 1998]. The serine protease inactivates Cardif, a caspase recruitment domain (CARD)-containing adaptor protein that interacts with the RNA helicase retinoic acid inducible gene 1 (RIG-1)-dependent antiviral pathway in infected cells [Foy et al., 2003; Meylan et al., 2005; Evans and Seeger, 2006]. This action inhibits phosphorylation and subsequent heterodimerization of interferon regulatory factor-3 (IRF-3), which is essential for activation of IFN signaling through translocation of IRF-3 heterodimers into the nucleus, and eventually blocks IFN-beta production. In addition, inactivation of IRF-3 is postulated to influence the therapeutic effect of IFN-based antiviral therapy, because the IRF-3 heterodimer translocates into the nucleus to bind to the IFN-stimulated response element that produces

many antiviral proteins, including 2′,5′-oligoadenylate synthetase and PKR [Nakaya et al., 2001; Grandvaux et al., 2002]. Collectively, these findings suggest that polymorphisms in HCV NS3 structure deduced from sequence variation may influence IFN-related signaling and the antiviral effect of IFN-based anti-HCV therapy.

We have focused on polymorphisms in the secondary structure of the viral polyprotein that interacts with host proteins involved in immunity, with the aim of identification of predictive viral markers for the response to Peg-IFN/RBV therapy. In this study, we examined the potential correlation between polymorphisms in the secondary structure of the HCV NS3 amino-terminal region and virological responses to Peg-IFN/RBV therapy in patients infected with HCV-1b with a high viral load.

PATIENTS AND METHODS

Patients and Treatment Regimen With Peg-IFN Plus Ribavirin

A total of 139 consecutive patients diagnosed with chronic hepatitis C were enrolled in the study from December 2004 to March 2007. These patients included 81 men and 58 women, and were aged from 31 to 75 years old (mean ± SD, 56.8 ± 8.7 years old). All patients were infected with HCV-1b with a high viral load of over 100 KIU/ml, and all received Peg-IFN/RBV therapy. Patients with alcoholic liver injury, autoimmune liver disease, and those who had symptoms of decompensated cirrhosis including ascites were excluded. Briefly, all patients were treated with a combination of Peg-IFN-alpha 2b (Pegintron®; Schering-Plough, Kenilworth, NJ) and RBV (Rebetol®; Schering-Plough) for 48 weeks. Peg-IFN was administered subcutaneously once a week and RBV was given orally twice a day for the total dose. The dosages were determined on the basis of body weight according to the Japanese standard prescription information supplied by the Japanese Ministry of Health, Labour and Welfare, and there was a limit for calculating the optimized dose: patients with body weights of 35–45, 46–60, 61–75, and 76–90 kg were given Peg-IFN at doses of 60, 80, 100, and 120 µg, respectively, and those with body weights of <60, 60–80, and >80 kg were given RBV at doses of 600, 800, and 1,000 mg, respectively. The dose of Peg-IFN or RBV was reduced according to the Japanese standard criteria based on the white blood cell count, neutrophil count, hemoglobin concentration and platelet count [Hiramatsu et al., 2008].

Virological Tests and Response to Peg-IFN Plus Ribavirin

Virological responses were evaluated at 12 weeks after the start of treatment with an early depletion of viremia referred to as an early virological response (EVR), at the end of treatment with depletion of viremia referred to as an end of treatment virological response (ETR), and at 24 weeks after completion of treatment,

with a clinical outcome of a sustained virological response (SVR) representing successful HCV eradication. All patients were negative for hepatitis B surface antigen. Quantification of serum HCV RNA was performed using an RT-PCR-based commercial kit (Amplicor HCV monitor test, ver. 2.0, Roche Diagnostics, Tokyo, Japan). This Amplicor HCV RNA assay has a lower limit of detection of 50 IU/ml. SVR was determined by monitering negativity for HCV RNA monthly for 6 months. The real-time PCR assay kit (COBAS TaqMan HCV Auto, Roche Diagnostics) for more precise quantitation of HCV viremia has recently become available and pre-treatment viral titers were re-evaluated using preserved serum samples. This real-time PCR assay has a lower limit of detection of 15 IU/ml. The study protocol was approved by the Ethics Committee of Yamagata University Hospital. Informed consent was obtained from all patients.

PCR Amplification of the Amino-Terminal Region of NS3

RNA was extracted from 50 µl of serum using an RNeasy Mini kit (Qiagen, Tokyo, Japan). To amplify the region of the HCV genome encoding the amino-terminal region of NS3 (1,027–1,206), a one-step PCR was performed in a tube using the Superscript One-Step RT-PCR kit with Platinum Taq (Gibco-BRL, Tokyo, Japan) and an outer set of primers: NS3-F1 (sense primer; 5'-ACA CCG CGG CGT GTG GGG ACA T-3'; nucleotides 3,295–3,316) and NS3-AS2 (antisense primer; 5'-GCT CTT GCC GCT GCC AGT GGG A-3'; nucleotides 4,040–4,019), as reported previously [Ogata et al., 2002a, 2003]. PCR was initially performed at 45°C for 30 min at RT and then at 94°C for 2 min, followed by the first-round PCR for forty 3-min cycles at 94°, 55°, and 72°C for 1 min each. The second-round PCR was performed with *Pfu* DNA polymerase (Promega, Tokyo, Japan) and an inner set of primers: NS3-F3 (sense primer; 5'-CAG GGG TGG CGG CTC CTT-3'; nucleotides 3,390–3,407) and NS3-AS1 (antisense primer; 5'-GCC ACT TGG AAT GTT TGC GGT A-3'; nucleotides 4,006–3,985). The second-round PCR was performed for 35 cycles, with each cycle consisting of 1 min at 94°C, 1.5 min at 55°C, and 3 min at 72°C. This method allowed amplification of the corresponding portion of the HCV genome from HCV-1b RNA-positive samples. The amplified fragments were purified with a QIAquick PCR purification kit (Qiagen) and directly sequenced (without being subcloned) in both directions using a dRhodamine Terminator Cycle Sequencing Ready Reaction kit and an ABI 377 sequencer (Applied Biosystems, Tokyo, Japan).

Classification of the Secondary Structure of the HCV-1b NS3 Amino-Terminal Region

The secondary structure of the amino-terminal region of HCV NS3 was predicted by computer-assisted Robson analysis [Garnier et al., 1978] with Genetyx-Mac software (ver.10.1; Software Development Co., Tokyo,
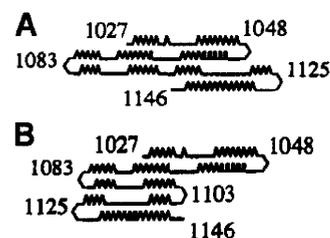
Fig. 1. Secondary structure of the 120 amino-terminal residues of HCV-1b nonstructural 3 (NS3) region classified into two major groups: A and B. The looped, zigzag, straight, and bent lines represent α-helix, β-sheet, coil, and turn structures, respectively. The numbers indicate amino acid positions. A: Group A, (B) Group B.

Japan). Previously, the full-length secondary structure of the HCV-1b NS3 region was analyzed, and this showed that the secondary structure deduced from the carboxy-terminal 60 residues was well conserved in terms of linear structure, without any turn structure [Ogata et al., 2002a]. We have shown that the secondary structure of the 120 residues in the amino-terminal region of HCV-1b NS3 can be classified into two major groups: A and B (Fig. 1) [Ogata et al., 2002a, 2003]. Briefly, the criteria for this classification are as follows: in group A isolates, the carboxy-terminal 20 residues (aa 1,125–1,146) are oriented leftward relative to a domain composed of the remaining amino-terminal region; whereas in group B isolates, the same 20 residues are oriented rightward relative to the rest of the amino-terminal domain.

Analysis of Amino Acid Substitutions in the Core Region

To amplify a region of the HCV genome encoding the core region including positions 70 and 91, reverse transcription and the first-round PCR were performed in a tube by the Superscript One-Step RT-PCR kit with Platinum *Taq* (Gibco-BRL) and an outer set of primers, followed by second-round PCR with an inner set of primers in accordance with procedures reported previously [Ogata et al., 2002b]. The sequences of the amplified fragments were determined by direct sequencing.

Statistical Analysis

Data were analyzed by a χ^2 test for independence with a two-by-two contingency table and a Student *t*-test. A *P*-value <0.05 was considered significant.

RESULTS

Virological Response *and Adherence* to the Peg-IFN Plus Ribavirin Regimen

Rates of virological responses in patients treated with PegIFN/RBV combination therapy for 48 weeks are shown in Figure 2. Of the 139 patients enrolled in the study, SVR, non-SVR and cessation of therapy occurred in 58 (42%), 62 (45%), and 19 (14%), respectively. Serious
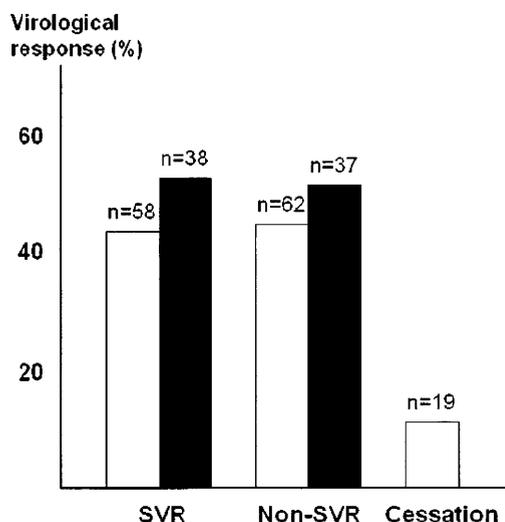
Fig. 2. Virological response in patients treated with peginterferon plus ribavirin for 48 weeks. The results are shown for all 139 subjects (open bars) and for 75 cases with good adherence of >80% of the scheduled dosages (closed bars). SVR, sustained virological response.

adverse events that necessitated discontinuation of this therapy were depression in one patient, thyroid function disorder in 2, general itching in 2, infection in 2, anorexia in 2, occurrence of hepatocellular carcinoma in 2, and a decreased neutrophil count in 2. Six patients also terminated this therapy at their own request. Of the 139 patients, 75 (54%) received >80% of the scheduled dosage of Peg-IFN and RBV designated before treatment, and of these 75 cases SVR and non-SVR occurred in 38 (51%) and 37 (49%), respectively.

Prevalence of Types of Secondary Structure of the Amino-Terminal Region of HCV NS3

The prevalence of the types of secondary structure of HCV NS3 in the 139 subjects is shown in Table I. Among these subjects, 43 (31%), 70 (50%), and 26 (19%) were classified into groups A, B, and others, including 3 of mixed type (A plus B) and 23 of non-A, non-B type. Of the 75 cases with good adherence to administration of >80% of the scheduled dosage, 28 (37%), 40 (53%) and 7 (9%) were classified into groups A, B, and others. The amino acid data of group A and B in the cases with good adherence to administration are available in the DDBJ/EMBL/GenBank databases with the accession numbers AB548070–AB548137. Our analysis revealed no specific correlations between amino acid sequences

TABLE I. Prevalence of the HCV NS3 Secondary Structure Type

	Group A (%)	Group B (%)	Others (%)
Enrolled cases (n = 139)	43 (31)	70 (50)	26 (19)
Adherent cases (n = 75)	28 (37)	40 (53)	7 (9)

and the secondary structure deduced by the Robson method, as we have reported previously [Ogata et al., 2003].

Characteristics of Adherent Patients Based on Different HCV NS3 Structure Types

The virological responses to Peg-IFN/RBV combination therapy for patients infected with group A and B isolates were assessed in the 68 subjects with good adherence to the scheduled dosage of Peg-IFN and RBV. The characteristics of patients infected with group A and B isolates are shown in Table II. Age, gender, pretreatment level of serum HCV RNA and ALT, and frequency of fibrosis stage did not differ significantly between the two groups. Peg-IFN/RBV combination therapy was completed in all the patients, and the total administered dosages of Peg-IFN and RBV was >95% of the scheduled dosage in both groups.

Relationship Between Virological Responses and Polymorphisms in the HCV NS3 Amino-Terminal Region

In the 68 patients who received >95% of the scheduled doses of Peg-IFN and RBV for 48 weeks, SVR and non-SVR occurred in 33 (49%) and 35 (51%), respectively. The EVR, ETR, and SVR rates in patients infected with group A and B isolates are shown in Table III. There was a significant difference in the rates of EVR between subjects infected with group A and B isolates: EVR was achieved in 19 of 28 (68%) patients with group A infection, compared to 17 of 40 (43%) with group B infection ($P < 0.05$). The final outcome also differed significantly between subjects infected with group A and B isolates: SVR was achieved in 18 of 28 (64%) patients with group A infection, compared to 15 of 40 (38%) with group B infection ($P < 0.05$).

Polymorphisms in Core Amino Acids 70/91 and in the HCV NS3 Secondary Structure

The wild-type core sequence (Arg70, Leu91) has been associated with SVR in Peg-IFN/RBV combination therapy, while the non-double wild-type containing one or two substitutions at positions 70 and/or 91 was associated with non-SVR [Akuta et al., 2007]. Therefore, we examined substitutions at positions 70 and 91 in the HCV core region in pre-treatment serum samples of 44 cases that were available for testing. The double wild-type 70/91 sequence was found in 22 of the 44 cases (50%), of which 12 were SVR and 10 were non-SVR. Combination analysis of polymorphisms of the HCV core 70/91 positions and the NS3 amino-terminal region showed that 10 (83%) of the 12 SVR cases and only 3 (30%) of the 10 non-SVR cases with the double wild-type core had a group A polymorphism in HCV NS3 (Table IV). Thus, combination analysis of the core and NS3 regions may improve prediction of the outcome of Peg-IFN/RBV therapy.

TABLE II. Characteristics of Adherent Patients Infected With HCV Group A and B Isolates

	Group A (n = 28)	Group B (n = 40)	P
Age (years)	55.5 ± 9.5	55.5 ± 8.9	NS[a]
Sex (men/women)	18/10	21/19	NS[b]
Pre-treatment HCV RNA (KIU/ml)	1,635 ± 930	2,087 ± 1,422	NS[a]
Alanine aminotransferase level (U/L)	80 ± 62	71 ± 47	NS[a]
Stage of liver fibrosis			
F1 or F2/F3 or F4	19/9	28/12	NS[b]
Drug adherence dosage (%)			
Pegylated interferon	97.7 ± 5.2	95.2 ± 7.3	NS[a]
Ribavirin	96.8 ± 6.4	95.3 ± 7.7	NS[a]

NS, not significant.
[a]t-test.
[b]χ^2 test.

Re-Evaluation of Pre-Treatment HCV Viremia Status Using Real-Time PCR

Since the viral titer before treatment is a major predictive marker of the outcome of Peg-IFN/RBV therapy, we re-evaluated the pre-treatment viral titers more precisely using preserved serum samples taken within 1 month before treatment, using a real-time PCR assay. The pre-treatment viral titers did not differ significantly between sera with group A and B isolates (5.98 ± 0.94 vs. 6.25 ± 0.62 logIU/ml) (Table V). The secondary structure polymorphisms of HCV NS3 were independent of the pre-treatment viral titers.

DISCUSSION

Antiviral therapy with Peg-IFN/RBV for 48 weeks fails to eradicate HCV in about half of patients infected with a high titer of HCV genotype 1b, and the severe adverse events and high costs associated with this therapy require outcome prediction to allow targeted treatment for chronic hepatitis C. The pre-treatment viral titer, viral factors that influence the virological response to IFN-based anti-HCV therapy have been widely investigated. Viral kinetics showing prompt seronegativity after the start of treatment is a critical factor for achieving SVR, and thus the possible correlation between an early virological response and genetic sequence variation of the HCV has been studied. In particular, amino acid substitutions in the HCV core region at positions 70 and 91 or multiple mutations detected in the IRRDR of the HCV NS5A region are useful markers for predicting EVR and subsequent SVR.

To date, the influence of several single amino acid substitutions and accumulation of these changes in the viral genome on the effect of IFN-based anti-HCV therapy has been examined. Since interactions between host and viral proteins in infected cells may influence the therapeutic effect of an antiviral agent, we focused on the association of structural polymorphism of a viral protein with the effect of Peg-IFN/RBV combination therapy in this study. Our results suggest that polymorphism analysis of secondary structure deduced from sequence variations in the HCV NS3 amino-terminal region can be used to predict viral responses to this therapy.

Amino acid sequences of the HCV NS3 amino-terminal region, which encodes a serine protease, vary greatly among HCV isolates. Interactions between HCV NS3 and host proteins may influence both oncogenesis and immunity, and thus elucidation of the biological significance of these interactions could result in a new prognostic marker for HCC or a predictive marker for anti-HCV therapy. First, HCV NS3 interacts with the p53 tumor suppressor to suppress p53-dependent apoptosis or p21 transcriptional activity [Ishido and Hotta, 1998; Kwun et al., 2001; Deng et al., 2006]. Transfection of a plasmid expressing the amino-terminal portion of HCV NS3 induces cell transformation in vitro, and transplanted cells proliferate with sarcoma-like features in vivo [Sakamuro et al., 1995]. These findings suggest that NS3 may be involved in the oncogenic pathway in HCV infection. We have shown that the secondary structure of the 120-residue amino-terminal region of NS3 (1,027–1,146) is classifiable into two major groups: A and B. This region encodes a serine protease and also includes p53-binding sites. Our

TABLE III. Virological Responses in Subjects With Different Polymorphisms in the Secondary Structure of HCV NS3

	EVR*	ETR**	SVR*
Group A (n = 28)	19 (68%)	23 (82%)	18 (64%)
Group B (n = 40)	17 (43%)	25 (63%)	15 (38%)

EVR: early virological response at 12 weeks after the start of treatment.
ETR: virological response at the end of treatment.
SVR: sustained virological response 24 weeks after completion of treatment.
*P < 0.05.
**P = 0.08; χ^2 test.

TABLE IV. Treatment Outcome of Cases With a Double Wild-Type Core Region and Different HCV NS3 Structural Polymorphism

	Group A (%)	Group B (%)	P
SVR (n = 12)	10 (83)	2 (17)	0.02[a]
Non-SVR (n = 10)	3 (30)	7 (70)	

SVR, sustained virological response.
[a]χ^2 test.

TABLE V. Pre-Treatment HCV RNA Levels Measured by
Real-Time PCR for Subjects With Different HCV NS3
Structural Polymorphism

	Group A	Group B	P
SVR (n = 33)	5.78 ± 1.05	6.13 ± 0.71	NS[a]
Non-SVR (n = 35)	6.33 ± 0.59	6.32 ± 0.55	NS[a]
Total (n = 68)	5.98 ± 0.94	6.25 ± 0.62	NS[a]

SVR, sustained virological response. NS, not significant.
[a]t test.

previous cross-sectional studies revealed that the prevalence of group B infection is significantly higher in HCC cases than in non-HCC cases [Ogata et al., 2003], and that the group B infection is an independent risk factor for development of HCC in patients with chronic HCV infection [Nishise et al., 2007]. Second, NS3 interacts with host proteins associated with IFN signaling and thus influences cellular immunity. Since the serine protease encoded by the amino-terminal region of NS3 inhibits the IFN-signaling pathway, polymorphism of this region is likely to influence the effect of Peg-IFN/RBV combination therapy.

Several factors associated with the virological response to this therapy are well known, with adherence to both IFN and RBV strongly influencing outcome [Pearlman, 2004; Arase et al., 2005; Yamada et al., 2008]. In this study, we analyzed 75 cases in which >80% of the scheduled dosage of both drugs was administered. Of these cases, 28 (37%) and 40 (53%) were infected with group A and B isolates, respectively, which were similar rates to those for the 139 cases in the overall study. Age, gender, viral load before treatment, ALT level, proportion of fibrosis stage and adherence to Peg-IFN and RBV did not differ between the group A and B cases. However, the frequencies of SVR and EVR were significantly higher in group A, and those for non-EVR and non-SVR were significantly higher in group B. The results suggest that infection with the group B isolate, which correlates with a higher rate of HCC, is resistant to Peg-IFN/RBV therapy. The pre-treatment viremia status in the 68 cases with group A or B isolates showed no significant differences between the two groups of patients. Therefore, these results suggest that the secondary structure of the HCV NS3 amino-terminal region may be useful for prediction of the outcome of Peg-IFN/RBV combination therapy. In this initial study setting, the relationship of these polymorphisms to the frequency of rapid viral response at 4 weeks after the start of treatment was not evaluated. It will be important to assess this relationship in a future study.

The polymorphism in HCV core region (Arg70/Leu91) is a useful predictive marker for virological responses in Peg-IFN/RBV therapy [Akuta et al., 2007]. Interestingly, a combined analysis of polymorphisms of the core region (which encodes a structural protein) and HCV NS3 (a nonstructural protein) improved the prediction rate. Therefore, analysis of NS3 polymorphism in combination with the core structural polymorphism
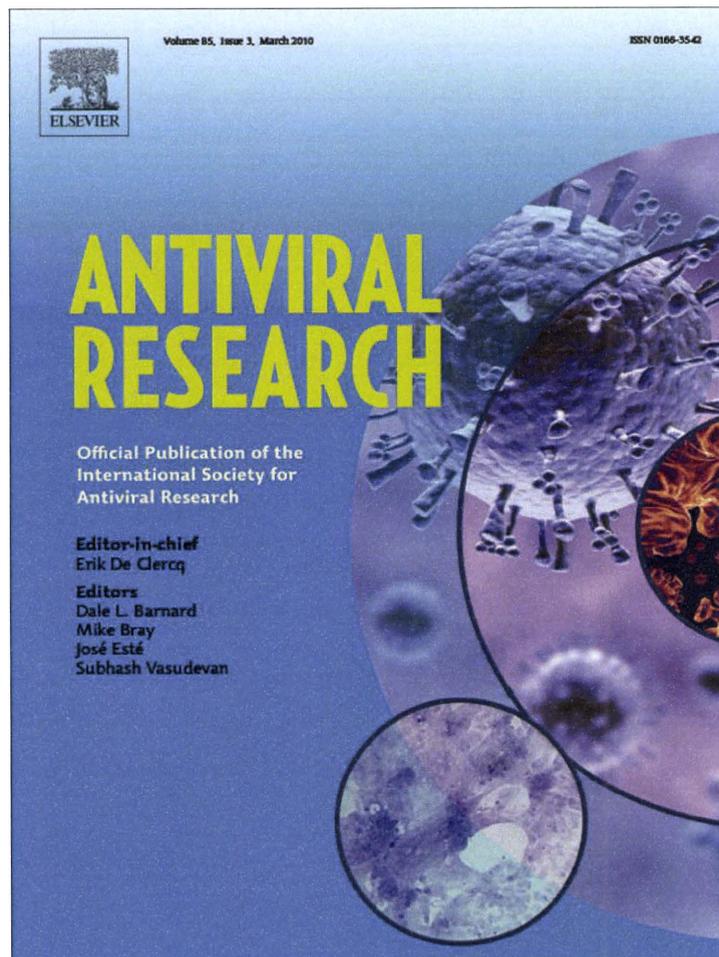
appears to improve prediction of the outcome of Peg-IFN/RBV therapy. A larger, multi-center prospective study would be necessary to validate the present results. In conclusion, the results of this study suggest that secondary structure polymorphism in the amino-terminal region of HCV NS3 is a useful predictive marker of the effect of Peg-IFN/RBV combination therapy for chronic hepatitis C. Although the present findings are clinically important, and will be helpful for predicting the outcome of Peg-IFN/RBV therapy, further in vitro studies will be needed to elucidate the molecular mechanism underlying the association of HCV NS3 polymorphisms with clinical outcome.

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Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin

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ABSTRACT

Ribavirin (RBV), a nucleoside analogue, is used in the treatment of hepatitis C virus (HCV) infection in combination with interferons. However, potential mechanisms of RBV resistance during HCV replication remain poorly understood. Serial passage of cells harboring HCV genotype 2a replicon in the presence of RBV resulted in the reduced susceptibility of the replicon to RBV. Transfection of fresh cells with RNA from RBV-resistant replicon cells demonstrated that the RBV resistance observed is largely replicon-derived. Four major amino acid substitutions: T1134S in NS3, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B region, were identified. Site-directed mutagenesis of these mutations into the replicon indicated that Y2471H plays a role in the reduced susceptibility to RBV and leads to decrease in replication fitness. The results, in addition to analysis of sequence database, suggest that HCV variants with reduced susceptibility to RBV identified are preferential to genotype 2a.

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1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, such as chronic hepatitis, cirrhosis and hepatocellular carcinoma, affecting approximately 170 million people worldwide (WHO, 2000). HCV belongs to the genus Hepacivirus of the family Flaviviridae, and its genome is a single-stranded, positive-sense RNA of 9.6 kb. HCV displays marked genetic heterogeneity and is currently classified into 6 major genotypes and more than 50 subtypes. HCV genotypes have regional distribution and, of those, genotypes 1 and 2 are detected worldwide (Simmonds et al., 2000). Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN-α) in combination with ribavirin (RBV). However, approximately 50% of treated patients infected with genotype 1 do not respond or show only a partial or transient response and treatment is limited by the adverse effects of both agents (Manns et al., 2001; Fried et al., 2002).

HCV replication is associated with a high rate of mutation that gives rise to a mixed and changing population of mutants, known as quasispecies (Martell et al., 1992; Domingo, 1996). The characteristic of HCV may have important implications concerning viral persistence, pathogenicity and resistance to antiviral agents (Domingo, 1996; Forns et al., 1999; Farci and Purcell, 2000). Most previous studies on the possible relationship between HCV quasispecies and response to chemotherapy have been carried out in HCV genotype 1 patients. In addition, several studies have successfully demonstrated that the HCV subgenomic replicon is derived from genotype 1, which typically contains HCV nonstructural genes placed downstream of the neomycin phosphotransferase gene, in selecting variants resistant to antiviral inhibitors. Two studies have demonstrated the identification of HCV genotype 1 mutants responsible for decreased sensitivity to RBV (Young et al., 2003; Pfeiffer and Kirkegaard, 2005). However, little is known about the generation of genotype 2 isolates resistant to antivirals including RBV, or the molecular mechanisms that confer resistance.

In this study, we report the generation and characterization of HCV genotype 2a replicon variants with reduced susceptibility to RBV. The impacts of major amino acid substitutions observed on RBV susceptibility and viral replication capacity were also examined.

2. Materials and methods

2.1. Compounds

RBV and IFN-α were purchased from MP Biomedicals (Eschwege, Germany) and Dainippon Sumitomo Pharma (Osaka, Japan), respectively.

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Table 1
Primers used for PCR and nucleotide sequencing.

Region	Primer name	Nucleotide sequence	Position[a]	Polarity
NS3–4A–4B region	PCR primers			
	JF1S	GAAAAACACGATGATACCATG	1756–1776	Sense
	JF1AS	AACCCAGTCCCACACGTC	4650–4633	Antisense
	Sequencing primers			
	JF5S	CACTTTCAGTGACAACAGCA	2322–2341	Sense
	JF6S	CGCCACCGACGCCCTCATGA	3003–3022	Sense
	JF4AS	CTGGTCGACAACGGACTGGT	4109–4090	Antisense
NS5A–NS5B region	PCR primers			
	JF2S	TGCTCCGGATCCTGGCTC	4612–4629	Sense
	JF2AS	TACCTAGTGTGTGCCGCTCTA	7786–7806	Antisense
	Sequencing primers			
	JF3S	TGAGGTCCATGCTAACAGA	5209–5228	Sense
	JF4S	TCGAGGGGGAGCCTGGAGAT	5870–5889	Sense
	JF3AS	GAGTGTCTAACTGTTTCCCAG	7220–7200	Antisense

[a] Reference strain: Gene Bank accession no. AB114136.

2.2. Cell culture

The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with MEM non-essential amino acids (Invitrogen) 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. HCV replicon cells JFH-1/4-1 (Miyamoto et al., 2006), which are Huh-7-derived cells carrying a subgenomic replicon of JFH-1 (Kato et al., 2003) were maintained in the Huh-7 medium as above, supplemented with 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan).

2.3. Quantification of HCV RNA

Total RNA was isolated from harvested cells using Trizol (Invitrogen). Copy numbers of the viral RNA were determined by real-time RT-PCR involving single-tube reactions and performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA), as described previously (Aizaki et al., 2003; Takeuchi et al., 1999).

2.4. Cell viability assay

Cells were seeded at density of 5×10^4 cells/well in 24-well plates and RBV at various concentrations was added on the next day. Cultures were further incubated for 3 days at 37 °C under a humidified 5% CO_2 atmosphere. Cytotoxicity assay was performed by Cell Titer-GLO™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were quantified with LUMAT LB 9501 (Berthold Technologies, Bad Wilbad, Germany).

2.5. Isolation and nucleotide sequencing of HCV nonstructural regions from replicon-containing cells

Total cellular RNA was isolated from replicon cells with or without RBV treatment as described above. cDNA synthesis was carried out by using Super ScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen) with primer JF1AS for NS34AB region and JF2AS for NS5AB region. Two cDNA fragments, corresponding to NS3–NS4B and NS5A–NS5B regions, were amplified by PCR using Takara EX Taq DNA polymerase (Takara BIO, Kyoto, Japan) and specific primers (Table 1; Date et al., 2004). PCR products were subcloned into pGEM-T vector (Promega) and inserts were sequenced using QIA prep[R] Spin Mini Prep kit (QIAGEN, Tokyo, Japan). Nucleotide sequences were analyzed with the 3100 Avant Genetic Analyzer (PE Applied Biosystems).

2.6. Plasmid constructions

pSGR-JFH1/luc, a subgenomic replicon construct with luciferase reporter derived from HCV genotype 2a JFH-1 isolate was reported previously (Miyamoto et al., 2006). Mutant replicons carrying T1134S, P1969S, V2405A, and Y2471H were created by PCR-based site-directed mutagenesis and cDNA fragments containing the above mutations were inserted into the corresponding sites of pSGR-JFH/luc. All plasmids were confirmed by sequencing the entire PCR-generated inserts. Each mutant is referred to by the original amino acid (one letter code) followed by the residue positions within the complete open reading frame of full-length JFH-1 and the substituted amino acid (one letter code).

2.7. RNA synthesis and transient replication assay

The transient replication assay method was described previously (Kato et al., 2005). Briefly, purified plasmids of pSGR-JFH1/Luc, -JFH1/Luc-T1134S, -JFH/Luc-P1969S, -JFH/Luc-V2405A and -JFH/Luc-Y2471H were linearized with XbaI and were treated with proteinase K and SDS, followed by phenol–chloroform extraction. RNA was synthesized with Ampliscribe™ T7 Transcription Kits (Epicentre BIO Technologies, Madison, WI, USA). Each transcribed RNA (5 μg) was electroporated into 2.5×10^6 of Huh7 cells pulsed at 290 mV, 975uFD with Gene pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transfected cells were resuspended in growth medium without selection antibiotics and were plated in 24-well plates at 6×10^4 cells per well. Cells were harvested at different time points post-transfection and were lysed in Passive Lysis Buffer (Promega). Luciferase activity in cells was determined using the Luciferase Assay System (Promega).

3. Results

3.1. Selection of replicon variants derived from genotype 2a with reduced susceptibility to RBV

It has been reported that RBV inhibits HCV RNA replication in Huh-7 cells bearing the viral subgenomic replicon RNAs with the EC_{50} (50% effective concentration) values of 15–225 μM (Zhou et al., 2003; Tanaka et al., 2004; Kato et al., 2005; aus dem Siepen et al., 2007). To select for RBV-associated replicon variants, cells bearing a genotype 2a HCV replicon were serially passed in the presence of 200 μM RBV as well as 1 mg/ml G418. After 20-week treatment, variant cells were then tested for RBV resistance. HCV RNA levels were determined after a 72-h incubation with various concentrations of RBV in the absence of G418, and about 5-fold-reduced susceptibility to RBV was observed in the variant replicon
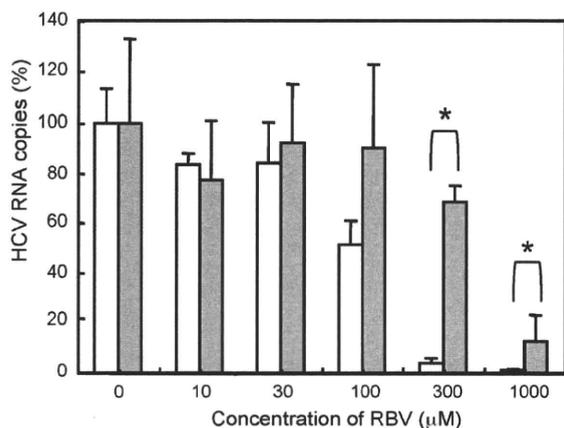
Fig. 1. Inhibitory effect of RBV on HCV RNA levels in genotype 2a replicon cells after long-term treatments with RBV. The replicon cells were serially passaged in 0 or 200 μM RBV for 20 weeks. The cells were then split and incubated with fresh RBV at various concentrations in the absence of G418 for 3 days, followed by the determination of HCV RNA. Clear bars, passage in the absence of RBV: gray bars, passage in the presence of RBV. HCV RNA copies per microgram of total RNA were normalized as percentages of those of untreated (RBV 0 μM). Each data point is presented as the mean of three independent determinations with standard deviation. *$p < 0.05$.

cells; the EC_{50} values for the variant and wild-type replicon cells were 470 and 102 μM, respectively (Fig. 1). Comparable cytotoxic effects of RBV were observed against wild-type and variant replicon cells, with the CC_{50} (50% cytotoxicity concentration) values of 151 and 156 μM, respectively (data not shown).

3.2. Mapping RBV resistance to cell line or replicon RNA

To test whether reduced susceptibility to RBV in the variant cells observed as above was due to the appearance of mutations within the viral RNA or was cell-derived, total RNAs from the variant and wild-type replicon cells were extracted and used for retransfection of naïve Huh7 cells. Retransfected cells resistant to G418 were established after 4 weeks of cultures in the presence of 1 mg/ml G418 and were assessed for HCV RNA replication sensitivity to RBV (Fig. 2A). HCV RNA levels in the cells obtained from the wild-type replicon were inhibited by 56, 89 and 97% with 100, 300 and 1000 μM RBV, respectively. By contrast, the culture retransfected with RNA derived from the variant replicon cells exhibited inhibition levels of 13, 29 and 89% with the corresponding concen-

trations of RBV. EC_{50} values were calculated to be 93 and 449 μM, respectively. We confirmed the presence of replicon mutations, as described below, in the cells retransfected with RNA derived from the variant replicon cells.

In order to explore the possibility for cell-derived resistance, both wild-type and variant replicon cells were cured of viral RNAs by IFN treatment; cells were passaged with media containing 100 IU/mL IFN-α in the absence of G418 for 2 months. To compare RBV sensitivity, cured cells were transiently transfected with the wild-type JFH-1 subgenomic replicon RNA and were treated with various concentrations of RBV for 72 h. Similar anti-HCV effects of RBV were observed in the cured cells derived from wild-type and variant replicons, with the EC_{50} values of 147 and 118 μM, respectively (Fig. 2B). Thus, the results suggest that the RBV resistance observed may arise by mutations in the replicon rather than by changes in the cells.

3.3. HCV mutations in replicon variant with reduced susceptibility to RBV

It has been reported that mutations in RNA virus genomes responsible for RBV resistance are mostly present in the coding region for the viral RNA-dependent RNA polymerase (RdRp). On the other hand, it is known that RBV works as an RNA mutagen to generate rapidly mutating viral RNA and that NS5B RdRp and other nonstructural proteins in HCV are involved in the viral replication complex, playing key roles in genome replication. Therefore, we sequenced the coding regions for NS3 through NS5B proteins of the replicon molecules in order to determine whether mutations associated with RBV resistance were generated. As shown in Table 2, there were numerically more synonymous and non-synonymous mutations in the RBV-resistant variant replicon cells (RBV treatment) when compared with untreated replicative conditions (No-treatment) across most regions examined. Mutation frequencies of NS3, NS4B and NS5A regions of RBV treatment were significantly higher than those of No-treatment. The total number of synonymous mutations in the RBV-resistant variant replicon cells was 3 times higher than that under untreated replicative conditions, and the number of non-synonymous mutations in the RBV-resistant variant replicon cells was 1.5 times higher than that under untreated replicative conditions. The number of both synonymous and non-synonymous mutations (NS3, NS4B, NS5A and NS5B regions) in the RBV-resistant replicon cells was greater than that in the control cells. We also found a large number of transition
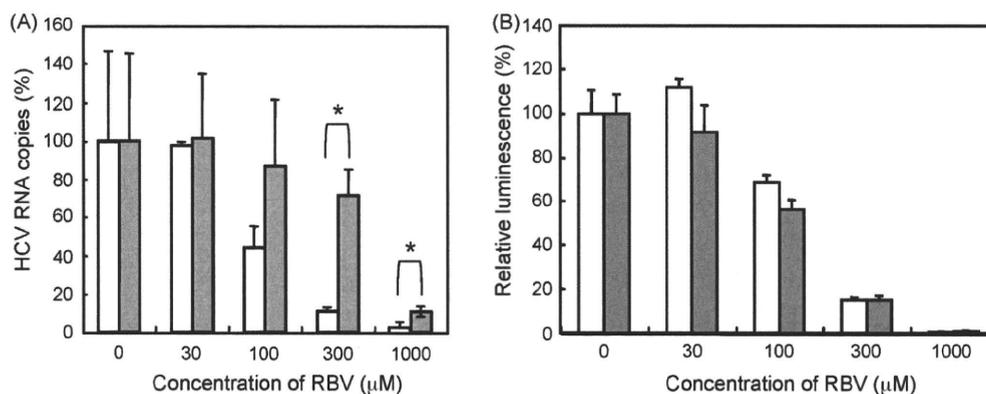

Fig. 2. Testing for replicon-derived resistance (A) or for cell-derived resistance (B). (A) Total RNA from RBV-resistant- or wild-type replicon cells was transfected into naïve Huh7 cells. After selection in 1 mg/ml G418 for 4 weeks, re-established replicon cells, wild-type derived (clear bars) and RBV resistance derived (gray bars), were treated with increasing concentrations of RBV in the absence of G418 for 3 days. HCV RNA copies per microgram total RNA were assessed and the levels from wild-type cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations. *$p < 0.05$. (B) RBV-resistant- or wild-type replicon cells were cured by passage in IFN-α in the absence of G418. Cured cells were transiently transfected with the replicon RNA derived from pSGR-JFH1/luc. Transient replication assay of transfectants derived from wild-type (clear bars) and RBV resistance (gray bars) was performed after treatment with various concentrations of RBV for 72 h. The values for wild-type-derived cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations.

Table 2
Mutation frequencies in HCV NS regions after 20-weeks culture with or without RBV treatment.

Region	nt length	No-treatment			RBV treatment		
		No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})	No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})
NS3	1893	1.7 ± 2.1	2.3 ± 1.5	2.1	4.7 ± 2.4	6.5 ± 2.5	5.9[b]
NS4A	165	1.0 ± 1.0	0.3 ± 0.6	8.1	0.3 ± 0.5	0.5 ± 0.9	4.4
NS4B	780	1.3 ± 1.2	0.3 ± 0.6	2.1	2.3 ± 1.5	2.5 ± 1.2	4.7[c]
NS5A	1380	4.0 ± 1.2	2.0 ± 1.2	4.3	5.9 ± 1.2	6.2 ± 2.4	12.2[c]
NS5B	1773	4.5 ± 1.5	2.3 ± 1.5	3.8	4.8 ± 1.8	4.2 ± 1.1	9.0
NS3–NS5B	5991	12.5 ± 2.7	7.3 ± 2.7	–	17.8 ± 4.5	20.1 ± 4.6	–

[a] Values are means ± standard deviations.
[b] $p < 0.05$ relative to No-treatment by the unpaired t-test.
[c] $p < 0.01$ relative to No-treatment by the unpaired t-test.

mutations in RBV-resistant cells, particularly G-to-A and C-to-U transitions, as expected from previous studies. Although mutations were distributed throughout nonstructural regions, four major amino acid substitutions; T1134S in the NS3 region, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B, not seen in wild-type cells were observed in most of the subclones among RBV-resistant replicon cells. T1134S, P1969S, V2405A, and Y2471H were present, respectively, in 7 of 11, 6 of 11, 8 of 13, and 7 of 13 PCR subclones sequenced.

3.4. Effects of T1134S, P1969S, V2405A, and Y2471H on RBV susceptibility

To test the possibility that any of the four mutations as identified confer resistance to RBV, we introduced these mutations individually into the JFH-1 subgenomic replicon containing a luciferase reporter gene. Cells transfected with mutant- or wild-type replicon RNA grown in the presence of various concentrations of RBV for 2 or 3 days. As demonstrated in Fig. 3A, the replication levels of all four mutant replicons (SGR-JFH1/Luc-T1134S, -P1969S, -V2405A, and -Y2471H) in the presence of 125 or 500 μM RBV were higher than those of the wild-type replicon. In particular, the Y2471H mutant significantly reduced susceptibility to RBV; replication levels of SGR-JFH1/Luc-Y2471H were 3–5-fold higher when compared to those of wild-type under the present assay conditions.

The relative replication activity of these mutant replicons was further determined in 3-day replication assay without drug treatment (Fig. 3B). All mutant replicons exhibited reduced efficiency

relative to the wild-type replicon. Levels of the Y2471H-mutated replicon were approximately 30% of those of the wild-type, thus suggesting that replicon mutants with reduced sensitivity to RBV are associated with decreased replication fitness.

4. Discussion

It is generally accepted that, during chemotherapy against viral infection, high rates of viral replication and high frequencies of mutation lead to generation of drug-resistant mutants. Although several potential mechanisms for the inhibition of HCV replication by RBV have been proposed, the molecular mechanisms involved in the generation of RBV-resistant HCV remain poorly understood.

This study found that long-term treatment of HCV JFH-1-derived replicon cells with RBV leads to selection of preferential mutations in NS3 (T1134S), NS4B (P1969S), NS5A (V2405A) and NS5B (Y2471H) genes. Each mutation only required a single nucleotide change, and P1969S, V2405A and Y2471H are transition mutations, which are known to be commonly caused by incorporated RBV. Site-directed mutagenesis of these mutations into the replicon demonstrated that Y2471H plays a role in reduced susceptibility to RBV.

Crystal structure information revealed that HCV RdRp is organized into an arrangement with palm, fingers, and thumb subdomains (Lesburg et al., 1999). Residue 2471 (the 33rd position of NS5B) is present in the N-terminal loop region that bridges the fingers. Although this site is apparently distant from the active site of the polymerase in the palm region, it has been reported
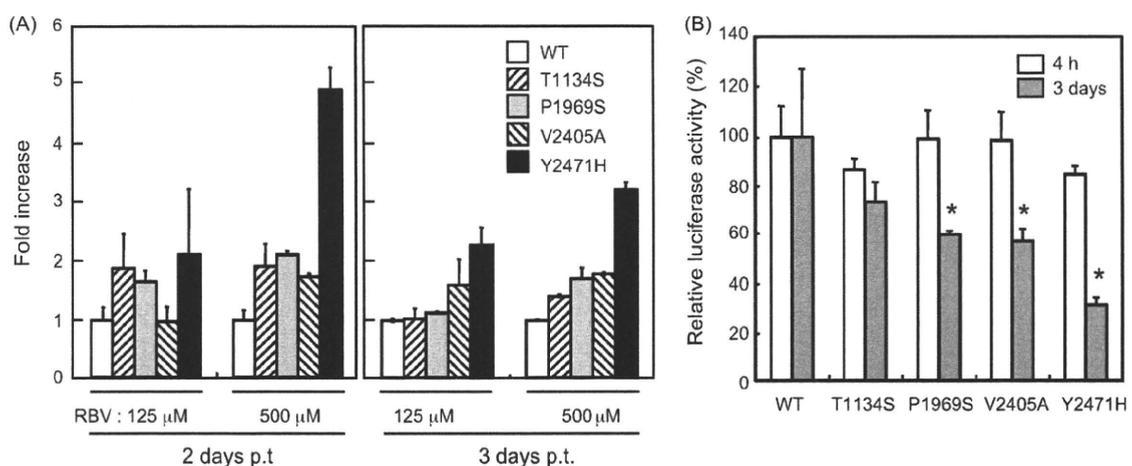

Fig. 3. Impact of major mutations in NS3–NS5B regions on RBV susceptibility (A) and replication capacity (B). Mutated replicons carrying single residue substitutions (T1134S, P1969S, V2405A, and Y2471H) were constructed and used for transient replication assay. Cells were transfected with either wild-type (WT) or with mutant replicon RNA in the absence or presence (125, 500 μM) of RBV. Luciferase activity was assessed at 4 h, 2 days and 3 days post-transfection (p.t.). (A) Luciferase activities of WT were set at 1, and the fold increases in the activities of mutants were plotted. (B) Luciferase activities in the absence of RBV at 4 h and 3 days post-transfection were shown. The activities of mutants were normalized as percentages of the WT activities. Data from triplicate samples were averaged and indicated with standard deviations. *$p < 0.05$ against WT.