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# Transient reappearance of serum hepatitis C virus RNA observed by real-time PCR during antiviral therapy with peginterferon and ribavirin in patients with HCV genotype 1b

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ABSTRACT

*Background:* The "response-guided therapy" based on response of hepatitis C virus (HCV) during antiviral combination therapy with peginterferon and ribavirin is important for patients with HCV genotype 1. However, the sensitivity of previous assays for serum HCV RNA is limited.
*Objectives:* We evaluated the changes in serum HCV RNA during the combination therapy using a novel method for measurement based on real-time PCR.
*Study design:* Changes in serum HCV RNA during the combination therapy were reanalyzed using TaqMan PCR assay in 144 patients with chronic HCV genotype 1b infection who underwent the therapy under HCV RNA monitoring with the Amplicor Monitor assay. Treatment duration was elongated from 48 weeks to 72 weeks in 17 patients based on the time when serum HCV RNA became negative.
*Results:* In 9 of 144 (6.3%) patients, serum HCV RNA transiently appeared again on the TaqMan PCR assay after having previously become negative. At the point of reappearance, the Amplicor Monitor assay gave a negative result in all patients, and no flare of alanine aminotransferase activity was observed. Each of the 9 patients achieved an end-of-treatment response but relapsed after the end of treatment, including 3 patients in whom the treatment duration was elongated to 72 weeks.
*Conclusions:* Attention should be paid to this phenomenon in the antiviral treatment for patients with HCV infection. The transient reappearance of HCV RNA in the serum indicates a high likelihood of relapse, and is likely to be missed without frequent measurements by a sensitive detection method.


## 1. Background

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin.[1] Although the rate of sustained virologic response (SVR), which indicates the eradication of hepatitis C virus (HCV), has increased with the use of the current regimen, it is still only around 50% in patients infected with HCV genotype 1.[2–8] The response of HCV during combination therapy, i.e., the changes in serum HCV RNA after the start of therapy have been reported to be predictors of the therapeutic outcome[9–12]; therefore "response-guided therapy" based on this response is now favored,[12,13] especially for patients with HCV genotype 1.

To improve outcome prediction and the selection of treatment duration for response-guided therapy, more precise and sensitive evaluation of serum HCV RNA is necessary. Serum HCV RNA concentration has previously been measured by the branched-DNA probe assay and, more recently, by the Amplicor Monitor assay.[14,15] However, the sensitivity of these assays is limited. Very recently, a novel method for measurement of serum HCV RNA, based on real-time PCR, has been established, and is reported to have high sensitivity for the detection of serum HCV RNA.[16–18]

## 2. Objectives

In the present study, we used the real-time PCR-based TaqMan assay to reanalyze the changes in serum HCV RNA from stored serum samples of patients with chronic HCV genotype 1 infection. These patients had undergone antiviral combination therapy with PEG-IFN and ribavirin under monitoring of serum HCV RNA using the Amplicor Monitor assay. In some patients, we observed a reappearance of serum HCV RNA during the treatment after hav-

*Abbreviations:* HCV, hepatitis C virus; PEG-IFN, peginterferon; SVR, sustained virologic response; RVR, rapid virologic response; cEVR, complete early virologic response; ETR, end-of-treatment response.

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**Table 1**
Baseline characteristics of study patients ($n = 144$).

| | |
|---|---|
| Age (years) | 58.3 ± 8.9 |
| Sex (female/male) | 71 (49.3)/73 (50.7) |
| History of interferon therapy (naive/retreatment) | 109 (75.7)/35 (24.3) |
| Body weight (kg) | 58.7 ± 10.1 |
| Alanine aminotransferase (IU/L) | 62.1 ± 58.2 |
| Aspartate aminotransferase (IU/L) | 52.7 ± 40.4 |
| Gamma-glutamyl transpeptidase (IU) | 52.6 ± 58.3 |
| Alkaline phosphatase (IU/L) | 260.6 ± 83.9 |
| Albumin (g/dL) | 4.15 ± 0.35 |
| Total bilirubin (mg/dL) | 0.68 ± 0.30 |
| White blood cell count (/μL) | 5130 ± 1327 |
| Hemoglobin (g/dL) | 14.2 ± 1.4 |
| Platelet count ($\times 10^3$/μL) | 167 ± 51 |
| Liver histology-activity (A0/A1/A2/A3)[a] | 3 (2.3)/73 (54.9)/45 (33.8)/12 (9.0) |
| Liver histology-fibrosis (F0/F1/F2/F3)[a] | 5 (3.8)/79 (59.4)/33 (24.8)/16 (12.0) |
| Pretreatment HCV RNA concentration ($\log_{10}$ IU/mL) | 6.28 ± 6.16 |
| Reduction of the peginterferon dose | 40 (27.8) |
| Reduction of the ribavirin dose | 71 (49.3) |

HCV, hepatitis C virus. Percentages are shown in parentheses.
[a] Liver biopsy was not performed in 11 patients.

ing previously gone negative. This reappearance could be observed only using real-time PCR, and all patients who showed the phenomenon relapsed after the completion of the therapy.

## 3. Study design

Between January 2006 and March 2008, a total of 156 patients with chronic HCV genotype 1b infection underwent antiviral combination therapy with PEG-IFN and ribavirin at our institution. Among these patients, 148 had pretreatment HCV RNA concentrations >100,000 IU/mL as assayed by quantitative Amplicor Monitor assay (AMPLICOR HCV MONITOR Test, version 2.0; Roche Molecular Systems, Pleasanton, CA). No patients with HCV genotype 1a were included because this type is not found in the general Japanese population. In this study, we included the 144 of these 148 patients who agreed to store serum samples and to have them used in the study. Table 1 shows the baseline characteristics of the 144 study patients. Although 35 patients had a history of previous antiviral monotherapy with conventional IFN or combination therapy with conventional IFN and ribavirin (retreatment cases), no patients had a history of combination therapy with PEG-IFN and ribavirin. Of 133 patients who underwent a pretreatment liver biopsy, the grade of liver fibrosis according to the METAVIR score[19] was F0 in 5 patients (3.8%), F1 in 79 patients (59.4%), F2 in 33 patients (24.8%), and F3 in 16 patients (12.0%), respectively. No patients were coinfected with hepatitis B virus or human immunodeficiency virus. No patients had histories of alcohol abuse or intravenous drug use. For combination therapy with PEG-IFN and ribavirin, all patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough) daily. The dose of PEG-IFN and ribavirin were adjusted by patient body weight. Patients weighing ≤45 kg were given 60 μg of PEG-IFN alpha-2b once a week, those weighing >45 kg and ≤60 kg were given 80 μg, those weighing >60 kg and ≤75 kg were given 100 μg, those weighing >75 kg and ≤90 kg were given 120 μg, and those weighing >90 kg were given 150 μg. Patients weighing ≤60 kg were given 600 mg of ribavirin per day, those weighing >60 kg and ≤80 kg were given 800 mg of ribavirin per day, and those weighing >80 kg were given 1000 mg of ribavirin per day. Dose modification or discontinuation of PEG-IFN or ribavirin was based on the manufacturer's recommendations. During the therapy, 40 patients (27.8%) had their PEG-IFN doses reduced and 71 patients (49.3%) had their ribavirin doses reduced. No patients discontinued the therapy. SVR was defined as undetectable serum HCV RNA throughout 24 weeks after the end of therapy. Relapse was defined as positive serum HCV RNA during the period between the end of treatment and 24 weeks thereafter, despite the disappearance of serum HCV RNA by the end of treatment. As for responses during the therapy, rapid virologic response (RVR) was defined as negative serum HCV RNA at 4 weeks after the start of the therapy. Complete early virologic response (cEVR) was defined as negative serum HCV RNA at 12 weeks after the start of the therapy.[20] Slow virologic response was defined as the disappearance of serum HCV RNA between 12 and 24 weeks after the start of the therapy. Non-response was defined as failure to clear serum HCV RNA until 24 weeks after the start of the therapy (null-response or partial response).[1] End-of-treatment response (ETR) was defined as negative serum HCV RNA at the end of the therapy.[1] HCV RNA in the serum was measured by the qualitative Amplicor Monitor HCV RNA assay (AMPLICOR Hepatitis C Virus (HCV) Test, version 2.0, Roche Molecular Systems)[21] to confirm the undetectability of serum HCV RNA, when it was unquantifiable (under the detection limit) by the quantitative Amplicor Monitor assay. Patients who showed slow virologic response were recommended to elongate the treatment duration from 48 to 72 weeks according to previously published reports.[22,23]

**Table 2**
Responses to combination therapy with peginterferon and ribavirin evaluated by Amplicor and TaqMan assay.

| | Evaluation by Amplicor | Evaluation by TaqMan |
|---|---|---|
| Rapid virologic response | 10 (6.9) | 9 (6.3) |
| Complete early virologic response[a] | 70 (48.6) | 54 (37.5) |
| Slow virologic response | 34 (23.6) | 39 (27.1) |
| Non-response[b] | 40 (27.8) | 51 (35.4) |
| End-of-treatment response | 104 (72.2) | 104 (72.2) |
| Sustained virologic response | 63 (43.8) | 63 (43.8) |
| Relapse | 41 (28.5) | 41 (28.5) |

Amplicor, measured by AMPLICOR HCV MONITOR Test, version 2.0; TaqMan, measured by COBAS AmpliPrep/COBAS TaqMan HCV Test.
[a] Includes patients with rapid virologic response.
[b] Patients with null-response and those with partial response.

After a patient gave consent, serum samples were obtained at the patient's regular visit to the hospital just prior to beginning treatment, and at every 4 weeks during the treatment and during the 24-week follow-up period after the treatment. Serum samples were stored at −80 °C. We measured the HCV RNA levels in these stored serum samples using a real-time PCR-based quantitation method for HCV (COBAS AmpliPrep/COBAS TaqMan HCV Test, Roche Molecular Systems), and compared the results with those from the Amplicor Monitor assays. When serum HCV RNA level was low and unquantifiable, the detection of HCV RNA was tested repeatedly and the presence or absence of serum HCV RNA was confirmed.

Quantitative values are reported as mean ± SD. Between-group differences were analyzed by Chi-square test. The study protocol was approved by the institutional review board and was in compliance with the Helsinki Declaration. Written informed consent was obtained from all patients prior to the study for use of the clinical data and serum samples.

## 4. Results

### 4.1. Response of HCV RNA during treatment and final outcomes

All patients completed the therapy. Table 2 shows the responses to the therapy evaluated by the Amplicor Monitor assay and by the TaqMan PCR assay. Based on the evaluation of serum HCV RNA by the Amplicor Monitor assay during the treatment, 10 patients (6.9%) showed RVR, 70 (48.6%) showed cEVR (including the 10 with RVR), and 34 (23.6%) showed slow virologic response. The elonga-

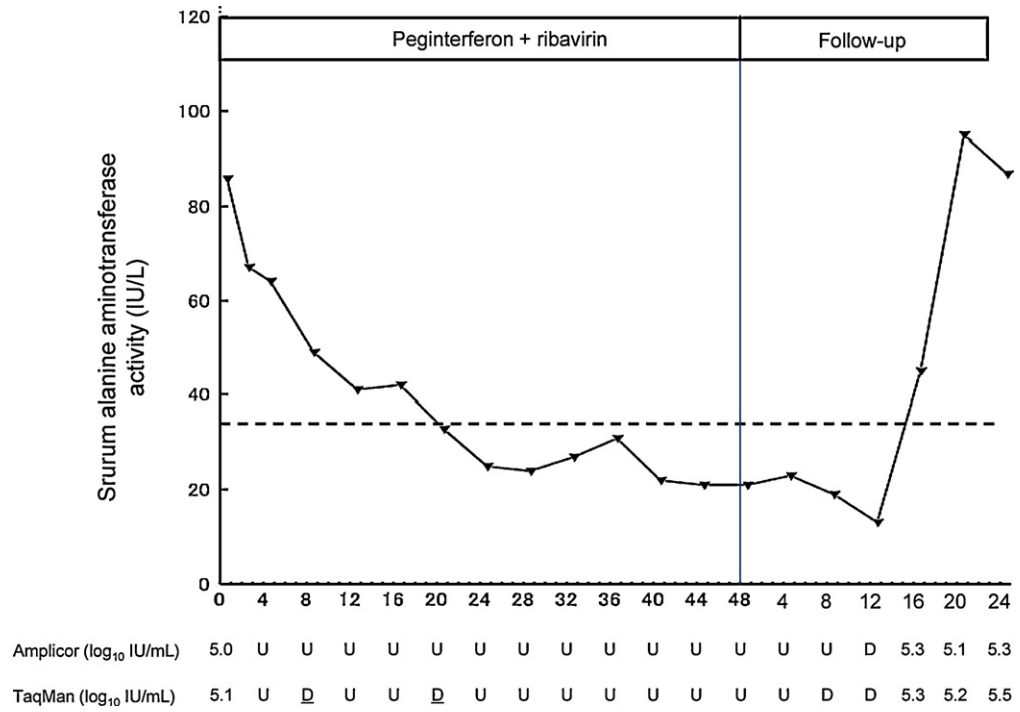


**Fig. 1.** Changes in serum alanine aminotransferase activity and serum HCV RNA concentration as detected by the Amplicor Monitor and TaqMan PCR assays during treatment and follow-up period (24 weeks) in a patient in whom serum HCV reappeared transiently during treatment after it had previously disappeared (patient 6). Pretreatment serum HCV RNA concentration was 5.05 $\log_{10}$ IU/mL. The Amplicor Monitor assay showed that serum HCV RNA disappeared at 4 weeks after the start of the therapy; therefore, the patient was classified as rapid virologic response. He relapsed after the end of the treatment with an increase in serum HCV RNA concentration and alanine aminotransferase activity. The TaqMan PCR assay also showed a disappearance of serum HCV RNA at 4 weeks, but this assay further showed that it reappeared transiently at 8 and 20 weeks after the start of the therapy (underline). Without the measurement of HCV RNA at 8 and 20 weeks this patient would be classified as rapid virologic response by the TaqMan PCR assay as well. If only the measurement at 20 weeks was omitted, he would be classified as complete early virologic response by the TaqMan PCR assay. "D" at Amplicor lane means that HCV RNA was under detection limit of quantitative Amplicor Monitor assay (3.70 $\log_{10}$ IU/mL) but was detected by qualitative Amplicor Monitor assay. "D" at TaqMan lane means that HCV RNA was under quantitation limit of TaqMan assay but was detected. "U" at Amplicor lane and TaqMan lane mean undetectable.

tion of the treatment duration from 48 weeks to 72 weeks was recommended for patients with slow virologic response; 17 of 34 patients (50.0%) followed the recommendation. As the final outcomes, 63 patients (43.8%) showed SVR and 41 (28.5%) relapsed. Among the 34 patients who showed the slow virologic response, the SVR rate was 5.9% (1 of 17) in the patients without the elongated treatment duration, and 41.2% (7 of 17) in the patients with it ($p = 0.0432$).

Under evaluation with the TaqMan PCR assay, 9 patients (6.3%) showed RVR, 54 (37.5%) showed cEVR (including 9 patients with RVR), and 39 (27.1%) showed slow virologic response. For 16 patients, the Amplicor Monitor assay gave the cEVR result, while the TaqMan PCR assay gave the slow virologic response result; elongation of treatment duration was not recommended for these patients because only the Amplicor Monitor assay was used during the treatment. At 24 weeks after the start of therapy, serum HCV RNA was detectable by the TaqMan PCR assay (non-response) in 11 patients for whom the Amplicor Monitor assay had given a result of slow virologic response.

### *4.2. Detection by TaqMan PCR assay of transient reappearance of serum HCV RNA during treatment*

Using the TaqMan PCR assay, serum HCV RNA was detected again in 9 (6.3%) patients after having previously disappeared from the serum. Table 3 summarizes the data from these 9 patients. Patients 2, 5, 6, 7, 8, and 9 were categorized as cEVR by the Amplicor Monitor assay during treatment and underwent 48-week treatment. Patient 6 showed RVR during treatment (Fig. 1). Patient 1 was categorized as cEVR during treatment but strongly desired the elongation of the treatment duration and underwent 72-week treatment (Fig. 2). Patients 3 and 4 showed slow virologic response and underwent 72-week treatment. Under reanalysis of the serum samples with the TaqMan PCR assay, patients 1, 2, 6, 7, 8, and 9 remained cEVR, patient 6 remained RVR, and patients 3 and 4 remained classified as slow virologic response, when the responses were determined by the first disappearance of serum HCV RNA. However, when the reappearance was considered, patients 1, 2, 5, 6, 7, 8, and 9 actually had a slow virologic response, and serum HCV RNA remained detectable at 24 weeks after the start of the therapy (non-response) in patients 3 and 4.

Reappearance of serum HCV RNA was found at only one measurement point in 7 patients and at 2 points in the remaining 2 patients. In all case, the level of reappeared HCV RNA was low and unquantifiable despite detection. Although patients 1, 4, and 9 experienced the reduction of ribavirin dose, the reduction was not concomitant with the reappearance of serum HCV RNA. HCV RNA reappeared transiently at these points and disappeared again thereafter. In 8 patients, the reappearance was observed at the measurement point just after the first disappearance of serum HCV RNA (i.e., 4 weeks after the previous measurement). In patient 9, HCV RNA reappearance was observed at 8 weeks after the initial disappearance. In patient 6, HCV RNA first disappeared at 4 weeks after the start of the therapy but reappeared at 8 weeks. It became negative again at 12 and 16 weeks, but reappeared again at 20 weeks. In the final outcome, all 9 patients relapsed after the end of treatment regardless of treatment duration. The prevalence of relapse in patients who experienced transient reappearance of serum HCV RNA were significantly higher than those without it, by the evaluation in patients with cEVR (100% vs. 2.1%, $p < 0.0001$), in those with cEVR and slow virolgic response (100% vs. 25.0%, $p < 0.0001$), and in those with ETR (100% vs. 33.7%, $p = 0.0004$).

**Table 3**
Patients in whom serum HCV reappeared transiently during treatment after having previously disappeared.

| | Age | Sex | History of IFN therapy | Pretreatment HCV RNA[a] ($log_{10}$ IU/mL) | HCV RNA disappearance by Amplicor | HCV RNA disappearance by TaqMan | HCV RNA reappearance by TaqMan[b] | ALT flare during therapy | Treatment duration | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 29 | F | No | 5.89 | 12 W | 12 W | 16 W | No | 72 W | Relapse |
| 2 | 57 | F | No | 6.46 | 12 W | 12 W | 16 W | No | 48 W | Relapse |
| 3 | 51 | F | No | 6.26 | 16 W | 20 W | 24 W | No | 72 W | Relapse |
| 4 | 65 | M | No | 5.66 | 20 W | 20 W | 24 W | No | 72 W | Relapse |
| 5 | 58 | M | Yes | 6.51 | 12 W | 16 W | 20 W | No | 48 W | Relapse |
| 6 | 65 | M | Yes | 5.05 | 4 W | 4 W | 8 W and 20 W | No | 48 W | Relapse |
| 7 | 58 | M | No | 6.97 | 12 W | 12 W | 16 W | No | 48 W | Relapse |
| 8 | 61 | F | No | 5.99 | 12 W | 12 W | 16 W and 20 W | No | 48 W | Relapse |
| 9 | 68 | F | No | 6.09 | 8 W | 12 W | 20 W | No | 48 W | Relapse |

M, male; F, female; W, weeks; Amplicor, measured by AMPLICOR HCV MONITOR Test, version 2.0; TaqMan, measured by COBAS AmpliPrep/COBAS TaqMan HCV Test.
[a] Measured by COBAS AmpliPrep/COBAS TaqMan HCV Test.
[b] HCV RNA reappeared transiently only at these measurement points and again disappeared thereafter.

## 5. Discussion

Measurement of HCV RNA with the real-time PCR-based TaqMan PCR assay has been reported to be superior to previous methods for the prediction of treatment outcome and the selection of a response-guided therapy regimen.[24,25] In the present study, we used the PCR-based TaqMan assay to reanalyze the changes in serum HCV RNA in patients who underwent antiviral combination therapy with PEG-IFN and ribavirin under the guidance of the Amplicor Monitor assay, in order to evaluate the usefulness of the newer technique. We found the TaqMan PCR assay to be a more sensitive detector of serum HCV RNA; it detected HCV RNA at 12 weeks after the start of the therapy in 24.3% of patients showing a cEVR result by the Amplicor Monitor assay. Umder the guidance of the TaqMan PCR assay, elongation of the treatment duration would have been recommended for these patients and their rate of SVR would presumably have increased.

More importantly, only by measurement with the TaqMan PCR assay did we observed the transient reappearance of serum HCV RNA after it had previously disappeared. Because this phenomenon was not accompanied by a flare of alanine aminotransferase and because serum HCV RNA continued to be negative by the Amplicor assay, it was missed during treatment. Breakthrough of HCV RNA during treatment is usually accompanied by an increase in serum HCV RNA concentration and a serum ALT flare, and is not transient. The phenomenon that we observed was, therefore, different from the typical breakthrough.

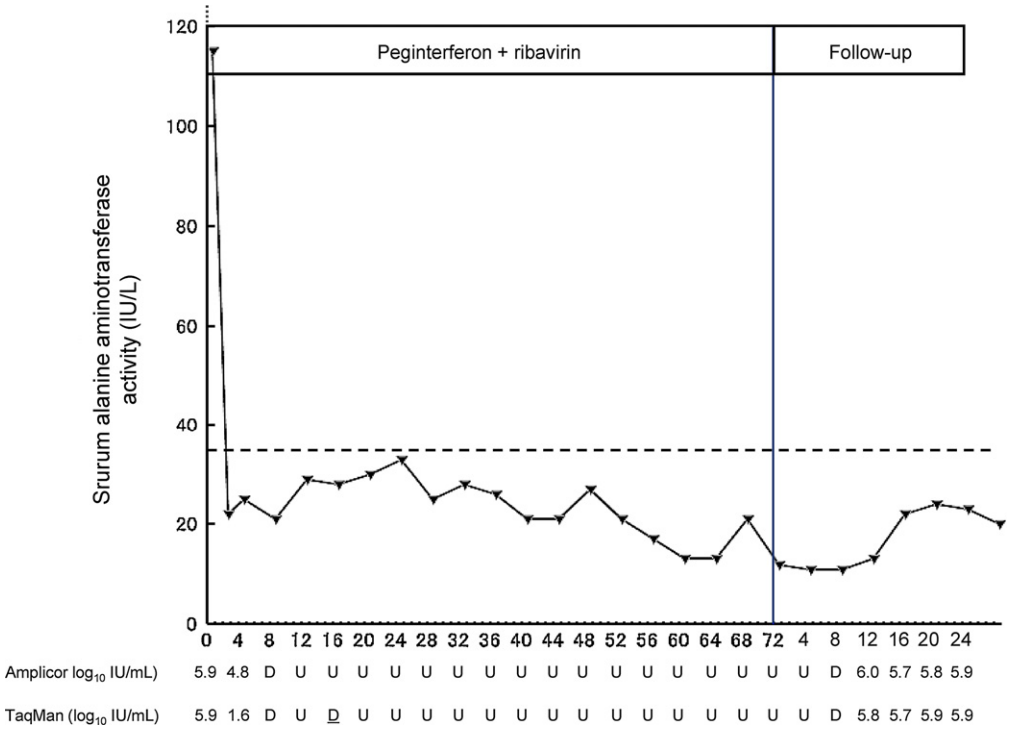


**Fig. 2.** Changes in serum alanine aminotransferase activity and serum HCV RNA concentration as measured by the Amplicor Monitor and TaqMan PCR assays during the treatment and follow-up period (24 weeks) in a patient in whom serum HCV reappeared transiently during treatment after having previously disappeared (patient 1). Her pretreatment serum HCV RNA concentration was 5.89 $log_{10}$ IU/mL. The Amplicor Monitor assay showed that her serum HCV RNA disappeared at 12 weeks after the start of the therapy; therefore, she was classified as complete early virologic response. She strongly desired to elongate the treatment duration, and completed 72 weeks treatment. However, she relapsed after the end of the treatment, showing an increase in serum HCV RNA concentration. The TaqMan PCR assay also showed the disappearance of serum HCV RNA at 12 weeks, but this assay further showed that it reappeared transiently at 16 weeks after the start of the therapy (underline). "D" at Amplicor lane means that HCV RNA was under detection limit of quantitative Amplicor Monitor assay (3.70 $log_{10}$ IU/mL) but was detected by qualitative Amplicor Monitor assay. "D" at TaqMan lane means that HCV RNA was under quantitation limit of TaqMan assay but was detected. "U" at Amplicor lane and TaqMan lane mean undetectable.

Because the phenomenon is transient, it is likely to be missed even under monitoring by the TaqMan PCR assay unless the measurement is frequently performed. In the 9 of our study patients who showed this phenomenon, reappearance of HCV RNA was found at 8, 16, or 20 weeks after the start of the therapy. Serum HCV RNA is usually measured at 4, 12, and 24 weeks after the start of the therapy[1]; therefore, any transient reappearance would be missed, unless the measurement was performed every 4 weeks. Patients 1, 2, 3, 8, and 9 would have remained cEVR and patient 6 would have remained RVR, even under measurement with the TaqMan PCR assay, if the measurement was performed only at the standard 4, 12, and 24 weeks after the start of the therapy.

In the final outcome, all these 9 patients relapsed. It is unclear why they all relapsed and no patient achieved SVR. Patients 2, 5, 6, 7, 8, and 9 would have been classified as slow virologic responders if the reappearance of HCV RNA had been detected; in these cases, the elongation of the treatment duration to 72 weeks might have resulted in SVR. In patients 3 and 4, HCV RNA was positive at 24 weeks after the start of therapy; this could explain the lack of SVR even with their 72-week treatment duration. Otherwise, a very low level of serum HCV RNA, close to the detection threshold for the TaqMan PCR assay was present throughout the treatment period, causing redetection of HCV RNA in the serum by this assay. For example, one of a few minor HCV strain that are resistant to PEG-IFN and ribavirin therapy could have been present throughout the treatment period. Further improvement of the sensitivity of the detection of serum HCV RNA will explain the results.

In conclusion, using the TaqMan PCR assay we observed a transient reappearance of serum HCV RNA after it had previously disappeared in patients with HCV genotype 1b undergoing antiviral combination therapy with PEG-IFN and ribavirin. This phenomenon is likely to be missed without frequent measurements of serum HCV RNA by sensitive detection method, and it may indicate a high likelihood of relapse after treatment even if the treatment duration is elongated. The possibility of this phenomenon should be considered during treatment in order to select the appropriate response-guided therapy. In addition, large-scale prospective studies will be needed to clarify the biological significance and clinical impact of this phenomenon.

## Conflict of interest

There is no conflict of interest and there is no grant support and other assistance on this study.

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