Case Report

Seven Patients with Chronic Hepatitis C Who Experienced Late Relapse After Sustained Virological Response to Pegylated Interferon Alpha Plus Ribavirin Combination Therapy

Hideki Fujii*[1], Toshihide Shima[2], Yoshito Itoh[3] and Yasuo Ohkawara[1]

[1]*Department of Internal Medicine, Aiseikai Yamashina Hospital*
[2]*Department of Gastroenterology and Hepatology, Saiseikai Suita Hospital*
[3]*Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine*

Abstract: Objective: Late relapse of hepatitis C virus (HCV) infection, defined as HCV RNA reappearance after achieving sustained virological response (SVR) to pegylated interferon alpha plus ribavirin combination therapy, is rare in Japan. In addition, true discrimination of late relapse from reinfection is difficult. We present a report on 7 patients with SVR who experienced late relapse.

Methods: The pretreatment clinical characteristics and clinical course of the 7 patients with late relapse are presented. In 5 cases, sera before therapy and after late relapse were available, and a phylogenetic study was performed after sequencing the E2 region of HCV RNA before therapy and after late relapse.

Results: Three patients were male and 4 were female; 4 patients were infected with HCV genotype 1b and 3 with genotype 2a. All patients achieved HCV RNA disappearance by treatment week 12. The latest HCV reappearance after confirming SVR was 2.1 years. Sequence analysis revealed high homology between HCV RNA before therapy and after late relapse, and phylogenetic analyses indicated that they were the same HCV strain.

Conclusion: We confirmed that 5 patients had once achieved SVR but suffered from late relapse. Although late relapse of HCV reappearance is rare, some cases certainly occur.

Key Words: HCV, Late relapse, Phylogenetic analysis, Sustained virological response.

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*Correspondence to Hideki Fujii 19-4 Shichouno-cho, Takehana, Yamashina-ku, Kyoto, 607-8086, Japan
hidekifuiii710810@yahoo.co.jp

Introduction

Hepatitis C virus (HCV) infection is an important cause of chronic liver disease worldwide, and more than 170 million people are assumed to be infected with it, including 1.5-2 million people in Japan[1]. Approximately 70% of Japanese patients with chronic hepatitis C (CHC) are infected with HCV genotype 1b, whereas the rest are infected with genotypes 2a or 2b. The primary goal of CHC therapy is sustained virological response (SVR), defined as undetectable HCV RNA at 24 weeks after therapy completion. Approximately 80% of patients infected with genotypes 2 or 3 achieve SVR after 24 weeks of treatment with pegylated interferon (PEG-IFN) alpha plus ribavirin (RBV), whereas approximately 50% of patients with genotype 1 can achieve SVR after 48 weeks of treatment[1]. Since 2011, the rate of SVR is increasing in Japan owing to use of the protease inhibitor telaprevir in addition to PEG-IFN alpha plus RBV.

Late relapse of HCV infection, defined as HCV RNA reappearance in serum after achieving SVR, is rare. The reported late relapse rate varies, ranging from 1% to 10%[2-4]. Patients of late relapse are rare in Japan[3]. Moreover, it is difficult to distinguish "true" relapse from reinfection. In our previous report, we described a woman infected with HCV genotype 2a who showed SVR after treatment with PEG-IFN alpha 2b plus RBV for 24 weeks but experienced late relapse 48 weeks after achieving SVR. The sequence comparisons using sera before therapy and after late relapse showed the same HCV strain[5]. Here we describe 7 patients of CHC who showed late relapse, including our previously reported case[5], among 224 patients with SVR who were treated with PEG-IFN alpha plus RBV. Analyses of sera before therapy and after late relapse in 5 patients showed high homology in the E2 region of HCV RNA. Phylogenetic analyses indicated that they were the same HCV strain, and we conclude that these 5 patients achieved SVR but truly suffered from late relapse.

Materials and Methods

Patients

Three hundred and ninety-six patients with CHC were treated with PEG-IFN alpha plus RBV combination therapy at Aiseikai Yamashina Hospital, Saiseikai Suita Hospital, JR Osaka Railway General Hospital and Notogawa Hospital between 2005 and 2012. The patients were diagnosed with CHC by board-certified hepatologists at each hospital. Patients with decompensated liver diseases, co-infection with hepatitis B virus or human immunodeficiency virus, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, or Wilson's disease were not included. Patients with severe hypertension or uncontrolled diabetes mellitus or those with a history of alcohol abuse were all excluded. One hundred and forty-nine patients with genotype 1 CHC and 75 patients with genotype 2 CHC achieved SVR after PEG-IFN alpha plus RBV combination therapy. In principle, these 224 patients were followed-up and were examined for hepatitis C relapse around every 6 to 12 months. Serum HCV RNA levels were determined using Amplicor HCV RNA kits, version 2 (Roche Diagnostics, Tokyo, Japan), presented as KIU/ml, or by real-time polymerase chain reaction (PCR) (COBAS TaqMan HCV test, Roche Diagnostics), presented as log IU/ml. SVR was defined as HCV RNA negativity at the end of combination therapy and 24 weeks later on the basis of the results of HCV RNA qualitative PCR assay.

The patients received weekly injections of 1.5 μg/kg body weight of PEG-IFN alpha 2b (PegIntron; MSD K.K., Tokyo, Japan) and p.o. administration of 600-1000 mg/day of RBV (Rebetol; MSD K.K.,

Tokyo, Japan), or weekly injections of 180 μg/body of PEG-IFN alpha 2a (Pegasys; Chugai Pharmaceutical CO., LTD, Tokyo, Japan) and p.o. administration of 600-1000 mg/day of RBV (Copegus; Chugai Pharmaceutical CO., LTD, Tokyo, Japan). The amount of RBV was adjusted on the basis of body weight (600 mg for＜60 kg, 800 mg for≥60 but＜80 kg, and 1000 mg for≥80 kg). Patients with a lower hemoglobin concentration, neutrophil count, or platelet count at baseline started combination therapy with reduced doses of PEG-IFN or RBV. Patients who had a lower hemoglobin concentration, neutrophil count, or platelet count during PEG-IFN and RBV therapy also received reduced drug dosages according to the manufacturer's recommendations.

Among 7 patients with late relapse, sera were available for 5 patients before therapy and after late relapse. Three patients were from Aiseikai Yamashina Hospital and 2 were from Saiseikai Suita Hospital. This study was approved by the ethics committee of the 2 hospitals, and it conformed to the provisions of the Declaration of Helsinki.

Virological analyses

To determine if HCV RNA that appeared after SVR was identical to that before therapy, we compared the nucleotide sequences of the HCV E2 region, as described previously[5]. To determine the interferon sensitivity, we investigated the nucleotide sequence of the amino acids in HCV core 70 and 91 and the interferon sensitivity determining region (ISDR) in the nonstructural region 5A (NS5A), as reported previously[6]. In brief, RNA was extracted using a commercially available kit (QIAamp viral RNA kit; QIAGEN, Valencia, CA, USA). This sample was used for reverse transcription with random hexamer primers (SuperScript III First-Strand Synthesis System for RT-PCR cDNA synthesis kit; Invitrogen, Carlsbad, CA, USA). The E2 region, core, and ISDR were amplified by nested PCR using Takara Ex Taq HS (Takara Ex Taq, Otsu, Japan). The first and second PCR primer sequences of core 70 and 91 and ISDR were the same as those in a previous report[6-8]. The first and second PCR primer sequences of the genotype 1b E2 region were newly determined, and those of the genotype 2a E2 region were the same as described in our previous report[5] (Table 1). The PCR products were separated by electrophoresis on 1% agarose gels. These were purified using QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sequenced with second-round PCR primers using a dye terminator sequencing kit (BigDye Terminator v 1.1 cycle sequencing kit; Applied Biosystems, Foster City, CA, USA) and ABI PRISM 310 genetic analyzer (Applied Biosystems).

Sequence analysis

Nucleotide and amino acid sequences were aligned, and phylogenetic analysis was performed using MEGA 5.2 software[9]. To evaluate evolutionary relationships of each patient's E2 sequences before therapy and after relapse, each isolated E2 region from case numbers 1-3 was compared with the 87 prototype genotype 1b sequences which entire coding region sequence has been determined and deposited in the GenBank database, and each isolated E2 region from case numbers 4 and 5 was compared with the 28 prototype genotype 2a sequences which entire coding region sequence has been determined and deposited in the GenBank database. A phylogenetic tree with 1000 bootstrap replicates was generated using the neighbor-joining method and Kimura 2-parameter model. Trees were rooted with respect to genotype 1a H77 sequences as an outgroup.

Table 1. PCR primer sequences for genotype 1b and 2a HCV E2 region

Name	Sense primers (5'-3')	Location[1]	Antisense primers (5'-3')	Location[1]	A.T.[2]	Amplicon size
E2 for genotype 1b						
First PCR	CAACTGCTCACTCTATCCCGG:	1253,1273	CCTATCCCTGTCCTCCAGGT	2318,2299	51	1066
Second PCR	TCCCACAAGCTATCGTGGAT	1360,1379	TTGACAGTGCAGGGGTAGTG	2209,2190	47	850
First PCR	GGCAACAACACCTTGACCTG	2064,2083	GTATGCTCGTGGTGGTAACG	2765,2746	52	702
Second PCR	ACCCCGAAGCCACTTACAC	2107,2125	GGCAGCACAGAAGAACACAAG	2666,2646	49	560
E2 for genotype 2a						
First PCR	ACTTCTCTATGCAGGGAGCG	1422,1441	GTTTTGGTGGAGGTGGAGAA	2437,2418	56	1016
Second PCR	CGTTGTCATCCTTCTGTTGG	1453,1472	CAACCCCTCCCACATACATC	2261,2242	56	809
First PCR	TGCCTGATCGACTACCCCTA	2171,2190	AGGCCAGTGAGGGAATAGGT	2730,2711	56	560
Second PCR	TACAGGCTCTGGCATTACCC	2189,2208	TACCCGACCCTTGATGTACC	2698,2679	56	510

PCR primers sequences and conditions used for genotype 1b and 2a HCV E2 region.

1 Numberling based on the reference sequence genotype 1b:accession no.AB442220.1, genotype2a:accession no. AF177036

2 denotes the annealing temperature(℃:Celsius) used for amplification.

Results

Clinical background and clinical course of the 7 patients with late relapse

Of the 7 patients with late relapse, 3 were male and 4 were female. Four patients were infected with genotype 1b and 3 with genotype 2a (1 case was previously reported [5]). Their clinical backgrounds are presented in Table 2a, and the clinical course is shown in Table 2b and Fig. 1. There were no patients with a family history of HCV infection. All cases achieved undetectable serum HCV RNA levels by treatment week 12, which was considered a complete early virological response (cEVR). In case numbers 1, 3, 5, and 7, adherences to PEG-IFN alpha and RBV were over 80% of the scheduled doses. Except case number 2, serum HCV RNA levels after therapy completion were monitored almost every month from the end of treatment to the 24 weeks after therapy completion. In case numbers 4 and 7, the time of HCV reappearance was relatively early after the diagnosis of SVR. Meanwhile, in case numbers 3, 5, and 6, it was delayed more than 1 year after the diagnosis of SVR. We re-treated case number 7 with PEG-IFN alpha 2a according to the extended treatment schedule for 48 weeks and finally achieved SVR. Sera from case numbers 1-5 (3 patients infected with genotype 1b and 2 with genotype 2a) were stored before treatment and later used for phylogenetic analysis.

Phylogenetic analysis

The nucleotide sequence homogeneity of the E2 region ranged from 94.7% to 99.5% between sera before therapy and after late relapse. The amino acid sequence homogeneity of the E2 region ranged from 92.3% to 99.5%. The nucleotide sequence homogeneity of the E2 region except hypervariable region (HVR) 1 ranged from 96.0% to 99.5%. The amino acid sequence homogeneity of the E2 region except HVR 1 region ranged from 94.6% to 99.7% (Table 2b).

Phylogenetic analysis was performed between the strains of the 5 patients of late relapse and the standard HCV strain (Fig. 2a and Fig. 2b). In each case, the strains before therapy and after late relapse showed a high bootstrap probability, indicating that they were derived from the same ancestral node and

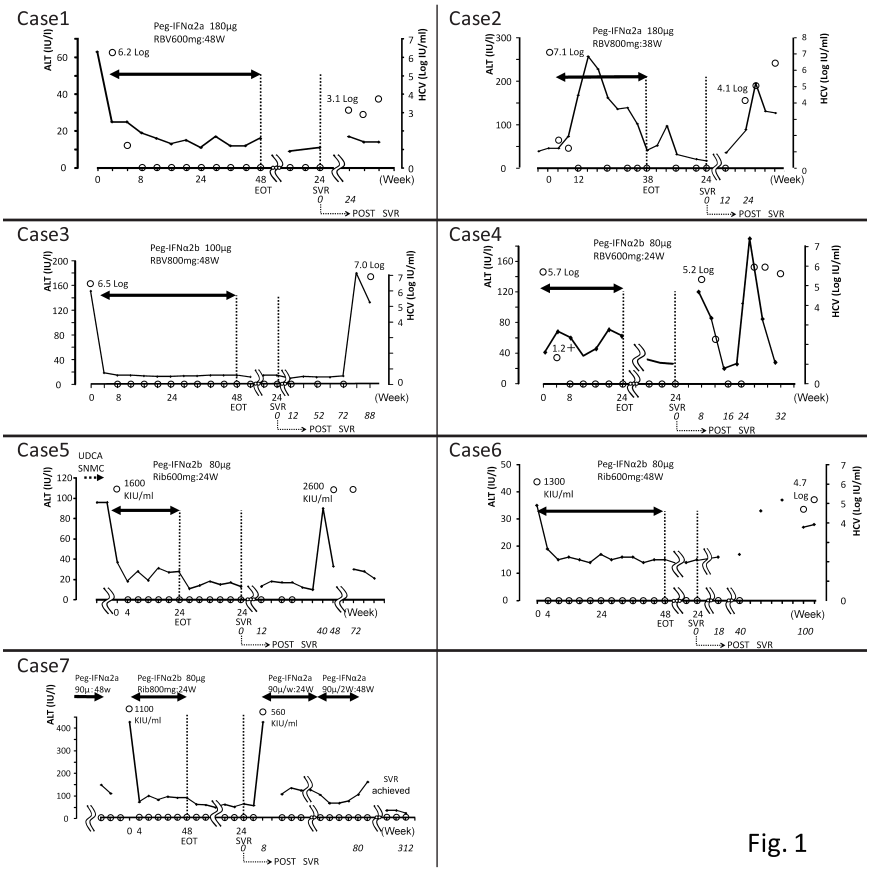


Fig. 1

Fig. 1. Clinical courses of the patients. The kinetics of ALT (IU/l) (black line) and HCV RNA (log IU/ml or KIU/ml) (white circle).

EOT: end of treatment, POST SVR: time after SVR (week), UDCA: ursodeoxycholic acid, SNMC: Stronger Neo-Minophagen C™

Case 5 has been reported in Hepatol Res. 2010; 40: 654-660(5).

Case 7 was retreated with PEG-IFN alpha 2a once a week for 24 weeks and once every 2 weeks for 48 weeks before she finally achieved SVR.

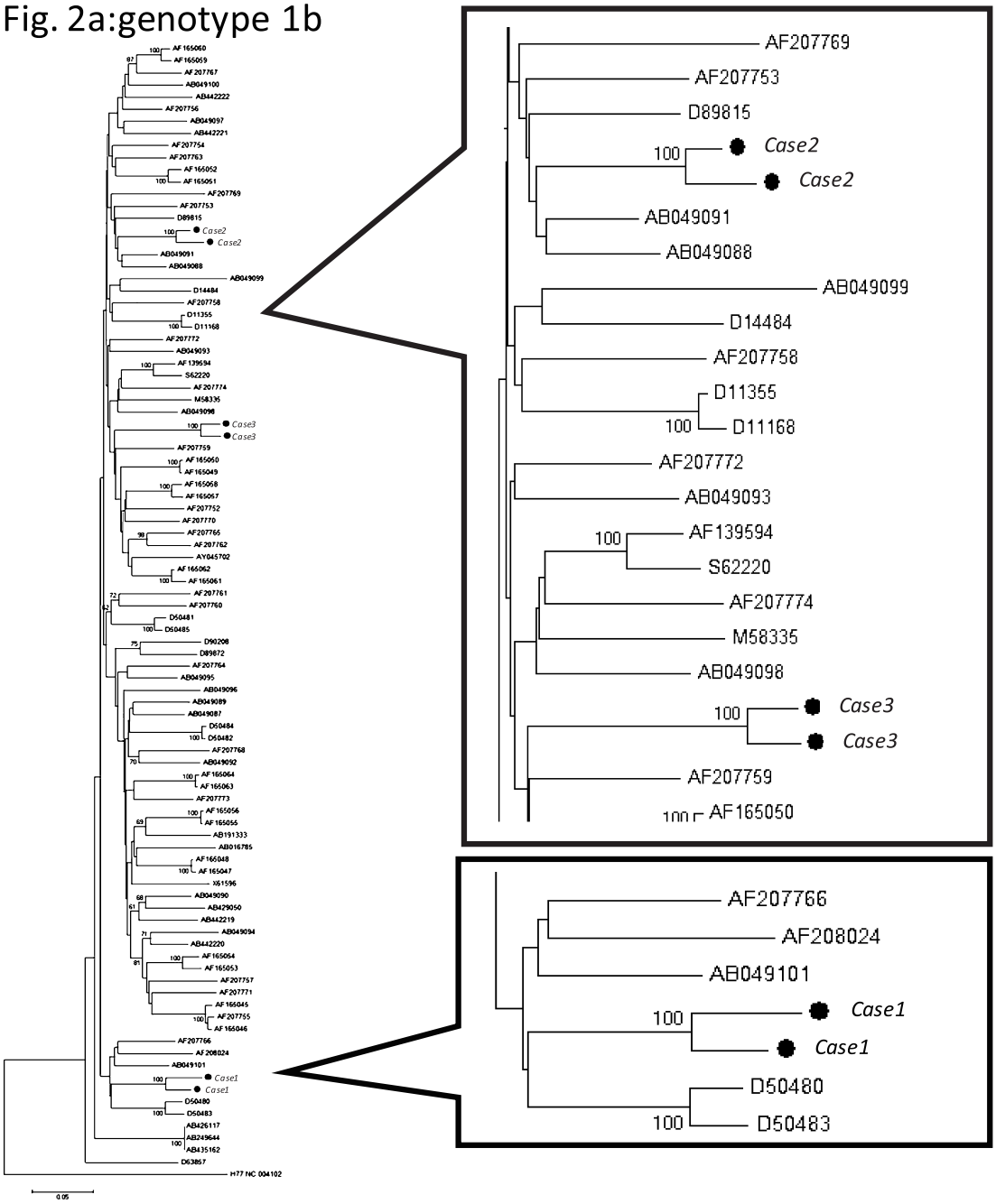
clustered discretely from other genotype 1b or genotype 2a strains. The strains before and after therapy were essentially identical.

Discussion

In this study, we identified 7 patients of late relapse. In 5 patients, sera before therapy and after achieving SVR were available. Comparison analysis of the E2 region of HCV RNA before therapy and after late relapse showed high homology, and phylogenetic analyses indicated that they were the same HCV strain. These results strongly indicate that these patients achieved SVR but suffered a relapse of hepatitis C viremia. Although late relapse is rare, virological examination may be necessary even after achieving SVR.

We first postulated that cases of late relapse may have some unfavorable or resistance factors for

Fig. 2a:genotype 1b



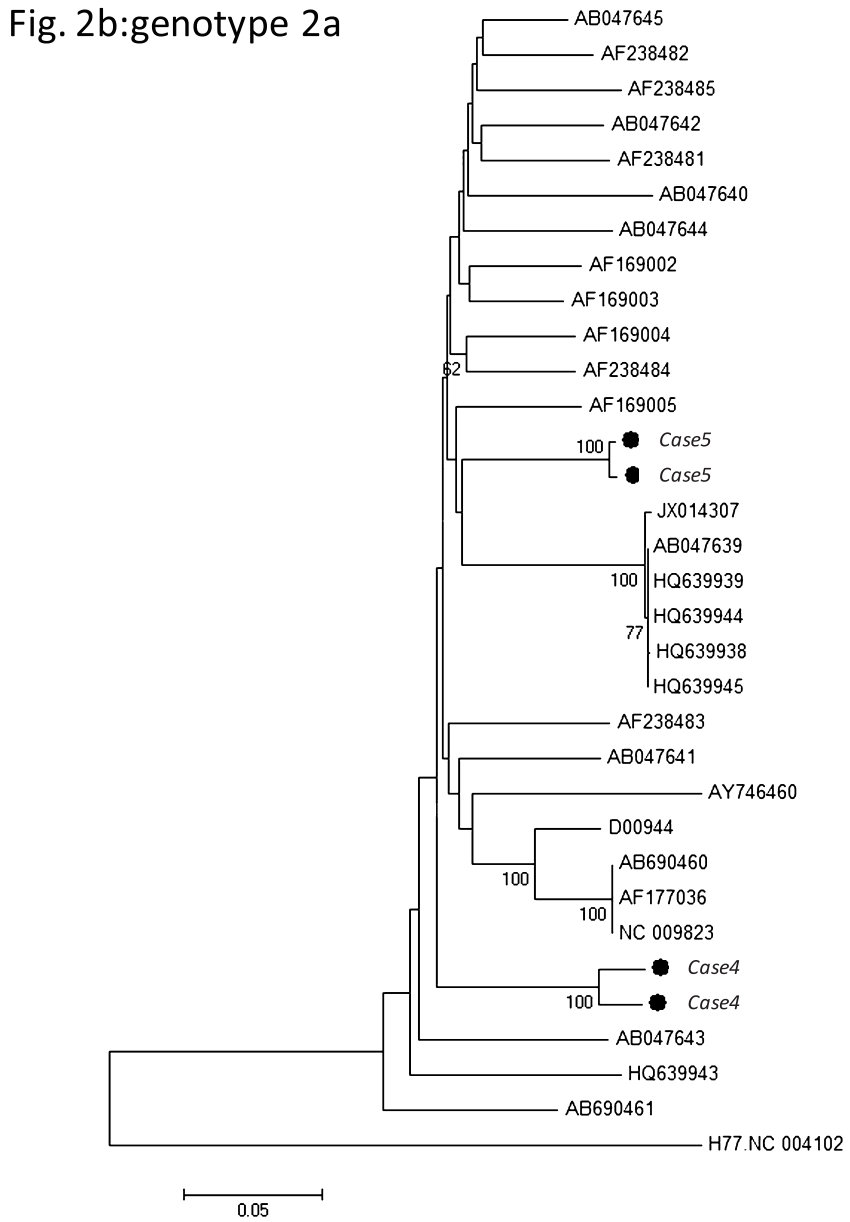


Fig. 2. A phylogenetic tree of (2a) genotype 1b cases and (2b) genotype 2a cases. Representative HCV strains were obtained from the National Center for Biotechnology Information (NCBI). Isolates obtained in the current patients' sequence before therapy and after relapse are indicated in bold and italics, with strain names designated by GenBank numbers/identification numbers. Bootstrap values greater than 60 are shown. The distance scale represents the number of nucleotide substitutions per position.

Table 2a. Clinical background of late relapse cases

Pretreatment evaluation

Case No	Age	Gender	BMI	Alcohol	HCV Genotype	Pretreatment	ALT (IU/l)	PLT $\times 10^4$/(μl)	HCV RNA (Log IU/ml or KIU/ml)	Core70/91	ISDR	IL-28B	ITPA
Case1	60	F	21.4	(-)	1b	(-)	60	15.4	6.2LogIU	Mutant/Wild	Inter-mediate	Major	Major
Case2	59	M	22.7	30g/day until 4 years ago	1b	(-)	45	16.2	7.1LogIU	Mutant/Wild	Wild	Major	Hetero
Case3	61	M	23.4	(-)	1b	Relapser[1)]	151	26.3	6.5LogIU	Wild/Mutant	Wild	Major	Hetero
Case4	68	M	21.2	(-)	2a	(-)	28	19.2	5.7LogIU	Wild/Wild	Inter-mediate	N.A.	N.A.
Case5	41	F	20.0	Social	2a	(-)	31	29.7	1600KIU	Wild/Wild	Inter-mediate	N.A.	N.A.
Case6	56	F	17.8	Social	1b	(-)	35	18.6	1300KIU	N.A.	N.A.	N.A.	N.A.
Case7	64	F	23.0	(-)	2a	Relapser[2)]	420	16.3	1100KIU	N.A.	N.A.	Major	Major

gender M: male F: female. BMI: body mass index. Social: persons who drink a little only on social occasions and do not usually drink in daily life. Core 70/91: the amino acid substitution of HCV core 70 and core 91. ISDR:the number of mutations in the interferon sensitivity determining region. IL28B: interleukin 28B gene polymorphism. ITPA: genetic polymorphism in inosine triphosphate

PEG-IFN alpha plus RBV combination therapy[10-13)] (Table 2a). As genetic factors, the IL28B genes were major homozygotes in all 3 patients with HCV genotype 1b. As course of treatment, even in cases of genotype 1b, the patients could achieve undetectable serum HCV RNA levels by treatment week 12 (cEVR) (Table 2b and Fig. 1) and were more likely to experience SVR and cure[14)]. Moreover, none of these patients were under immune suppression after SVR, with immunosuppressive therapy and opportunity for alcohol abuse or drug abuse. In addition, case number 1 and 6 remained normal ALT levels after late relapse. Some cases, as cases number 4 and 5, showed transiently abnormal liver function. These varied clinical courses may make it difficult to find out the late relapse. Therefore, it is presumably impossible to predict late relapse cases on the basis of clinical background and responsiveness to the PEG-IFN alpha plus RBV therapy.

The timing of HCV RNA re-emergence in sera is another point of interest. In case numbers 4 and 7, HCV RNA re-emergence was observed relatively early after the diagnosis of SVR (after 8 weeks). Although real-time PCR is a highly sensitive method, the detection limit is 1.2 log IU/ml[15)]. We postulate that HCV RNA may have existed below the detection limit when SVR was diagnosed in these patients. Meanwhile, in case numbers 3, 5, and 6, HCV RNA re-emergence was observed more than a year after diagnosing SVR.

Several reports have indicated that low levels of HCV RNA exist in some cells and organs such as lymphocytes, monocytes/macrophages, and liver after achieving SVR despite constantly undetectable levels in sera[16-23)]. This "occult" persistence of HCV could potentially play a role in HCV RNA re-emergence after the diagnosis of SVR. However, the significance/mechanism of HCV RNA persistence remains uncertain[24)], and data regarding occult persistence of HCV in organs are controversial.

Table 2b. Clinical course of late relapse cases

Course of treatment

Case No	Regimen	Adherence PEG/RBV (%)	Initial HCV RNA negativity (W)	Reappearance time after achieving SVR(W)	HCV-RNA after SVR(Log IU/ml or KIU/ml)	Nucleotide variation (%)	Nucleotide variation except HVR 1 region(%)	Amino acid Variation (%)	Amino acid variation except HVR 1 region(%)
Case1	PEGIFNα2a180μg+ RBV600mg:48w	(100/100)	8	24	3.1LogIU	58/1086 (94.7)	40/1005 (96.0)	28/362 (92.3)	18/335 (94.6)
Case2	PEGIFNα2a180μg+ RBV800mg:38W	(73/100)	12	24	4.1LogIU	39/1089 (96.4)	30/1008 (97.0)	12/363 (96.7)	6/336 (98.2)
Case3	PEGIFNα2b100μg+ RBV800mg:48W	(100/95)	8	88	7.0LogIU	38/1089 (96.5)	29/1008 (97.1)	14/363 (96.1)	9/336 (97.3)
Case4	PEGIFNα2b80μg+ RBV600mg:24W	(66/66)	8	8	5.2LogIU	6/1101 (99.5)	5/1020 (99.5)	2/367 (99.5)	1/340 (99.7)
Case5	PEGIFNα2b80μg+ RBV600mg:24W	(100/100)	4	48	2600KIU	39/1101 (96.5)	29/1020 (97.2)	11/367 (97.0)	2/340 (99.4)
Case6	PEGIFNα2b80μg+ RBV600mg：48W	(100/69)	4	100	4.7LogIU	N.A.	N.A.	N.A.	N.A.
Case7	PEGIFNα2b80μg+ RBV800mg：24W	(100/100)	4	8	560KIU	N.A.	N.A.	N.A.	N.A.

Adherence PEG/RBV：Adherence to PEG-IFN and RBV were assessed by separately calculating the actual doses of PEG-IFN and RBV received as percentages of the intended dosages according to the duration of therapy. HVR: the hypervariable region. N.A.: not available

We used pairwise HCV E2 gene sequences for virological analysis. HCV is an RNA virus belonging to the genus Hepacivirus of the Flaviviridae family. HCV HVR1, which is composed of 27 amino acids and is located at the 5′-terminus of the E2 gene, changes rapidly over time. It is highly variable among and within infected patients[25-27], thus, it can be used to identify individual HCV isolates[28][29]. In our results, pairwise sequences were not completely identical but shared a high homology, which indicated that they were identical strains.

Late relapse rates have been reported to range from 1% to 10%[2-4], and the intervals to late relapse and each as well as the clinical courses were not clear in these reports. Therefore, we believe that without virological analysis, it is impossible to distinguish how many of these patients with late relapse were "true" relapsers and how many were reinfected. Nakayama et al. reported that compared with reports from other countries, cases of late relapse were very rare in Japan, particularly after IFN plus RBV combination therapy, and the interval to relapse was principally restricted to the term within 2 years after therapy completion[3]. In this study, the number of late relapse cases was 7 among 224 patients with SVR, and the interval to relapse was principally restricted to within 2 years after therapy completion. We recommend that patients with SVR should be examined for HCV RNA persistence at least 2 or 3 years after SVR. In addition, there are a few case reports on the late relapse during immunosuppressive therapy[30]. To prevent de novo hepatitis during immunosuppressive therapy, we have already made the clinical guideline for the management of the patients with persistent occult HBV infection. Our present study suggests that we propose a similar guideline for the patients after SVR by accumulating more clinical data on HCV relapse in immunosuppressive conditions and further clarifying its basic mechanisms[19].

The present study has several limitations. We cannot deny re-exposure to the same HCV source,

although all patients denied the risk of re-infection, such as casual sex or illegal drug use. The other limitation is the difficulty in performing regular follow-up. In our limited hospital research, follow-up interval varied after SVR. Further, it was difficult to find HCV viremia in patients whose ALT levels remained stable below 30 IU/L during the follow-up after SVR.

Treatment of CHC is changing rapidly worldwide[31]. At present, the regimen to use the protease inhibitor simeprevir combined with PEG-IFN plus RBV and also the IFN-free regimen using daclatasvir and asunaprevir are clinically available in Japan[32)33)]. Late relapse in patients with SVR treated with these new therapies is another important issue to be addressed in the near future.

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1 Department of Gastroenterology and Hepatology, Saiseikai Suita Hospital, Suita, Japan
2 Department of Gastroenterology, JR Osaka Railway General Hospital, Osaka, Japan
3 Notogawa Hospital, Higashioumi City, Japan
4 Department of Internal Medicine, Aiseikai Yamashina Hospital, Kyoto, Japan

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〈和文抄録〉

ペグインターフェロンα＋リバビリン治療で Sustained Virological Response を達成後，遅発再燃した7名の患者の検討

藤井　秀樹[1]，島　　俊英[2]，伊藤　義人[3]，大川原康夫[1]

[1]愛生会山科病院内科
[2]大阪府済生会吹田病院消化器内科
[3]京都府立医科大学大学院医学研究科消化器内科学

【対象】C型肝炎でペグインターフェロン（PEG-IRN）＋リバビリン（RBV）治療後でSVR達成後の遅発再燃は日本ではまれである．また真に遅発再燃なのか，再感染なのか鑑別は難しい．今回我々は7例の遅発再燃を経験した．【方法】7例の遅発性再燃の臨床背景と臨床経過を提示する．5症例は治療前と遅発再燃後の血清が利用できたのでHCVのE2領域の系統樹解析を治療前と再燃後で行った．【結果】7例中3例は男性，4例は女性であった．4例はgenotype 1bで3例はgenotype 2aだった．すべての患者は治療開始12週までにウイルスが消失した．もっとも遅い再燃は2.1年だった．治療前と遅発再燃のE2領域のシークエンス解析では高い相同性を示した．系統樹解析では同一株であった．【結果】5例は一旦SVRを達成したが，再燃したと判断した．遅発再燃はまれであるが，確かに存在する．

キーワード：HCV，遅発再発，系統樹解析，SVR.