

Mutational diversity of NS5A and NS3 during triple therapy (telaprevir, pegylated-interferon- α 2b and ribavirin) for genotype 1b chronic hepatitis C: The Kobe Hepatitis Therapeutic Group

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Received November 2, 2013; Accepted February 26, 2014

DOI: 10.3892/ijmm.2014.1706

Abstract. Telaprevir, a non-structural (NS)3/4A protease inhibitor, is a direct-acting antiviral drug that inhibits viral replication. Triple therapy with telaprevir, pegylated interferon, and ribavirin is a standard therapeutic regimen for patients with genotype 1b chronic hepatitis C virus (HCV) infection and a high viral load. Several factors, including mutations in the NS5A gene, are important predictors of the efficacy of interferon therapy. In this study, we examined the mutational diversity of NS5A and its impact on the efficacy of triple therapy. We enrolled patients with genotype 1b chronic HCV infection and a high viral load (31 males/17 females; mean age, 57.6 years), who were treated with triple therapy. This study was conducted at Kobe University Hospital and at three affiliated hospitals in Hyogo prefecture, Japan, between November 2011 and June 2013. A sustained viral response after 12 weeks (SVR12) was achieved in 37/48 patients (77%). Based on intent-to-treat analysis, SVR12 was significantly greater in patients with the major allele than in those with the minor allele for the IL28B single nucleotide polymorphism (SNP; 88 vs. 56%; $P<0.05$). The prevalence of the V2334I mutation in NS5A was significantly higher in patients who achieved SVR12, while that of

G2356E was significantly higher in patients who did not achieve SVR12 ($P<0.05$). Mutations in the NS3 region that are thought to confer resistance to telaprevir were detected in 3/27 patients who achieved SVR12 (Val36, $n=3$) and in 5/10 patients who did not achieve SVR12 (Val36, $n=4$; Thr54, $n=1$). In conclusion, the IL28B SNP and mutations in the NS5A region were associated with the therapeutic response to triple therapy. Half of the patients who did not achieve SVR12 had mutations conferring resistance to telaprevir. However, pre-existing mutations in NS3 did not affect the efficacy of triple therapy.

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease. An estimated 170 million individuals worldwide are infected with HCV, including 1.5 million individuals in Japan (1,2). The main goal of treatment for chronic HCV is to prevent the progression to cirrhosis and the development of hepatocellular carcinoma by eradicating the virus. Interferon (IFN)-based therapy was first used in 1992 (3), and the treatment regimens have improved since then. In Japan, a combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) has been used since 2004 (4). However, almost half of the patients treated with this combination do not achieve a sustained viral response (SVR), despite long-term therapy (5).

Several factors have been reported to influence the efficacy of PEG-IFN/RBV therapy. Host factors associated with unfavorable outcomes, include advanced age, the female gender and advanced fibrosis. Additionally, the IL28B single nucleotide polymorphism (SNP) has been reported to be a strong genomic predictor of the efficacy of PEG-IFN/RBV therapy, such that the IL28B SNP is routinely examined before beginning any treatment regimen (6). As regards viral factors, a mutation at amino acid 70 of the core region of HCV (core 70) is an important predictor of the therapeutic efficacy (7). The

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Key words: chronic hepatitis C, telaprevir, pegylated interferon/ribavirin therapy, NS5A

non-structural (NS)5A protein combines with the core protein in the formation of the viral particle. It has been reported that several regions in domain II of NS5A are associated with the therapeutic efficacy of IFN monotherapy and PEG-IFN/RBV combination therapy. For example, in 1996, it was reported that a large number of mutations in the IFN sensitivity-determining region (ISDR) (amino acids, 2209-2248) were associated with the SVR to IFN monotherapy in Japanese patients with genotype 1b HCV (8). In 2008, it was reported that mutations in the IFN-RBV resistance-determining region (IRRDR) (amino acids, 2334-2379) were also associated with the SVR to combined PEG-IFN/RBV therapy (9).

Telaprevir (TPV), an NS3/4A protease inhibitor, is the first commercially available direct-acting antiviral (DAA) that directly inhibits viral replication. Although TPV/PEG-IFN/RBV combination therapy has achieved a viral eradication rate of up to 70-80% (10), 20-30% of patients did not achieve SVR during treatment due to side-effects (e.g., skin rash and renal dysfunction), loss of compliance and the development of antiviral resistance.

Thus, taking the above data into consideration, we conducted a collaborative study in Kobe, Japan to identify which factors, including NS5A mutations, are associated with the SVR in patients with genotype 1b HCV and a high viral load who were treated with TPV/PEG-IFN/RBV.

Materials and methods

Patients and sample collection. Serum samples were collected from patients with genotype 1b chronic HCV infection and a high viral load. Overall, 48 patients (31 males/17 females; mean \pm standard deviation age, 57.7 \pm 8.3 years) were enrolled in this study. The patients were treated with TPV/PEG-IFN/RBV combination therapy for 12 weeks followed by PEG-IFN/RBV for 12 weeks. TPV (Mitsubishi Tanabe Pharma Corp., Osaka, Japan) was orally administered at doses of 750 or 500 mg 3 times daily (every 8 h). PEG-IFN (Pegintron®; Schering-Plough, Innishannon, County Cork, Ireland) was subcutaneously injected once weekly (1.5 μ g/kg). RBV was orally administered at 400-800 mg daily.

The serum HCV-RNA status was assessed at 4 weeks, at the end of treatment, and at 6 months after the end of treatment. Serum samples were collected and stored at -80°C until virological examination. The rapid virological response (RVR) was defined as undetectable HCV-RNA at 4 weeks. SVR12 was defined as persistent undetectable serum HCV-RNA and normal serum alanine aminotransferase levels at 12 weeks after the end of treatment.

This study was conducted between November 2011 and June 2013 at Kobe University Hospital and at 3 affiliated hospitals in Hyogo prefecture. The study was approved by the Ethics Committee of Kobe University Hospital. Written informed consent was obtained from each patient prior to enrollment in the study.

NS3 and NS5A sequence analysis. HCV-RNA was extracted from 140 μ l of serum using a commercial kit according to the manufacturer's instructions (QIAamp viral RNA kit; Qiagen, Tokyo, Japan). The NS3 and NS5A regions of the HCV genome were amplified and sequenced by nested RT-PCR

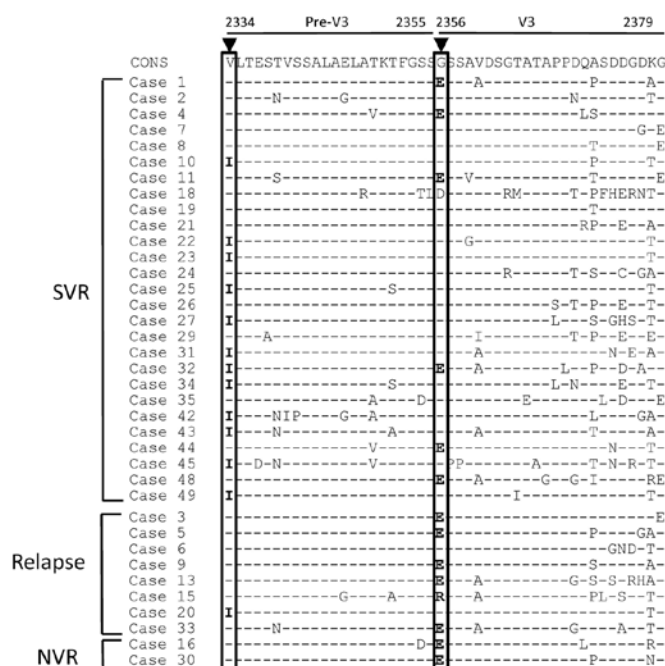


Figure 1. Amino acid alignment in the IFN-RBV resistance-determining region (IRRDR) region. V2334I and G2356E mutations were frequent in patients who achieved SVR12 and in those who did not achieve SVR12, respectively.

using primer sets, as previously described (9,11). The amino acid sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan).

Statistical analysis. All statistical analyses were performed using SPSS software version 16 (SPSS Inc., Chicago, IL, USA). P-values of <0.05 were considered to indicate statistically significant differences.

Results

Baseline characteristics and on-treatment responses. Among the 48 patients, 22 were treatment-naïve and 26 were receiving IFN-based therapy. Of these latter 26 patients, 17 experienced relapse on prior therapy and 9 were non-responders. As regards the IL28B SNP (rs8099917), 32 patients had the major allele (TT) and 16 patients had the minor allele (TG/GG). Overall, 37/48 patients (77%) achieved SVR12 following triple therapy. The mean age of the patients who achieved SVR12 was less than that of those who did not achieve SVR12, although the difference was not significant. The SVR12 rate was significantly lower in non-responders to previous therapy (44%) than in relapsed patients (82%) and treatment-naïve patients (86%) (P<0.05). The SVR12 rate was significantly higher in patients with the IL28B major allele than in patients with the minor allele (88 vs. 56%; P<0.05).

Effect of mutations in the core protein or NS5A region on therapeutic outcomes. We then sought to identify factors associated with the SVR12 by intent-to-treat analysis. The frequency of mutations amino acid 70 in the core region of HCV and the number of mutations in the ISDR did not differ significantly between patients who achieved SVR12 and

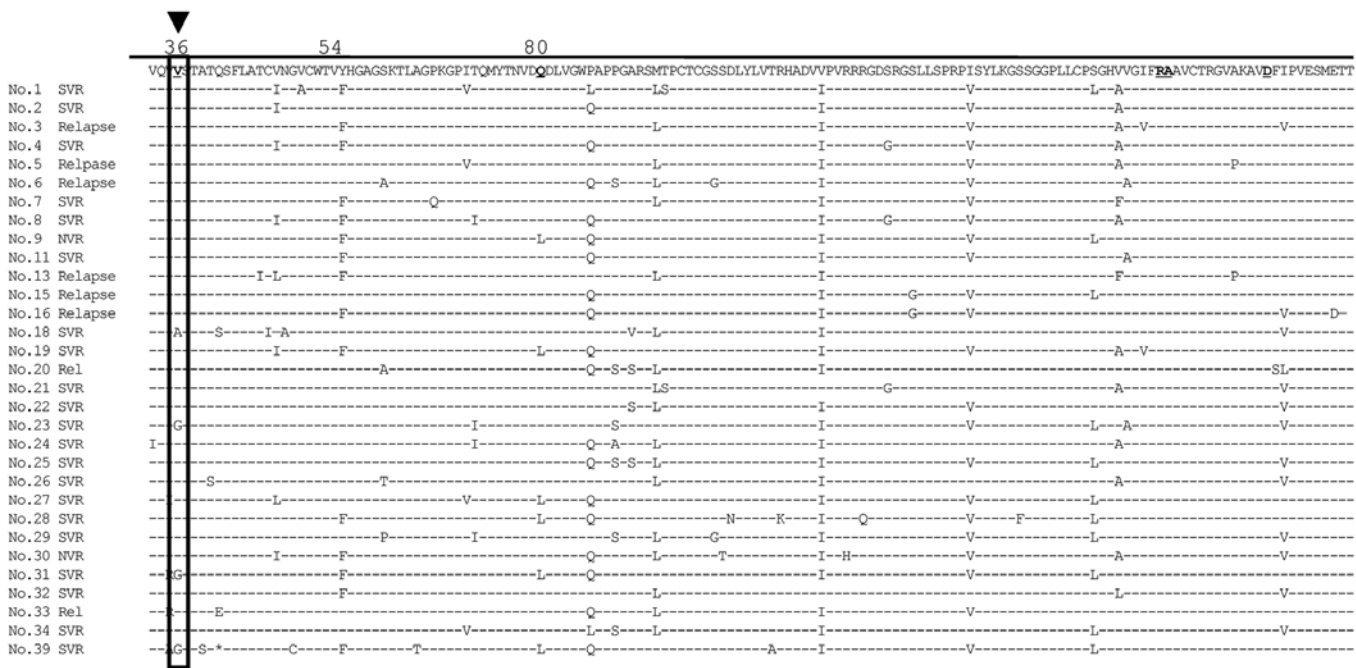


Figure 2. Amino acid alignment in the NS3 region before treatment. Three patients who achieved SVR12 had the Val36 mutation in the NS3 region.

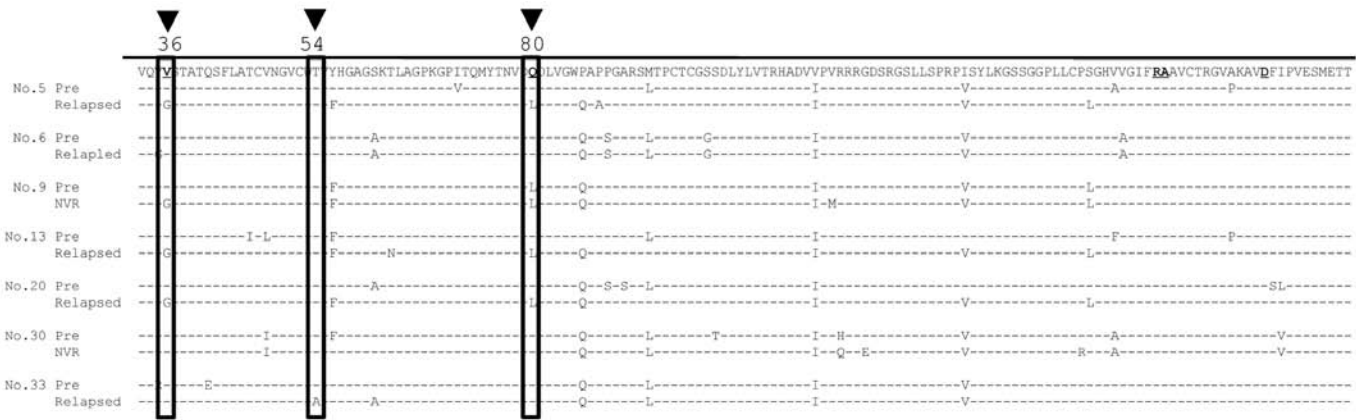


Figure 3. Amino acid alignment in the NS3 region of 7 patients is shown. Alignment before and after treatment was arranged in 2 lines. Four patients who did not achieve SVR12 (case nos. 5, 9, 13 and 20) had Val36 mutations and 1 patient (case 33) had a Thr54 mutation, which are thought to confer resistance to TVR.

those who did not achieve SVR12 (Table I). The amino acid alignment in IRRDR is shown in Fig. 1. The mean number of mutations in the IRRDR did not differ significantly between patients who achieved SVR12 and those who did not achieve SVR12 (5.1 ± 2.9 vs. 4.4 ± 2.2). However, the frequencies of 2 amino acid mutations differed significantly the 2 groups of patients. The V2334I mutation was significantly more frequent in patients who achieved SVR12 than in those who did not achieve SVR12 (44 vs. 10%; $P < 0.05$), and the frequency of the G2356E mutation was lower in patients who achieved SVR12 than in those who did not achieve SVR12 (22 vs. 70%; $P < 0.05$).

Mutations in the NS3 region before and after treatment. We then examined whether mutations in the NS3 region were associated with antiviral resistance, using sera obtained before and after therapy. Three patients who achieved SVR12 had the Val36 mutation in the NS3 region, which is thought to confer

resistance to TVR (Fig. 2). In addition, 5/10 patients who did not achieve SVR12 had mutations conferring resistance to TVR, including 4 patients with Val36 and 1 with Thr54 (Fig. 3).

Discussion

In recent years, many DAAs have been developed and thus clinical trials are examining their efficacy for the treatment of chronic HCV infection (12,28). TPV was approved in Japan in November 2011, and the triple therapy (TVR/PEG-IFN/RBV) has become a standard regimen for patients with genotype 1b chronic HCV infection with a high viral load. In the US, TVR was approved by the Food and Drug Administration in May 2011 for use in combination with PEG-IFN/RBV for the treatment of genotype 1b chronic HCV infection (13). The SVR rate in patients with genotype 1 HCV treated with triple therapy was 61-69% in the PROVE1 and PROVE2 studies published in

Table I. Comparison between patients who achieved SVR and those who did not (no SVR).

Factor	SVR	No SVR	P-value
Number of patients	37 (77%)	11 (23%)	
Age	56.0±9.2	59.5±12.4	NS
Gender (M/F)	24/13	7/4	NS
Previous therapy			
Naïve	19 (86%) ^a	3 (14%)	
Relapse	14 (82%) ^a	3 (18%)	
NVR	4 (44%)	5 (56%)	
IL28B SNP			
TT	28 (88%)	4 (12%)	<0.05
TG/GG	9 (56%)	7 (44%)	
Core 70 wild	17/28	5/11	
ISDR ≥1	5/16 (31%)	4/9 (44%)	NS
IRRDR ≥4	19/27 (70%)	6/10 (60%)	NS
V2360A and/or K2378T	17/27 (63%)	5/10 (50%)	NS
V2334I	12/27 (44%)	1/10 (10%)	0.039
G2356E	6/27 (22%)	7/10 (70%)	0.016

^aP<0.05, compared to NVR. SVR, sustained viral response; SNP, single nucleotide polymorphism; NS, not significant; NVR, no viral response; ISDR, IFN sensitivity-determining region; IRRDR, IFN-RBV resistance-determining region.

2009 (10,14). Since then, several clinical studies have confirmed the effectiveness of TVR-based therapy and it has now become a standard component of therapeutic regimens worldwide (15,16). In our study, the SVR12 rate was 77%, which is as high as that in earlier studies. However, 31% of patients classified as non-responders to prior therapy achieved SVR during treatment with a TVR-based regimen in the REALIZE trial published in 2012 (17). We obtained similar results in our study, as the SVR12 was significantly lower in non-responders (44%) than in relapsed patients (82%) and treatment-naïve patients (86%) (P<0.05).

The NS3 protein is approximately 67 kDa and has serine proteinase activity. The NS3 protein forms a complex with NS4 and serves to process NS4 and NS5 proteins. NS3/4A protease inhibitors exhibit strong antiviral effects as the NS3/4A protease activity is necessary for the lifecycle of HCV. Although TVR is very effective, it is frequently associated with side-effects, including skin rash and renal dysfunction, which lead to treatment discontinuation. Therefore, it is essential to increase compliance to improve the therapeutic efficacy of TPV. In this study, 7/48 patients discontinued treatment due to side-effects and 4 of these patients experienced disease relapse. Accordingly, it will be important to investigate how to maintain compliance, particularly among older patients.

Although TVR-based therapy is highly effective, approximately 30% of patients do not achieve SVR, despite triple therapy. In trials of boceprevir-based therapy, it was found

that RVR and the IL28B SNP were predictive of SVR (18). Similarly, Chayama *et al* reported that RVR, the IL28B SNP, and the response to prior therapy were predictors of SVR during triple therapy (19). In terms of viral factors, it has been reported that substitutions of amino acid 70 in the core region of HCV genotype 1b were significant predictors of SVR (20). In our study, IL28B and the response to prior therapy were significant predictors of SVR.

Mutations in several amino acids in the NS5A protein have been reported and appear to play an important role in the response to IFN. HCV NS5A is a zinc-containing phosphoprotein that regulates HCV RNA replication and particle production. A previous study using bioinformatics-assisted modeling suggested that there were 3 principal domains (21), with domain I (amino acids, 1973-2185) located in the N-terminal region, and domain II (amino acids, 2222-2314) and domain III (amino acids, 2328-2419) in the C-terminal region. Another study revealed that domain III was important for HCV particle formation (22). We have also previously reported that IRRDR in domain III is strongly associated with SVR (23). Although the number of ISDRs and IRRDRs did not affect the therapeutic efficacy of triple therapy in this study, 2 novel mutations were potentially associated with SVR. V2334 is located in the putative nuclear localization signal sequence (PPRKKRTVV; amino acids, 2326-2334) within the C-terminal region of NS5A (24). This mutation may affect the sensitivity to antiviral therapy by changing the transition of HCV during intracellular localization. Another study suggested that a specific C-terminal region (amino acids, 2350-2419) is involved in basal phosphorylation (25). G2356 is located in this region and may affect cellular signaling mechanisms by altering NS5A phosphorylation.

TPV has a covalent linear structure and is a first-generation NS3/4A inhibitor. Several mutations, including V36A/M/L, T54A/S, R155K/M/S/T, A156S and A156T/Y, have been reported to confer resistance to TPV (26). In this study, we also examined mutations associated with resistance to antiviral drugs. Using direct sequencing analysis, we found that 3 patients had the Val36 mutation in NS3 before therapy, which may confer resistance to TVR. However, all of these patients achieved SVR12, which suggests that antiviral therapy should not be contraindicated in patients with mutations conferring low levels of resistance, such as Val36 and Thr54. Simeprevir, which should soon be available for the treatment of genotype 1 chronic HCV infection (27), is a non-covalent macrocyclic inhibitor and is classified as a second-generation inhibitor with a different resistant profile to first-generation inhibitors. While the Q80 and D168 mutations are specific to non-covalent peptidomimetic inhibitors, Arg155 and A156 confer cross-resistance to all proteinase inhibitors (28). Although the Val36 and Thr54 mutations were detected in relapsed patients and in non-responders of the present study, we found none of the cross-resistant mutations. These patients may benefit from second-generation DAAs, but it is important to determine the presence of mutations that may confer resistance to these drugs.

Although TVR-based IFN therapy is effective, this treatment regimen is often limited by the side-effects of IFN. It is necessary that future therapies should be associated with greater SVR, greater compliance, shorter treatment duration, less viral resistance and better safety profiles than existing

drugs. Previous studies demonstrated that dual oral therapy with daclatasvir, a NS5A inhibitor, and asunaprevir, an NS3 protease inhibitor, was well tolerated and the SVR was high (29,30). Based on these results, we predict that a combination of two or more DAAs could achieve complete viral clearance in all patients with chronic HCV infection.

In conclusion, the IL28B SNP is strongly associated with SVR in patients receiving TVR/PEG-IFN/RBV triple therapy. Mutations in V2334 and G2356 are potential viral factors associated with the therapeutic efficacy of this regimen. Mutations in NS3 were found in approximately half of patients who did not achieve SVR and may confer resistance to second-generation proteinase inhibitors.

Acknowledgements

This study was supported by a Grant-in-Aid from the Japan Initiative for Global Research Network on Infectious Disease (J-GRID) supported by The Ministry of Education, Culture, Sports, Science and Technology, Japan and a SATREPS Grant from Japan Science and Technology Agency and Japan International Cooperation Agency.

References

1. Seeff LB: Natural history of chronic hepatitis C. *Hepatology* 36: S35-S46, 2002.
2. Lavanchy D: The global burden of hepatitis C. *Liver Int* 29: 74-81, 2009.
3. Hagiwara H, Hayashi N, Mita E, *et al*: Detection of hepatitis C virus RNA in serum of patients with chronic hepatitis C treated with interferon-alpha. *Hepatology* 15: 37-41, 1992.
4. National Institutes of Health: National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C. *Hepatology* 36 (Suppl 1): S3-S20, 2002.
5. Fried MW, Shiffman ML, Reddy KR, *et al*: Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 347: 975-982, 2002.
6. Tanaka Y, Nishida N, Sugiyama M, *et al*: Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 41: 1105-1109, 2009.
7. Akuta N, Suzuki F, Kawamura Y, *et al*: Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 46: 403-410, 2007.
8. Enomoto N, Sakuma I, Asahina Y, *et al*: Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 334: 77-81, 1996.
9. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR and Hotta H: Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. *Hepatology* 48: 38-47, 2008.
10. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, McNair L, Alam J and Muir AJ; PROVE1 Study Team: Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 360: 1827-1838, 2009.
11. Sanjo M, Saito T, Ishii R, *et al*: Secondary structure of the amino-terminal region of HCV NS3 and virological response to pegylated interferon plus ribavirin therapy for chronic hepatitis C. *J Med Virol* 82: 1364-1370, 2010.
12. Welsch C, Jesudian A, Zeuzem S and Jacobson I: New direct-acting antiviral agents for the treatment of hepatitis C virus infection and perspectives. *Gut* 61 (Suppl 1): i36-i46, 2012.
13. Jesudian AB, Gambarin-Gelwan M and Jacobson IM: Advances in the treatment of hepatitis C virus infection. *Gastroenterol Hepatol* 8: 91-101, 2012.
14. Hézode C, Forestier N, Dusheiko G, *et al*; PROVE2 Study Team: Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 360: 1839-1850, 2009.
15. Yee HS, Chang MF, Pocha C, *et al*: Update on the management and treatment of hepatitis C virus infection: recommendations from the department of veterans affairs hepatitis C resource center program and the national hepatitis C program office. *Am J Gastroenterol* 107: 669-689, 2012.
16. Chayama K, Hayes CN, Ohishi W and Kawakami Y: Treatment of chronic hepatitis C virus infection in Japan: update on therapy and guidelines. *J Gastroenterol* 48: 1-12, 2013.
17. Forestier N and Zeuzem S: Triple therapy with telaprevir: results in hepatitis C virus-genotype 1 infected relapsers and non-responders. *Liver Int* 32: 44-50, 2012.
18. Poordad F, Bronowicki JP, Gordon SC, *et al*; SPRINT-2 and RESPOND-2 Investigators: Factors that predict response of patients with hepatitis C virus infection to boceprevir. *Gastroenterology* 143: 608-618, 2012.
19. Chayama K, Hayes CN, Abe H, *et al*: IL28B but not ITPA polymorphism is predictive of response to pegylated interferon, ribavirin, and telaprevir triple therapy in patients with genotype 1 hepatitis C. *J Infect Dis* 204: 84-93, 2011.
20. Akuta N, Suzuki F, Seko Y, *et al*: Determinants of response to triple therapy of telaprevir, peginterferon, and ribavirin in previous non-responders infected with HCV genotype 1. *J Med Virol* 84: 1097-1105, 2012.
21. Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE and Rice CM: The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J Biol Chem* 279: 48576-48587, 2004.
22. Moradpour D, Penin F and Rice CM: Replication of hepatitis C virus. *Nat Rev Microbiol* 5: 453-463, 2007.
23. Yano Y, Seo Y, Miki A, *et al*: Mutations in non-structural 5A and rapid viral response to pegylated interferon- α -2b plus ribavirin therapy are associated with therapeutic efficacy in patients with genotype 1b chronic hepatitis C. *Int J Mol Med* 30: 1048-1052, 2012.
24. Ide Y, Zhang L, Chen M, *et al*: Characterization of the nuclear localization signal and subcellular distribution of hepatitis C virus nonstructural protein NS5A. *Gene* 182: 203-211, 1996.
25. Tanji Y, Kaneko T, Satoh S and Shimotohno K: Phosphorylation of hepatitis C virus-encoded nonstructural protein NS5A. *J Virol* 69: 3980-3986, 1995.
26. Vermehren J and Sarrazin C: The role of resistance in HCV treatment. *Best Pract Res Clin Gastroenterol* 26: 487-503, 2012.
27. Hayashi N, Seto C, Kato M, Komada Y and Goto S: Once-daily simeprevir (TMC435) with peginterferon/ribavirin for treatment naïve hepatitis C genotype 1-infected patients in Japan: the DRAGON study. *J Gastroenterol* 49: 138-147, 2014.
28. Sarrazin C, Hézode C, Zeuzem S and Pawlotski JM: Antiviral strategies in hepatitis C virus infection. *J Hepatol* 56 (Suppl 1): S88-S100, 2012.
29. Chayama K, Takahashi S, Toyoda J, *et al*: Dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protease inhibitor, asunaprevir, in hepatitis C virus genotype 1b-infected null responders. *Hepatology* 55: 742-748, 2012.
30. Suzuki Y, Ikeda K, Suzuki F, *et al*: Dual oral therapy with daclatasvir and asunaprevir for patients with HCV genotype 1b infection and limited treatment options. *J Hepatol* 58: 655-662, 2013.