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Prevention of Disease Progression with Anti-Inflammatory Therapy in Patients with HCV-Related Cirrhosis: A Markov Model

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Key Words

Hepatitis C · Hepatocellular carcinoma · Interferon · Glycyrrhizin · Carcinogenesis · Markov model · Anti-inflammatory therapy

Abstract

Background: The significance of anti-inflammatory therapy has not been fully evaluated in hepatitis C virus (HCV)-related cirrhosis. ***Patients and Methods:*** We analyzed stepwise progression rates from cirrhosis to hepatocellular carcinoma (HCC) and to death using a Markov model in 1,280 patients with HCV-related cirrhosis. During the observation period, 303 patients received interferon and 736 received glycyrrhizin injections as anti-inflammatory therapy. ***Results:*** In the entire group, annual progression rates from cirrhosis to HCC and from cirrhosis to death were 6.8 and 1.9%, and the rate from HCC to death was 19.0%. When sustained virological response (SVR) or biochemical response (BR) was attained with interferon, the annual rate to HCC decreased to 2.6%. On the contrary, the progression rates to HCC and to death in the patients without SVR and BR were 7.2 and 2.0%, respectively ($p < 0.0001$). Continuous interferon administration significantly decreased the carcinogenesis rate to 5.5% ($p = 0.0087$). In the analysis of the remaining patients with high alanine transaminase of 75 IU/l or more but without interferon response or without interferon administration, glycyrrhizin injection significantly decreased annual non-progression probability (no glycyrrhizin 88.0% vs. glycyrrhizin therapy 92.3%, $p = 0.00055$). ***Conclusion:*** Glycyrrhizin injection therapy is useful in the prevention of disease progression in interferon-resistant or intolerant patients with HCV-related cirrhosis.

Introduction

Hepatitis C virus (HCV) is one of the principal etiologies of hepatocellular carcinoma (HCC), with high morbidity and mortality rates in many countries [1–5]. Because interferon has anti-viral, anti-fibrotic and anti-inflammatory properties, it is still a main agent in the treatment of chronic hepatitis C [6, 7]. Many authors have described interferon as capable of preventing hepatocarcinogenesis and prolonging patient survival [8–13]. The radical eradication of HCV by interferon greatly depends on viral load, HCV subtype, certain mutations of the hepatitis virus gene, liver histology, mode of interferon administration and various host factors, including

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the patient's age [10, 13, 14]. When a significant side effect occurs during interferon therapy, cessation or early withdrawal of the therapy often leads to an unsuccessful result. Early withdrawal and treatment failure is usually more common in patients with an advanced stage of liver disease.

Adverse effects of interferon are more commonly found in patients with cirrhosis, and hematological disorders often necessitate cessation of interferon before the therapy is complete. As a result, interferon is considered less effective in the advanced stage of hepatitis. Liver cirrhosis is usually associated with patients aged 55–60 years or older; the adverse effects of interferon-based anti-viral therapy are prevalent in this age group, resulting in low overall compliance for long-term therapy. Because the severity of chronic liver disease is closely associated with the response to interferon therapy [14–16], the sustained response rate is often low in patients with cirrhosis. Furthermore, an older patient with cirrhosis has a very high risk of carcinogenesis and mortality because fibrotic stage is correlated with a patient's age. The role of interferon in suppression of the carcinogenesis rate is therefore likely to be less significant in patients with cirrhosis en masse. There have been several clinical attempts to administer interferon for HCV-related cirrhosis to suppress the hepatocellular carcinogenesis rate [8, 9, 11, 12, 17–19]. However, there have been conflicting reports about the therapeutic value of interferon for this purpose. Some studies have shown a beneficial effect of interferon in reducing carcinogenesis [8, 9, 12, 18], but other reports have not [11, 17, 19].

When interferon fails to eliminate HCV RNA in a patient, long-term administration of interferon often shows anti-carcinogenic action through stabilization of alanine transaminase (ALT) and suppression of the necro-inflammation of hepatocytes [20]. For patients who do not respond to long-term interferon therapy, as shown by persistently high ALT values, glycyrrhizin injection therapy is available in several countries, including some countries in Asia and Europe. A glycyrrhizin-containing product, Stronger Neo-Minophagen C™ (SNMC; Minophagen Pharmaceutical Co. Ltd., Tokyo, Japan), is widely used in Japan for suppression of hepatitis activity and for prevention of disease progression in patients with hepatitis B virus- and HCV-induced chronic hepatitis. Glycyrrhizin has been reported to mitigate hepatic inflammation by suppressing elevated ALT levels and preventing disease progression [21–24]. We previously reported the favorable effects of long-term administration of glycyrrhizin against hepatocellular carcinogenesis in patients with interferon-naïve and interferon-resistant chronic hepatitis C [25, 26].

In order to elucidate whether long-term glycyrrhizin injection therapy suppresses hepatocarcinogenesis and mortality rates in patients with interferon-resistant cirrhosis, we retrospectively analyzed a large cohort of patients with HCV-related cirrhosis in a single institution. The principal aims of our study were to show the clinical role of glycyrrhizin in advanced liver disease, and to determine whether glycyrrhizin can be used as an anti-inflammatory therapy.

Patients and Methods

Study Population and Analyzed Cohorts

A total of 1,358 consecutive patients with hepatitis C were diagnosed as having liver cirrhosis at the Department of Hepatology, Toranomon Hospital, Tokyo, Japan, from 1974 to 2007. They had positive anti-HCV antibody, detectable HCV RNA (nested PCR), and negative hepatitis B surface antigen. Anti-HCV and HCV RNA were assayed using stored frozen sera. Among the 1,358 consecutive patients with hepatitis C, 78 patients were excluded from the study based on meeting one or more of the following exclusion criteria: (1) possible association with HCC; (2) association with hemochromatosis, autoimmune liver disease, primary biliary cirrhosis, α1-antitrypsin deficiency or Wilson disease; (3) daily alcohol ingestion of 75 g or more; (4) α-fetoprotein of 400 ng/ml or higher; (5) a short follow-up period of 6 months or less, or (6) Child-Pugh stage C liver disease because of the substantial difference in carcinogenesis in these patients [27–29].

The remaining 1,280 patients with HCV-positive liver cirrhosis were retrospectively analyzed for hepatocellular carcinogenesis and mortality. Among them, 754 patients (59.4%) were diagnosed as having cirrhosis by histopathological findings with peritoneoscopy and biopsy, and the remaining 526 (40.6%) were diagnosed with clinical findings: rough-surfaced liver on imaging (ultrasonography or computerized tomography, CT), plus endoscopic finding of esophageal varices, overt ascites or indocyanine green retention rate at 15 min of 30% or more. There were 744 men and 536 women, with a median age of 59 years (range 22–86). They were observed for a median of 8.1 years (table 1). A total of 231 patients (18.0%) were lost to follow-up during the observation period.

Interferon Treatment and Evaluation of Effects

Among the 1,280 patients with cirrhosis, 303 patients (23.7%) received interferon therapy with or without ribavirin. Among the 303 patients receiving interferon therapy, 252 received interferon-α and 51 received interferon-β therapy. For dosages, 258 patients received at least 6 million IU/day, and the other 45 patients received no more than 3 million IU/day as their initial anti-viral therapy. Of 303 patients receiving interferon, 52 patients received interferon daily for the first 2–8 weeks and then 2–3 times per week for the following 24–72 weeks. The other 251 patients received interferon 3 times per week for 24–72 weeks. The median administration period was 26.0 weeks (range 4–548).

Table 1. Clinical features of the study group: 1,280 patients with liver cirrhosis caused by hepatitis C

Demography	
Male	744 (58.1)
Female	536 (41.9)
Age, years	59 (22–86)
Decompensated cirrhosis	134 (10.5)
History of blood transfusion	549 (42.9)
Total alcohol intake >500 kg	200 (15.6)
Presence of diabetes mellitus	249 (19.5)
Observation period, years	8.1 (0.5–30.6)
Laboratory data	
ICG R15, %	27 (2–96)
Bilirubin, mg/dl	1.0 (0.2–7.7)
Albumin, g/dl	3.7 (1.6–5.1)
Aspartate transaminase, IU/l	66 (14–1313)
ALT, IU/l	62 (4–570)
Platelet count, $\times 10^3/mm^3$	104 (20–398)
Prothrombin time, %	82 (11–117)
Hepatitis C subtype	
1	821 (75.7)
2	254 (23.4)
Other	9 (0.8)
Treatment after diagnosis of cirrhosis	
Interferon with/without ribavirin	303
Glycyrrhizin injection	736
Ursodeoxycholic acid	615

Data are presented as the median value with range in parentheses, or n with percentages in parentheses. ICG R15 = Indocyanine green retention rate at 15 min.

Almost all patients who received interferon therapy showed varying degrees of influenza-like symptoms, leukocytopenia and thrombocytopenia. Eight patients discontinued interferon therapy because of significant adverse reactions: depression in 2 patients, severe cytopenias in 2, marked anorexia in 1, malaise in 2 and retinopathy in 1 patient. No patients developed decompensated liver disease with ascites, encephalopathy, jaundice or variceal bleeding.

The effects of interferon therapy were classified according to the elimination of HCV RNA and the levels of ALT for 6 months after the end of the treatment. Sustained virological response (SVR) was defined as persistent disappearance of HCV RNA after therapy. Biochemical response (BR) was defined as normal ALT values without elimination of HCV RNA for at least 6 months after therapy. No response (NR) was defined as persistently abnormal or only transient normalization of ALT for a period of less than 6 months. Because 73 patients (24.1%) were still undergoing their course of interferon therapy, the evaluation was conducted in 230 (75.9%) of the 303 patients.

Glycyrrhizin Injection (SNMC) Therapy

Glycyrrhizin therapy was performed using intravenous injections of SNMC™ (Minophagen Pharmaceutical Co. Ltd.). The preparation contains 0.2% (40 mg) glycyrrhizinic acid as the main active constituent, 2% (400 mg) glycine and 0.1% (20 mg) L-cysteine in a 20-ml ampule.

Of 376 chronic hepatitis patients with interferon resistance or who did not receive interferon injection therapy, 264 patients underwent glycyrrhizin injection therapy and the remaining 112 patients did not receive therapy until the end of observation. The purpose of glycyrrhizin injection therapy was to suppress elevated ALT levels and to prevent disease progression in all the patients. In patients for whom the treatment was regarded as effective with respect to ALT levels, the treatment was usually continued for as long a period as possible. As a result, a daily dose of 100 ml of SNMC was administered three times a week for a median period of 4.9 years (range 0.1–24.1) in the glycyrrhizin-treated group.

Certain patients with a high ALT value did not receive glycyrrhizin injection for a variety of reasons. These included the refusal of intravenous treatment, a difficulty in frequently visiting the clinic for the injection, inappropriate superficial veins for repeated injection, negativism towards the handling of intravenous therapy by the doctors in charge, and so on. Those patients who did not receive glycyrrhizin injection therapy in spite of a high ALT often received pills of ursodeoxycholic acid as an anti-inflammatory therapy.

Follow-Up of Patients and Diagnosis of HCC

Follow-up of the patients was made on a monthly to tri-monthly basis after the initial visit. Imaging diagnosis was made one or more times per year with ultrasonography, CT or magnetic resonance imaging. HCC was diagnosed by its typical hypervascular characteristics on CT, magnetic resonance imaging or angiography. When combined use of imaging modes could not demonstrate a typical image of HCC, a fine-needle biopsy was obtained for microscopic examination. The imaging diagnosis was similarly performed among those patients with interferon therapy, glycyrrhizin therapy and without therapy.

Statistical Analysis and Markov Model

Standard statistical measures and procedures were used. The χ^2 test, Fisher exact test and Mann-Whitney U test were used to analyze the differences in demography and laboratory findings. Progression and survival rates were analyzed using the Kaplan-Meier technique [30] with the log-rank test. A Markov model [31] was used to analyze the transition rates from liver cirrhosis to appearance of HCC, and to death. A homogenous Markov chain consisted of three states (fig. 1). These were liver cirrhosis, appearance of HCC and death as an absorbing state from where no transitions to the other states occurred. The model was based on the following principles: (1) the three states are mutually exclusive and collectively exhaustive; (2) the Markov assumption is that the current state has no memories of prior states; (3) the time intervals are uniform, and (4) the transition probabilities are constant and time independent. The first and second items here define a Markov chain, whereas the third and fourth items characterize a homogenous Markov chain [32]. Patient data were regarded as censored at the time of the last date of observation, in the evaluation of survival analysis and Markov analysis.

A p value <0.05 in the two-tailed test was considered significant. Data analysis was performed using IBM SPSS Statistics version 18 [33]. The Human Ethics Review Committee of Toranomon Hospital approved the study protocol.

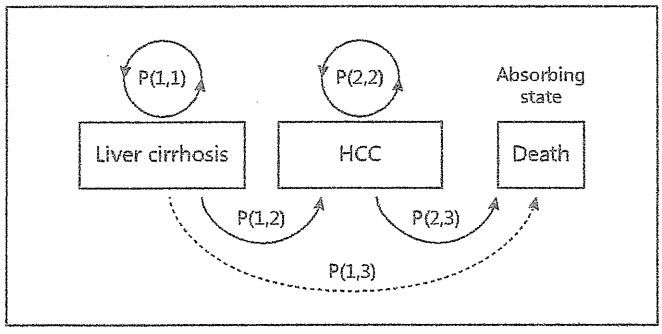

Fig. 1. Markov state transition diagram of liver cirrhosis. Three states were defined: liver cirrhosis without development of HCC, liver cirrhosis-associated HCC, and death. Of these, death was the absorbing state from which no transitions to the other states occurred. The transition in one cycle (1 year) is shown. Arrows connecting two different states indicate observed transitions. The figure represents a probability diagram of the entire study group. All patients were initially at the stage of liver cirrhosis, but transitions to HCC stages gradually increased with time.

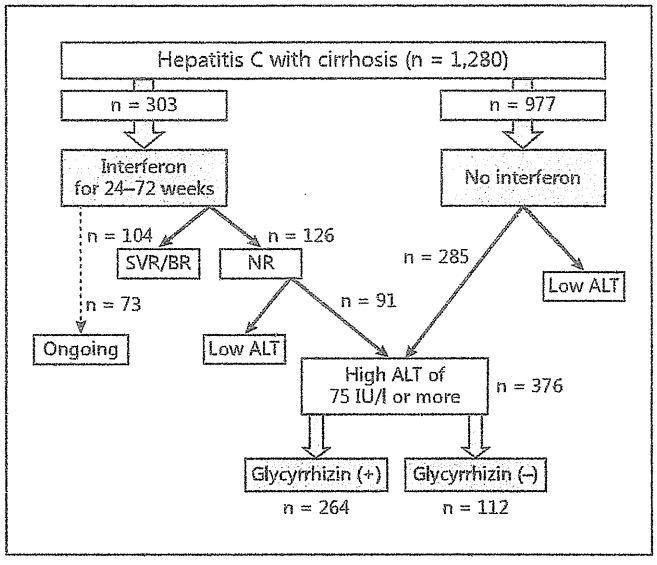

Fig. 2. Clinical courses of patients with cirrhosis. Among 303 patients who received interferon therapy, there were 104 patients who had SVR or BR, and 126 patients who had NR. The remaining 73 patients continued to receive long-term interferon therapy. Among 376 patients with a high ALT value of 75 IU/l or more, with or without a history of interferon therapy, 264 patients underwent glycyrrhizin injection therapy and 112 did not receive glycyrrhizin.

Results

Effects of Interferon and Anti-Inflammatory Treatment

Among the 303 patients who underwent interferon therapy with or without ribavirin, 79 patients (26.1%) showed HCV RNA clearance (SVR effect), and 25 patients (8.3%) showed a BR with normal ALT values for 6 months or longer. One hundred and twenty-six patients (41.6%) showed NR after cessation of interferon. The remaining 73 patients (24.1%) continued intermittent interferon administration for 1 year or longer.

Among the 977 patients who did not receive interferon therapy, plus the 126 patients who received interferon with NR, a high ALT value of 75 IU/l or more was found in 376. Of these patients, 264 (70.2%) underwent long-term glycyrrhizin injection therapy and the other 112 (29.8%) did not receive glycyrrhizin (fig. 2).

Crude Hepatocellular Carcinogenesis and Survival Rates in the Entire Study Group

Cumulative hepatocellular carcinogenesis rates were calculated in all 1,280 study patients with HCV-related cirrhosis. The carcinogenesis rates were 16.4, 29.2, 37.3, 51.6, 65.0 and 69.5% at the end of the third, fifth, seventh, tenth, fifteenth and twentieth years, respectively (fig. 3a). The cumulative survival rates were 93.0, 86.3, 77.1, 61.9, 39.3 and 25.4% at the same time points (fig. 3b).

Probabilities for Transition among the Three Disease States according to the Results of Interferon and Anti-Inflammatory Treatment

In the matrix of the entire study group, 6.8% (562/8,273) of the patients with liver cirrhosis progressed to HCC annually, and 1.9% (157/8,273) died. The remaining 91.3% (7,554/8,273) of the patients remained in the stage of liver cirrhosis after 1 year. Similarly, 19.0% (423/2,228) of the patients in the stage of HCC died, and 81.0% (1,805/2,228) of the patients remained in the stage of HCC annually (table 2).

The results are shown in table 3 as a matrix of transition probabilities for three subsets composed of treatments (SVR or BR, NR or no interferon, and continual interferon) stratified by three states (cirrhosis, HCC, and death). The probabilities for transition from liver cirrhosis to HCC and from liver cirrhosis to death were significantly lower in patients who achieved SVR or BR [2.6% (20/778) and 0.6% (5/778)] than in patients with NR or no interferon therapy [7.2% (542/7,494) and 2.0% (151/7,494); $\chi^2 = 32.4$, $p < 0.0001$]. The probabilities for transition from liver cirrhosis to HCC and from liver cirrhosis to death were significantly lower in patients who

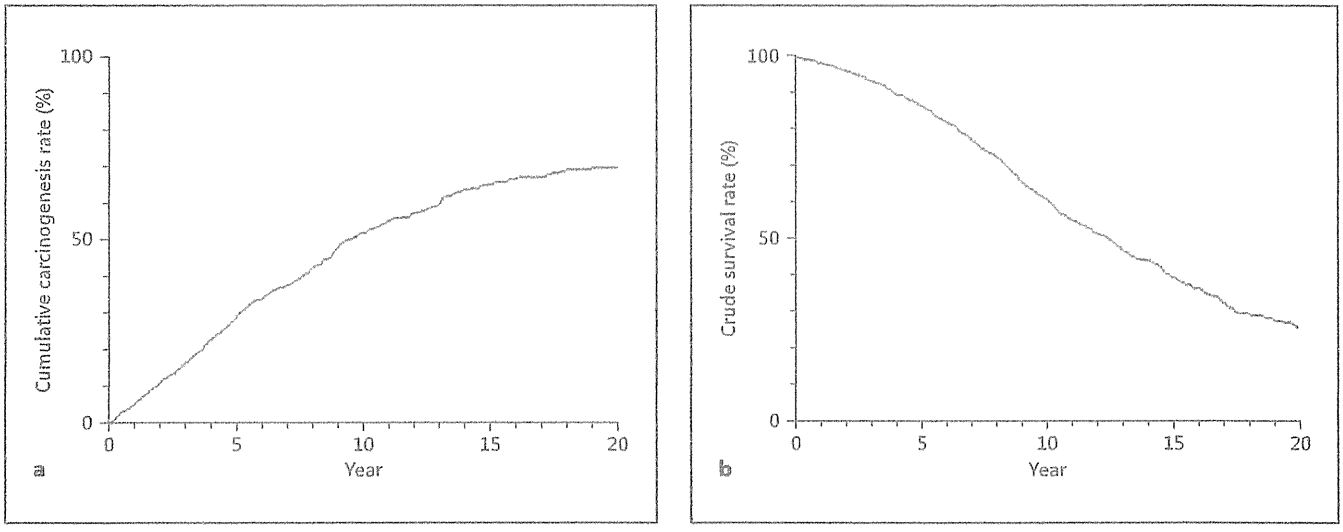

Fig. 3. HCV-positive chronic hepatitis patients with cirrhosis were retrospectively analyzed for hepatocellular carcinogenesis and mortality. **a** Cumulative hepatocellular carcinogenesis rate in the entire group of patients with cirrhosis. **b** Crude survival rate in the entire group of patients with cirrhosis.

Table 2. One-year state-transition probability matrix of the entire study group (n = 10,501 person years)

	Cirrhosis	HCC	Death
Liver cirrhosis (n = 8,273)	7,554 (91.3)	562 (6.8)	157 (1.9)
HCC (n = 2,228)		1,805 (81.0)	423 (19.0)

Figures in parentheses are percentages.

received continuous interferon therapy [5.5% (39/714) and 0.7% (5/714)] than in patients with NR or no interferon therapy [7.2% (542/7,494) and 2.0% (151/7,494); $\chi^2 = 7.59$, $p = 0.0059$].

Probabilities for Transition among the Remaining Patients with High ALT

Among 376 patients without SVR/BR effect and continuous interferon injection and with a high ALT value of 75 IU/l or more, 264 patients (70.2%) received glycyrrhizin injection as anti-inflammatory therapy. Among 692 patients without SVR/BR effect and continuous interferon injection and with relatively low ALT of less than 75 IU/l, glycyrrhizin injection was performed only in 253 patients (36.6%).

We evaluated the transition probabilities among the three states in the remaining patients with high ALT levels of 75 IU/l or more. In the matrix of patients without glycyrrhizin injection therapy, the transition probability from liver cirrhosis to HCC was 6.8% (85/1,245), and the probability of transitioning from cirrhosis to death was 2.0% (25/1,245). In the patients who received glycyrrhizin injection therapy, the transition probability from liver cirrhosis to HCC was 5.9% (45/764), and the probability of transitioning from cirrhosis to death was 0.8% (6/764). Glycyrrhizin injection therapy slightly improved the transition probability both from liver cirrhosis to HCC and from liver cirrhosis to death, but statistical significance was not observed ($\chi^2 = 5.5$, $p = 0.06$; table 4).

Disease Control Rates (Annual Non-Progression Probability) of Anti-Viral and Anti-Inflammatory Treatment

The disease control rates depended on the probabilities for transition between progression and non-progression of disease at a specific time interval, which was set at 1 year. The yearly transition probabilities were calculated based on the data of 10,501 person years of the 1,280 study patients with HCV-positive liver cirrhosis.

The disease control rate of the patients with SVR or BR (874/910, 96.0%) was significantly higher than that of the

Table 3. One-year state-transition probability matrices according to initial treatment

	Cirrhosis	HCC	Death
Patients with SVR or BR (n = 910 person years)			
Liver cirrhosis (n = 778)	753 (96.8)	20 (2.6)	5 (0.6)
HCC (n = 132)		121 (91.7)	11 (8.3)
Patients with no response or no interferon therapy (n = 9,590 person years)			
Liver cirrhosis (n = 7,494)	6,801 (90.8)	542 (7.2)	151 (2.0)
HCC (n = 2,096)		1,684 (80.3)	412 (19.7)
Patients with continuous interferon therapy (n = 856 person years)			
Liver cirrhosis (n = 714)	670 (93.8)	39 (5.5)	5 (0.7)
HCC (n = 142)		132 (93.0)	10 (7.0)

Figures in parentheses are percentages.

Table 4. One-year state-transition probability matrices according to glycyrrhizin injection therapy for patients with high ALT values

	Cirrhosis	HCC	Death
Patients without glycyrrhizin therapy (n = 1,637 person years)			
Liver cirrhosis (n = 1,245)	1,135 (91.2)	85 (6.8)	25 (2.0)
HCC (n = 392)		305 (77.8)	87 (22.2)
Patients with glycyrrhizin therapy (n = 913 person years)			
Liver cirrhosis (n = 764)	713 (93.3)	45 (5.9)	6 (0.8)
HCC (n = 149)		130 (87.2)	19 (12.8)

Figures in parentheses are percentages.

Table 5. One-year non-progression probability matrix of anti-viral and anti-inflammatory treatment

	Non-progression	Progression
Entire study group (n = 10,501)	9,359 (89.1)	1,142 (10.9)
Patients with SVR or BR (n = 910)	874 (96.0)	36 (4.0)
Patients with NR or no interferon therapy (n = 9,590)	8,485 (88.5)	1,105 (11.5)
Patients without glycyrrhizin therapy (n = 1,637)	1,440 (88.0)	197 (12.0)
Patients with glycyrrhizin therapy (n = 913)	843 (92.3)	70 (7.7)

Figures in parentheses are percentages.

patients with NR or the patients without interferon therapy (8485/9590, 88.5%; $\chi^2 = 49.1$, $p < 0.0001$).

We also evaluated disease control rates according to glycyrrhizin injection therapy in the subgroups of patients who either failed or did not receive interferon therapy with a high ALT of 75 IU/l or more. Anti-inflammation therapy with glycyrrhizin injections significantly increased the disease control rates, as shown by the rate of 92.3% (843/913) in the patients who received glycyrrhizin injection therapy versus 88.0% (1,440/1,637) in the patients without glycyrrhizin therapy ($\chi^2 = 11.9$, $p < 0.0001$; table 5).

Discussion

Based on our epidemiological data obtained from long-term observations of patients with chronic hepatitis [34] and patients with cirrhosis [35], we found that the life expectancy of patients with HCV-related liver cirrhosis heavily depends on the development of HCC. The probability of patients with HCV-related liver cirrhosis eventually developing HCC is staggeringly high at 75% [35]. In the present study, interferon administration significantly decreased the probability for transition from liver cirrhosis to HCC in the patients who achieved SVR or BR. However, there were some background varieties between the patients with SVR or BR and NR or no interferon therapy with respect to stage of fibrosis, sex, platelet count and age, which can affect the carcinogenesis rate.

From the standpoint of anti-inflammatory effects and cancer prevention [8–10, 13, 14, 19], interferon is effective in patients with chronic liver disease caused by HCV. Although the carcinogenesis rate is noticeably reduced when the ALT level becomes normal with or without HCV RNA eradication [10, 13, 14] after therapy, ALT levels become normal after interferon therapy in approximately half of the patients with a high viral load and group 1 HCV subtype. Furthermore, the anti-carcinogenic capacity of interferon has been demonstrated not only in patients with persistent ALT normalization, but also in patients with transient normalization of ALT for at least 6 or 12 months [20].

Many authors have already described that the activity of interferon in suppressing the development of HCC in patients with HCV RNA clearance (SVR) is similar to that in patients with ALT normalization in the absence of elimination of HCV RNA (BR) [13, 36–38]. Based on these compelling lines of evidence, the anti-carcinogenic activity of interferon is ascribed to the suppression of in-

flammatory and regenerative processes in hepatocytes. Moreno and Muriel [39] reported that interferon reverses liver fibrosis and, therefore, control of the necro-inflammatory process can suppress the growth of HCC.

An SVR improves clinical symptoms in decompensated cirrhosis [40], but interferon often induces severe complications, even in young patients with decompensated cirrhosis [41]. A patient with compensated cirrhosis can be a candidate for interferon therapy if careful, close hematologic monitoring is performed.

Because patients with liver cirrhosis generally experience some difficulties with interferon treatment, our present study demonstrated practical information about carcinogenesis and the life expectancy of patients with HCV-related liver cirrhosis and the order of priority in the management of interferon for these patients. Interferon administration is considered and initiated in patients with HCV-related liver cirrhosis preferably to reduce the probability for the transition from liver cirrhosis to HCC.

Because carcinogenesis is not a single-step event, but rather a complex, multi-step process, the exact mechanism of the role of glycyrrhizin in suppressing liver carcinogenesis remains unknown. One of the principal functions of long-term administration of glycyrrhizin in decreasing the carcinogenesis rate is considered to be anti-inflammation, which blocks the active carcinogenic process of continuous hepatic necro-inflammation and cell damage. In the treated group of the present study, the median ALT values markedly decreased after initiation of the glycyrrhizin injections, suggesting that the pathological process of hepatocyte necrosis or apoptosis was significantly suppressed by glycyrrhizinic acid. The actions of the amino acids, glycine and cysteine contained in SNMC have not been completely explained, but these substances have been demonstrated to suppress increased aldosterone levels that are induced by glycyrrhizinic acid. Tarao et al. [42] reported that a high ALT level resulted in an increased HCC recurrence rate in patients with HCC. From the standpoint of these anti-inflammatory activities, SNMC may be considered to only postpone the time of HCC appearance in the clinical course of cirrhosis. Since the entire process of hepatocellular carcinogenesis from the initial transformation of a hepatocyte to a detectable growth of cancer is considered to take at least several years, the influence of glycyrrhizin on the carcinogenesis rate cannot be evaluated over a short period.

Because the data in the present study were obtained from a retrospective cohort analysis, glycyrrhizin doses, times of injection per week and duration of therapy varied in each patient in the treated group. In order to elucidate the cancer preventive effect of glycyrrhizin therapy in active HCV-related liver disease, we should further stratify the treated patients or perform much more detailed statistical procedures. Future studies should aim at defining the basic oncogenic mechanisms and roles of long-term administration of glycyrrhizin in carcinogenesis in chronic hepatitis patients with cirrhosis caused by HCV.

In conclusion, the results of the present study demonstrated that long-term intermittent glycyrrhizin (SNMC) therapy for a few years or more successfully reduced disease progression probability (progression to carcinogenesis plus progression to death) in patients with HCV-related cirrhosis. A randomized controlled trial with a larger number of cases, with or without glycyrrhizin therapy, is expected to confirm the effectiveness of this therapy.

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Evolution of Simeprevir-Resistant Variants Over Time by Ultra-Deep Sequencing in HCV Genotype 1b

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Using ultra-deep sequencing technology, the present study was designed to investigate the evolution of simeprevir-resistant variants (amino acid substitutions of aa80, aa155, aa156, and aa168 positions in HCV NS3 region) over time. In Toranomon Hospital, 18 Japanese patients infected with HCV genotype 1b, received triple therapy of simeprevir/PEG-IFN/ribavirin (DRAGON or CONCERT study). Sustained virological response rate was 67%, and that was significantly higher in patients with *IL28B* rs8099917 TT than in those with non-TT. Six patients, who did not achieve sustained virological response, were tested for resistant variants by ultra-deep sequencing, at the baseline, at the time of re-elevation of viral loads, and at 96 weeks after the completion of treatment. Twelve of 18 resistant variants, detected at re-elevation of viral load, were de novo resistant variants. Ten of 12 de novo resistant variants become undetectable over time, and that five of seven resistant variants, detected at baseline, persisted over time. In one patient, variants of Q80R at baseline (0.3%) increased at 96-week after the cessation of treatment (10.2%), and de novo resistant variants of D168E (0.3%) also increased at 96-week after the cessation of treatment (9.7%). In conclusion, the present study indicates that the emergence of simeprevir-resistant variants after the start of treatment could not be predicted at baseline, and the majority of de novo resistant variants become undetectable over time. Further large-scale prospective studies should be performed to investigate the clinical utility in detecting simeprevir-resistant variants. ***J. Med. Virol. 86:1314–1322, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: HCV; ultra-deep sequence; simeprevir; resistant variants

INTRODUCTION

New strategies have been introduced for the treatment of chronic hepatitis C virus (HCV) infection based on inhibition of protease in the NS3/NS4 of the HCV polyprotein. Of the first-generation NS3/4A protease inhibitors, telaprevir had been approved for the treatment of patients infected with HCV genotype 1. The inclusion of this agent in HCV treatment regimens had led to large improvements in sustained virological rates, though this agent requires dosing three times daily and the use is associated with increased incidence and, in some cases, severity of adverse events such as anemia and rash [Hézode et al., 2009; McHutchison et al., 2009, 2010; Hayashi et al., 2012; Kumada et al., 2012].

Simeprevir (TMC435) is an investigational, once-daily oral NS3/4A protease inhibitor currently under clinical development globally. Phase II trials of simeprevir in combination with peginterferon (PEG-IFN) and ribavirin for patients infected with HCV genotype 1 demonstrated that simeprevir was generally well tolerated, had a pharmacokinetic profile that supports once-daily dosing and resulted in high virologic response rates. Treatment-naive patients, who received simeprevir for 12 or 24 weeks (SMV12 or SMV24) with PEG-IFN/ribavirin for 24 or 48 weeks (PR24 or PR48) (according to response-guided therapy), could achieve sustained virological response rates of 74–86%

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(PILLAR study) [Fried et al., 2013] and 77–92% (DRAGON study) [Hayashi et al., 2014]. Furthermore, treatment-experienced patients, who received simeprevir for 12, 24, or 24 weeks (SMV12, SMV24, or SMV24) with PEG-IFN/ribavirin for 48 weeks (PR48), could achieve sustained virological response rates of 61–80% (ASPIRE study) [Zeuzem et al., 2014].

Along with a high sustained virological response, NS3/4A protease inhibitor-based therapies are reported to induce resistant variants in patients, who could not achieve sustained virological response [Hézode et al., 2009; McHutchison et al., 2010; Fried et al., 2013; Hayashi et al., 2014; Zeuzem et al., 2014]. Hence, there is a need to predict at baseline the appearance of NS3/4A protease inhibitor-resistant variants. Previous reports have described the advantages of ultra-deep sequencing technology, including faster processing and large-scale sequencing, in addition to providing a better understanding of the dynamics of variants in HCV quasispecies [Hiraga et al., 2011; Nasu et al., 2011; Ninomiya et al., 2012]. Recent study based on telaprevir-based therapy showed that it was difficult to predict at baseline the emergence of telaprevir-resistant variants during triple therapy, even with the use of ultra-deep sequencing [Akuta et al., 2013]. However, it is not clear at this stage whether such technology is useful for the prediction of the emergence of simeprevir-resistant variants during or after the administration of simeprevir-based therapy.

The aim of this study using ultra-deep sequencing technology was to investigate the evolution of simeprevir-resistant variants over time after commencement of triple therapy of simeprevir/PEG-IFN/ribavirin, in adult Japanese patients infected with HCV genotype 1.

PATIENTS AND METHODS

Study Design

The Dose and duration Ranging study of Antiviral agent TMC435 in Genotype One HCV treatment-Naive patients (DRAGON) was a Phase II study conducted across Japan to evaluate the efficacy, safety, and pharmacokinetics of simeprevir and PEG-IFNα-2a/ribavirin in treatment-naive patients infected with HCV genotype 1. Simeprevir dose of 50 or 100 mg once-daily was administered orally for evaluation in this dose-ranging study. Patients were randomized to one of five treatment groups (SMV12/PR24 50 mg, SMV24/PR24 50 mg, SMV12/PR24 100 mg, SMV24/PR24 100 mg, and PR48) [Hayashi et al., 2014]. Furthermore, Clinical Optimization of New treatment strategy with TMC435 in Combination with pEginterferon plus Ribavirin for Treatment-naive and treatment-experienced patients infected with HCV genotype 1 (CONCERT) was a Phase III study conducted across Japan to evaluate the efficacy and safety of simeprevir and PEG-IFNα-2a/ribavirin in treatment-naive patients (CONCERT-1), simeprevir and PEG-IFNα-2a/ribavirin in treatment-experienced patients (CONCERT-2 for prior non-response, and CONCERT-3 for prior relapse), and simeprevir and PEG-IFNα-2b/ribavirin in treatment-naive and experienced patients (CONCERT-4). In CONCERT-1, patients were randomized to one of two treatment groups (SMV12/PR24 100 mg and PR48). In CONCERT-2, patients were randomized to one of two treatment groups (SMV12/PR24 100 mg and SMV24/PR24 100 mg). In CONCERT-3, patients were randomized to one treatment group (SMV12/PR24 100 mg). In CONCERT-4, treatment-naive and relapse patients were assigned to one treatment group (SMV12/PR24 100 mg), and patients of prior non-response were assigned to one treatment group (SMV12/PR48 100 mg) [Drafting Committee for Hepatitis Management Guidelines, the Japan Society of Hepatology, 2014]. In DRAGON and CONCERT-1,2,3, at week 24, patients either stopped or continued treatment with PEG-IFN/ribavirin up to week 48, according to response-guided therapy criteria. These studies protocols were in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and were approved by Toranomon Hospital Ethics Committee. Each patient gave an informed consent before participation in these trials.

In DRAGON and CONCERT-1,2,3, PEG-IFNα-2a (Pegasys®; Chugai, Tokyo, Japan) was administered as a subcutaneous injection (180 μg once weekly) and ribavirin (Copegus®; Chugai, Tokyo, Japan) as oral tablets (600–1,000 mg total daily dose, depending on body weight) in accordance with the manufacturer's prescribing information for both medication. In CONCERT-4, PEG-IFNα-2b (PegIntron®; MSD, Tokyo, Japan) was administered as a subcutaneous injection (1.5 μg/kg once weekly, depending on body weight), and ribavirin (Rebetol®; MSD, Tokyo, Japan) as oral tablets (600–1,000 mg total daily dose, depending on body weight) in accordance with the manufacturer's prescribing information for both medication.

The efficacy of treatment was evaluated by HCV-RNA negativity at 24 weeks after the completion of therapy (sustained virological response), based on the COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan). Failure to achieve sustained virological response was classified as non-response (HCV-RNA detected during or at the end of treatment), viral breakthrough (re-elevation of viral loads before the end of treatment, even when HCV-RNA was temporarily negative during treatment), and relapse (re-elevation of viral loads after the end of treatment, even when HCV-RNA was negative at the end of treatment). Especially, non-response was defined as null response (a reduction of less than 2 $\log_{10}$ in HCV RNA at 12 weeks after the start of therapy) or partial response (a reduction of 2 $\log_{10}$ or more in HCV RNA at 12 weeks).

Study Patients

Between October 2009 and June 2011, 20 patients infected with HCV met the following inclusion and exclusion criteria in one of these two studies (DRAGON or CONCERT) at the Department of Hepatology, Toranomon Hospital: (1) HCV genotype 1 confirmed by sequence analysis. (2) HCV RNA levels of ≥5.0 log IU/ml determined by the COBAS TaqMan HCV test (Roche Diagnostics). (3) Age at study entry of 20–70 years. (4) Absence of liver cirrhosis or hepatic failure, or other liver disease. (5) No infection/co-infection with HIV-1, HIV-2, hepatitis B, or HCV non-genotype 1. (6) No treatment for malignancy within 5 years prior to study. (7) No history of hepatocellular carcinoma. (8) No conditions that required caution with PEG-IFN or ribavirin treatment. (9) No history of any clinically significant disease. (10) No history of organ transplant. Absence of defined laboratory abnormalities during screening. In DRAGON, four treatment-naive patients were randomized to one of five treatment groups (two patients of SMV12/PR24 50 mg, none of SMV12/PR24 100 mg, one of SMV24/PR24 100 mg, and one of PR48). In CONCERT-1, six treatment-naive patients were randomized to one of two treatment groups (five patients of SMV12/PR24 100 mg, and one of PR48). In CONCERT-2, four patients of prior non-response were randomized to one of two treatment groups (three patients of SMV12/PR24 100 mg, and one of SMV24/PR24 100 mg). In CONCERT-4, three patients of prior relapse were assigned to one treatment group (SMV12/PR24 100 mg), and three patients of prior non-response were assigned to one treatment group (SMV12/PR48 100 mg).

The present study based on the 18 patients, who were assigned to triple therapy of simeprevir and PEG-IFN/ribavirin, was performed to investigate the evolution of simeprevir-resistant variants over time. Especially, patients, who did not achieve sustained virological response, was investigated whether the presence of low frequency resistant variants at baseline could predict the emergence of simeprevir-resistant variants after the start of triple therapy, using ultra-deep sequencing.

Measurement of HCV RNA

The antiviral effects of the triple therapy on HCV were assessed by measuring plasma HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every month before, during, and after therapy. HCV RNA concentrations were determined using the COBAS TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2–7.8 log IU/ml, and the undetectable samples were defined as negative.

Determination of *IL28B* Genotype

IL28B rs8099917 were genotyped by the Invader assay, TaqMan assay, or direct sequencing, as described previously [Ohnishi et al., 2001; Suzuki et al., 2003].

Detection of Amino Acid Substitutions in Core Regions of HCV Genotype 1

With the use of HCV-J (accession no. D90208) as a reference [Kato et al., 1990], the sequence of 1–191 aa in the core protein of HCV genotype 1b was determined and then compared with the consensus sequence constructed in a previous study to detect substitutions at aa 70 of arginine (Arg70) or glutamine/histidine (Gln70/His70) and aa 91 of leucine (Leu91) or methionine (Met91) [Akuta et al., 2005, 2007, 2010].

Assessment of Simeprevir-Resistant Variants

The genome sequence of the N-terminal 609 nucleotides (203 amino acids) in the NS3 region of HCV isolates from the patients was examined. HCV RNA was extracted from 100 μl of serum, and simeprevir-resistant variants were determined by ultra-deep sequencing. The primers used to amplify the NS3 region were NS3-F1 (5′-ACA CCG CGG CGT GTG GGG ACA T-3′; nucleotides 3295–3316) and NS3-AS2 (5′-GCT CTT GCC GCT GCC AGT GGG A-3′; nucleotides 4040–4019) as the first (outer) primer pair and NS3-F3 (5′-CAG GGG TGG CGG CTC CTT-3′; nucleotides 3390–3407) and NS3-AS2 as the second (inner) primer pair [Suzuki et al., 2012]. Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 sec at 95°C, annealing of primers for 1 min at 63°C, extension for 1 min at 72°C, and final extension was performed at 72°C for 7 min. The PCR-amplified DNA was purified after agarose gel electrophoresis, and then used for ultra-deep sequencing. Patients, who did not achieve sustained virological response, were tested for simeprevir-resistant variants by ultra-deep sequencing, at the baseline, at the time of re-elevation of viral loads, and at 96 weeks after the completion of treatment. simeprevir-resistant variants included Q80K/R/H/G/L, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, and D168A/V/E/G/N/T/Y/H/I [Romano et al., 2010].

Ultra-deep sequencing was performed using the Ion Personal Genome Machine™ (PGM™) Sequencer (Life Technologies, Carlsbad, CA). An Ion Torrent adapter-ligated library was prepared using an Ion Xpress Plus Fragment Library Kit (Life Technologies). Briefly, 100 ng of fragmented genomic DNA was ligated to the Ion Torrent adapters P1 and A. The adapter-ligated products were nick-translated and PCR-amplified for a total of eight cycles. Subsequently, the library was purified using AMPure beads (Beckman Coulter, Brea, CA) and the concentration determined using the StepOne Plus RealTime PCR (Life Technologies) and Ion Library Quantitation Kit, according to the instructions provided by the manufacturer. Emulsion PCR was performed using Ion OneTouch (Life Technologies) in conjunction with Ion OneTouch 200 Template Kit v2 (Life Technologies).

Enrichment for templated Ion spheres particles (ISPs) was performed using Ion OneTouch Enrichment System (Life Technologies), according to the instructions provided by the manufacturer. Templated ISPs was loaded onto an Ion 314 chip, and subsequently sequenced using 130 sequencing cycles according to the Ion PGM 200 Sequencing Kit user guide. Total output read length per run is over 10 Mbase (0.5M-tag, 200 base read) [Elliott et al., 2012]. The results were analyzed with the CLC Genomics Workbench software (CLCbio, Aarhus, Denmark) [Vogel et al., 2012].

A control experiment was also included to validate the error rates in ultra-deep sequencing of the viral genome. In this study, amplification products of the second-round PCR were ligated with plasmid and transformed in *Escherichia coli* in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). A plasmid-derived NS3 sequence was determined as the template, by the control experiment. The fold coverage evaluated per position for aa 80, aa 155, aa 156, and aa 168 in NS3 region, were 332,062×, 106,435×, 105,979×, and 33,725×, respectively. Thus, using the control experiment based on plasmid encoding HCV NS3 sequence, amino acid mutations were defined as amino acid substitutions at frequency of more than 0.2% among the total coverage. This frequency ruled out putative errors caused by ultra-deep sequence platform used in this study [Akuta et al., 2013].

Statistical Analysis

Non-parametric tests (chi-squared test and Fisher's exact probability test) were used to determine those factors that significantly contributed to sustained virological response. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL).

RESULTS

Virological Response to Therapy

Table I summarizes the profiles and laboratory data of the 18 patients at commencement of triple therapy, treatment regimen, treatment duration, and efficacy. They included 6 males and 12 females, aged 38–70 years (median, 60 years). The sustained virological response rate was 66.7% (12 of 18 patients). Taking into consideration the response to prior treatment, sustained virological response was achieved by 75.0% (6 of 8 patients), 100% (3 of 3 patients), and 42.9% (3 of 7 patients) by treatment-naive patients, patients who showed relapse to prior treatment, and non-responders to prior treatment, respectively. Of the six patients who did not show sustained virological response, the relapse, breakthrough, and non-response rates were 66.7% (Cases 2,3,11,17), 16.7% (Cases 12), and 16.7% (Cases 18), respectively. Cases 1 stopped simeprevir before the completion of 12-week treatment, due to the increase of total bilirubin levels (48-week PEG-IFN and ribavirin continued), and Case 5 stopped PEG-IFN and ribavirin before the completion of 24-week treatment, due to a fall in Hb concentration. Case 18 stopped the triple therapy at 12 weeks before the completion of the 48-week regimen, due to null response.

The sustained virological response rate was significantly higher in patients with *IL28B* rs8099917 TT (100% [8 of 8 patients]) than in those with non-TT (40% [4 of 10]) ($P = 0.013$). However, the present study based on the small numbers of patients did not identify the other factors that significantly contributed to sustained virological response.

Simeprevir-Resistant Variants Detected by Ultra-Deep Sequencing at Re-Elevation of Viral Load

Table II indicates the evolution of simeprevir-resistant variants over time by ultra-deep sequencing in patients, who did not achieve sustained virological response.

In Case 3,11,18, very low frequency variants at baseline persisted during treatment as very low frequency variants, but de novo resistant variants also emerged during treatment. In Case 3 (relapse), very low frequency variants of Q80R (0.2% of 141,326× coverage) at baseline persisted during treatment as very low frequency variants of Q80R (0.2% of 174,968× coverage), but de novo resistant variants (D168V [0.8% of 200,756× coverage] and D168G [0.2% of 200,756× coverage]) also emerged during treatment. In Case 11 (relapse), very low frequency variants of R155Q (0.2% of 29,454× coverage) at baseline persisted during treatment as very low frequency variants of R155Q (0.4% of 34,796× coverage), but de novo resistant variants (D168V [77.4% of 80,708× coverage]) also emerged during treatment. In Case 18 (null response), very low frequency variants of Q80R (0.3% of 143,435× coverage) at baseline persisted during treatment as very low frequency variants of Q80R (0.4% of 152,564× coverage), but de novo resistant variants (D168V [99.5% of 131,749× coverage] and D168E [0.3% of 131,749× coverage]) also emerged during treatment.

In Case 2 (relapse), very high frequency variants of Q80L (99.8% of 213,853× coverage) at baseline persisted during treatment as very high frequency variants of Q80L (99.9% of 188,910× coverage), but de novo resistant variants (D168G [61.3% of 137,720× coverage] and R155Q [2.4% of 165,980× coverage]) also emerged during treatment.

In Case 12 (breakthrough), very low frequency variants (Q80L [1.0% of 151,968× coverage] and Q80R [0.2% of 151,968× coverage]) at baseline increased during treatment (Q80L [76.0% of 158,451× coverage] and Q80R [1.7% of 158,451× coverage]), and de novo resistant variants (D168V [25.8% of 184,963× coverage] and D168E [2.0% of 184,963× coverage]) also emerged during treatment.

TABLE I. Profile at Commencement of Triple Therapy, Treatment Regimen, Treatment Duration, and Efficacy

Case	Sex	Age	HCV genotype	*IL28B* rs8099917	Core aa70	BMI (kg/m^2)	Previous response	Treatment regimen					Treatment duration		Efficacy
								Study	SMV (mg)	PEG type	PEG dose (μg)	RBV/BW (mg/kg)	SMV (wk)	PEG/RBV (wk)	
1[a]	M	60	1b	TT	Arg70	26.3	Naive	DRAGON	50	α2a	180	10.9	3	48	SVR
2	F	46	1b	TG	Arg70	20.9	Naive	DRAGON	50	α2a	180	10.6	12	24	Relapse
3	F	63	1b	TG	Gln70	20.9	Naive	DRAGON	100	α2a	180	11.4	24	24	Relpase
4	M	69	1b	TT	Arg70	23.2	Naive	CONCERT1	100	α2a	180	13.2	12	24	SVR
5[a]	F	63	1b	TT	Arg70	21.4	Naive	CONCERT1	100	α2a	180	11.2	12	22	SVR
6	F	52	1b	TT	Arg70	21.3	Naive	CONCERT1	100	α2a	180	11.2	12	24	SVR
7	M	53	1b	TT	Arg70	24.2	Naive	CONCERT1	100	α2a	180	11.2	12	24	SVR
8	F	55	1b	TG	Arg70	19.5	Naive	CONCERT1	100	α2a	180	12.5	12	24	SVR
9	F	70	1b	TG	Arg70	19.6	Partial	CONCERT2	100	α2a	180	12.9	12	24	SVR
10	F	61	1b	TG	Gln70	22.5	Partial	CONCERT2	100	α2a	180	10.5	24	24	SVR
11	M	45	1b	TG	Gln70	21.1	Null	CONCERT2	100	α2a	180	13.2	12	24	Relapse
12	F	67	1b	TG	Gln70	23.4	Null	CONCERT2	100	α2a	180	10.9	12	24	Breakthrough
13	F	59	1b	TT	Arg70	21.6	Relapse	CONCERT4	100	α2b	80	11.2	12	24	SVR
14	F	43	1b	TT	Arg70	22.4	Relapse	CONCERT4	100	α2b	80	10.0	12	24	SVR
15	M	38	1b	TT	Gln70	27.5	Relapse	CONCERT4	100	α2b	120	11.7	12	24	SVR
16	F	51	1b	TG	Gln70	22.9	Partial	CONCERT4	100	α2b	80	11.3	12	24	SVR
17	F	64	1b	TG	Arg70	20.1	Partial	CONCERT4	100	α2b	60	13.6	12	48	Relapse
18[a]	M	62	1b	TG	Gln70	26.2	Partial	CONCERT4	100	α2b	100	12.2	12	12	Null

[a]Cases 1 stopped simeprevir before the completion of 12-week treatment, due to the increase of total bilirubin levels (48-week PEG-IFN and ribavirin continued), and Case 5 stopped PEG-IFN and ribavirin before the completion of 24-week treatment, due to a fall in Hb concentration. Case 18 stopped the triple therapy at 12 weeks before the completion of the 48-week regimen, due to null response.

TABLE II. Evolution of Simeprevir-Resistant Variants Over Time by Ultra-Deep Sequencing in Patients, Who Did Not Achieve Sustained Virological Response

		At point of baseline			At point of re-elevation of viral loads			At point of 96Wk after the stop of therapy			
Case	Position	Frequencies (%)	Coverage	Viral loads	Frequencies (%)	Coverage	Viral loads	Frequencies (%)	Coverage	Viral loads	Efficacay
2	aa80	L (99.8%)	213,853×	6.0	L (99.9%)	188,910×	5.3	L (99.8%)	266,418×	6.1	Relapse
	aa155	—	197,250×		Q (2.4%)	165,980×		—	187,727×		
	aa156	—	176,208×		—	159,755×		—	179,884×		
	aa168	N (0.2%)	174,675×		G (61.3%)	137,720×		—	192,342×		
3	aa80	R (0.2%)	141,326×	6.8	R (0.2%)	174,968×	3.8	R (0.2%)	138,981×	6.7	Relapse
	aa155	—	114,101×		—	145,934×		—	92,640×		
	aa156	—	129,256×		—	165,294×		—	99,197×		
	aa168	—	166,778×		V (0.8%)·G (0.2%)	200,756×		G (0.2%)	115,136×		
11	aa80	—	141,147×	6.8	—	122,613×	7.3	—	86,092×	6.8	Relapse
	aa155	Q (0.2%)	29,454×		Q (0.4%)	34,796×		Q (0.2%)	20,149×		
	aa156	—	46,117×		—	47,303×		—	30,053×		
	aa168	—	91,622×		V (77.4%)	80,708×		—	65,050×		
12	aa80	L (1.0%)·R (0.2%)	151,968×	7.3	L (76.0%)·R (1.7%)	158,451×	7.3	R (0.2%)	189,759×	7.3	Breakthrough
	aa155	—	177,405×		—	194,176×		—	184,982×		
	aa156	—	189,503×		—	202,505×		—	194,138×		
	aa168	—	176,868×		V (25.8%)·E (2.0%)	184,963×		—	182,671×		
17	aa80	—	175,466×	7.0	R (0.2%)	156,143×	7.1	—	231,206×	6.9	Relapse
	aa155	—	135,348×		Q (2.2%)	161,895×		—	149,245×		
	aa156	—	150,908×		—	196,537×		—	178,579×		
	aa168	—	86,237×		V (79.0%)	92,466×		—	96,975×		
18	aa80	R (0.3%)	143,435×	6.4	R (0.4%)	152,564×	4.0	R (10.2%)	153,700×	6.8	Null
	aa155	—	88,537×		—	85,458×		Q (0.2%)	89,740×		
	aa156	—	109,437×		—	106,712×		—	114,061×		
	aa168	—	120,870×		V (99.5%)·E (0.3%)	131,749×		E (9.7%)	116,052×		

In Cases 17 (relapse), resistant variants were not detected at baseline, but de novo resistant variants (D168V [79.0% of 92,466× coverage], R155Q [2.2% of 161,895× coverage], and Q80R [0.2% of 156,143× coverage]) were detected at re-elevation of viral load.

Thus, the present study using ultra-deep sequencing indicates that the majority of resistant variants detected at re-elevation of viral load was de novo resistant variants (12 of 18 variants), and that the emergence of variants after the start of treatment could not be predicted at baseline.

Simeprevir-Resistant Variants Detected by Ultra-Deep Sequencing at 96-Week after the Cessation of Treatment

At 96-week after the cessation of treatment, de novo resistant variants detected at re-elevation of viral load, were undetected (R155Q of Case 2,17) (D168G of Case 2) (D168V of Case 3,11,12,17,18) (D168E of Case 12) (Q80R of Case 17), or detected as very low frequency variants of 0.2% (D168G of Case 3). However, very high (≥99.0%) or low (0.2%) frequency variants at baseline persisted as very high or low frequency variants at 96-week after the cessation of treatment (Q80L of Case 2) (Q80R of Case 3,12) (R155Q of Case 11).

In Case 18, variants of Q80R at baseline (0.3% of 143,435× coverage) persisted as very low frequency variants at re-elevation of viral load (0.4% of 152,564× coverage), but increased at 96-week after the cessation of treatment (10.2% of 153,700× coverage). Furthermore, de novo resistant variants of D168E (0.3% of 131,749× coverage) were detected at re-elevation of viral load, but increased at 96-week after the cessation of treatment (9.7% of 116,052× coverage).

Thus, the present study using ultra-deep sequencing indicates that the majority of de novo resistant variants become undetectable over time (10 of 12 variants), and that the majority of resistant variants detected at baseline persisted over time (5 of 7 variants).

DISCUSSION

Previous study based on telaprevir-based therapy showed that de novo telaprevir-resistant variants emerged regardless of variants frequencies at baseline, and that the emergence of variants after the start of triple therapy could not be predicted at baseline [Akuta et al., 2013]. The present study also indicated that the majority of simeprevir-resistant variants at re-elevation of viral load was de novo resistant variants, and that the emergence of variants could not be predicted at baseline. Hence, patients, who failed to achieve sustained virological response to simeprevir/PEG-IFN/ribavirin triple therapy, need to be identified to avoid the emergence of simeprevir-resistant variants. *IL28B* genotype have been useful as pretreatment predictors of poor virological response to telaprevir/PEG-IFN/ribavirin triple therapy [Akuta et al., 2010]. The present study based on the small numbers of patients indicated that sustained virological response rate was significantly higher in patients with *IL28B* rs8099917 TT than in those with non-TT, but did not identify the other factors that significantly contributed to sustained virological response. Recently, a polymorphism (ss469415590) within the gene that encodes a novel IFN-lambda 4 (*IFNL4*) protein has been found to be strongly associated with outcome of PEG-IFN/ribavirin dual therapy or telaprevir/PEG-IFN/ribavirin triple therapy [Fujino et al., 2013; Kawakami et al., 2013; Prokunina-Olsson et al., 2013]. In this study, the effect of *IFNL4* genotype could be assessed in 8 of 18 patients. Interestingly, sustained virological response rate for patients with the *IFNL4* TT/TT of the treatment-sensitive genotype was higher than patients with non-TT/TT of the treatment-resistant genotype in preliminary study based on the small numbers of patients (100% and 20%). Furthermore, one patient with the *IFNL4* TT/TT of the treatment-sensitive genotype, could achieve sustained virological response, regardless *IL28B* rs8099917 TG of the treatment-resistant genotype. Further studies of larger number of patients should be performed to investigate the pretreatment predictive factors of sustained virological response to simeprevir-based therapy, including host genetic factors (e.g., *IL28B* and *IFNL4* genotype) and viral factors (e.g., amino acid substitutions in the core region) [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; Akuta et al., 2010; Fujino et al., 2013; Kawakami et al., 2013; Prokunina-Olsson et al., 2013].

It is not clear at this stage whether the emergence of NS3/4A protease inhibitors-resistant variants might affect the second course of NS3/4A protease inhibitors-based treatment. The present study indicates that the majority of simeprevir-resistant variants become undetectable over time. However, in Case 18, variants of Q80R at baseline (0.3%) increased at 96-week after the cessation of treatment (10.2%), and de novo resistant variants of D168E (0.3%) also increased at 96-week after the cessation of treatment (9.7%). Recent report based on telaprevir-based therapy showed that one patient, who did not achieve sustained virological response by the first course of telaprevir/PEG-IFN/ribavirin, could achieve sustained virological response by the second course of telaprevir/PEG-IFN/ribavirin despite the persistence of de novo telaprevir-resistant variants (98.1% for V36C) [Akuta et al., 2014]. This finding may be due to one or more reasons. One reason is probably related to the high susceptibility of telaprevir-resistant variants to IFN. One previous study indicated that mice infected with the resistant strain (A156F [99.9%]) developed only low-level viremia and the virus was successfully eliminated with IFN therapy [Hiraga et al., 2011]. In the other clinical report, telaprevir-

resistant variants, which emerged during telaprevir monotherapy for 24 weeks, could be eliminated by PEG-IFN/ribavirin [Ozeki et al., 2011]. Furthermore, this finding probably suggests that a small number of mutant type viral RNA may be incomplete or defective, since a large proportion of viral genomes are thought to be defective due to the high replication and mutation rates of the virus [Bartenschlager and Lohmann, 2000]. However, recent report based on simeprevir-based therapy indicated persistence of simeprevir-resistant variants in patients infected with HCV genotype 1 at approximately 1.5 years after the cessation of simeprevir monotherapy, and which might affect response to re-treatment with simeprevir/PEG-IFN/ribavirin [Lenz et al., 2012]. Further studies of larger number of patients should be performed to evaluate whether the emergence of NS3/4A protease inhibitors-resistant variants affects treatment efficacy by the second course of NS3/4A protease inhibitors-based treatment.

In conclusion, the present study indicates that the emergence of simeprevir-resistant variants after the start of treatment could not be predicted at baseline, and the majority of de novo resistant variants become undetectable over time. One limitation in the present Japanese study is that the significance of preexisting resistant variants, especially HCV genotype 1a with variants of Q80K, could not be investigated. Patients with variants of Q80K at baseline indicated the lower rates of sustained virological response (46.7%) in PROMISE phase III trial with simeprevir/PEG-IFN/ribavirin [Forns et al., 2013]. Another limitation is that it could not be investigated at this stage whether the emergence of simeprevir-resistant variants (especially, variants of aa168) might affect the interferon-free regimens, including direct-acting antiviral agents, in future (e.g., Oral dual therapy of daclatasvir and asunaprevir, without PEG-IFN/ribavirin [Chayama et al., 2012; Karino et al., 2013]). Further large-scale prospective studies should be performed to investigate the clinical utility in detecting simeprevir-resistant variants on the response to treatment, and to help in the design of more effective therapeutic regimens.

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Utility of Detection of Telaprevir-Resistant Variants for Prediction of Efficacy of Treatment of Hepatitis C Virus Genotype 1 Infection

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The clinical usefulness of detecting telaprevir-resistant variants is unclear. Two hundred fifty-two Japanese patients infected with hepatitis C virus (HCV) genotype 1b received triple therapy with telaprevir–peginterferon (PEG-IFN)–ribavirin and were evaluated for telaprevir-resistant variants by direct sequencing at baseline and at the time of reelevation of the viral load. An analysis of the entire group indicated that 76% achieved a sustained virological response. Multivariate analysis identified a PEG-IFN dose of <1.3 μg/kg of body weight, an *IL28B* rs8099917 genotype (genotype non-TT), detection of telaprevir-resistant variants of amino acid (aa) 54 at baseline, nonresponse to prior treatment, and a leukocyte count of <5,000/mm^3 as significant pretreatment factors for detection of telaprevir-resistant variants at the time of reelevation of the viral load. In 63 patients who showed nonresponse to prior treatment, a higher proportion of patients with no detected telaprevir-resistant variants at baseline (54%) achieved a sustained virological response than did patients with detected telaprevir-resistant variants at baseline (0%). Furthermore, 2 patients who did not have a sustained virological response from the first course of triple therapy with telaprevir received a second course of triple therapy with telaprevir. These patients achieved a sustained virological response by the second course despite the persistence of very-high-frequency variants (98.1% for V36C) or a history of the emergence of variants (0.2% for R155Q and 0.2% for A156T) by ultradeep sequencing. In conclusion, this study indicates that the presence of telaprevir-resistant variants at the time of reelevation of viral load can be predicted by a combination of host, viral, and treatment factors. The presence of resistant variants at baseline might partly affect treatment efficacy, especially in those with nonresponse to prior treatment.

New strategies have been introduced recently for the treatment of chronic hepatitis C virus (HCV) infection based on the inhibition of protease in the nonstructural 3 (NS3)/NS4 region of the HCV polyprotein. Of the new agents currently available, telaprevir (VX-950) is used for the treatment of chronic HCV infection (1). Three studies (PROVE1, PROVE2, and a Japanese study [2–4]) showed that a 24-week regimen of triple therapy (telaprevir, peginterferon [PEG-IFN], and ribavirin) for 12 weeks followed by dual therapy (PEG-IFN and ribavirin) for 12 weeks (also called the T12PR24 regimen) achieved sustained virological response (SVR) (negative for HCV RNA for >24 weeks after the withdrawal of treatment) rates of 61%, 69%, and 73%, respectively, in patients infected with HCV genotype 1 (HCV-1). However, another study (PROVE3) found lower SVR rates to the T12PR24 regimen (39%) in nonresponders to previous PEG-IFN–ribavirin therapy infected with HCV-1 who did not achieve HCV RNA negativity during or at the end of the initial triple therapy course (5).

Telaprevir-based therapy is reported to induce resistant variants of HCV (6, 7). A recent report indicated that resistant variants are observed in most patients after failure to achieve an SVR by telaprevir-based treatment and that they tend to be replaced with wild-type viruses over time, presumably due to the lower fitness of those variants (8). However, the clinical usefulness of detecting telaprevir-resistant variants is still unclear. First of all, pretreatment factors associated with the detection of telaprevir-resistant variants at the time of reelevation of viral load have not been investigated. Furthermore, it is not clear at this stage whether the detection of telaprevir-resistant variants at baseline is useful for predicting the efficacy of telaprevir-based treatment and whether a history of the emergence of telaprevir-resistant variants affects treatment efficacy with the second course of telaprevir-based treatment.

Based on the above background, there is a need to investigate the clinical usefulness of detecting telaprevir-resistant variants. The aim of this study was to determine the pretreatment factors associated with the subsequent detection of telaprevir-resistant variants at the time of reelevation of viral load and the importance of telaprevir-resistant variants for predicting the efficacy of telaprevir-based treatment in patients infected with HCV-1b.

MATERIALS AND METHODS

Study population. From May 2008 through August 2013, 340 consecutive patients infected with HCV were selected for triple therapy with telaprevir (MP-424 or Telavic; Mitsubishi Tanabe Pharma, Osaka, Japan), PEG-IFN-α2b (PegIntron; MSD, Tokyo, Japan), and ribavirin (Rebetol; MSD, Tokyo) at the Department of Hepatology, Toranomon Hospital (located in metropolitan Tokyo, Japan). Subsequently, 252 of these patients received the triple therapy based on the following inclusion and exclusion criteria: (i) diagnosis of chronic hepatitis C, (ii) HCV-1b confirmed by sequence analysis, (iii) HCV RNA level of ≥5.0 log IU/ml as determined

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by the Cobas TaqMan HCV test (Roche Diagnostics, Tokyo, Japan), (iv) follow-up duration of ≥24 weeks after the completion of triple therapy, (v) no history of treatment with NS3/4A protease inhibitors, (vi) absence of decompensated liver cirrhosis and hepatocellular carcinoma (HCC), (vii) negative for hepatitis B surface antigen (HBsAg), (viii) no evidence of human immunodeficiency virus infection, (ix) negative history of autoimmune hepatitis, alcohol liver disease, hemochromatosis, and chronic liver disease other than chronic hepatitis C, (x) negative history of depression, schizophrenia, or suicide attempts, angina pectoris, cardiac insufficiency, myocardial infarction, severe arrhythmia, uncontrolled hypertension, uncontrolled diabetes, chronic renal dysfunction, cerebrovascular disorders, thyroidal dysfunction uncontrolled by medical treatment, chronic pulmonary disease, allergy to medication, or anaphylaxis at baseline, and (xi) pregnant or breastfeeding women or those willing to become pregnant during the study and men with a pregnant partner were excluded. The study protocol was in compliance with the guidelines for good clinical practice and the 1975 Declaration of Helsinki and was approved by the institutional review board of the Toranomon Hospital. Each patient received ample information about the goals and potential side effects of the treatment and their right to withdraw from the study at any time. Each patient provided a signed consent form before participating in this trial.

The efficacy of treatment was evaluated by the presence or absence of an HCV RNA-negative result at 24 weeks after the completion of therapy (i.e., SVR), as determined by the Cobas TaqMan HCV test (Roche Diagnostics). Furthermore, failure to achieve an SVR was classified as nonresponse (HCV RNA detected during or at the end of treatment) or relapse (at the time of reelevation of viral load after the end of treatment, even when HCV RNA result was negative at the end of treatment).

Twenty patients (8%) were assigned to a 12-week regimen of triple therapy (the T12PR12 group) and were randomly divided into two groups (10 patients each) treated with either 1,500 mg/day or 2,250 mg/day of telaprevir to evaluate the treatment efficacy during 12 weeks on treatment. Sixty patients (24%) were allocated to a 24-week regimen of the same triple therapy described above followed by dual therapy of PEG-IFN and ribavirin for another 12 weeks (the T12PR24 group) to evaluate treatment efficacy according to the response to prior treatment, and they were treated with 2,250 mg/day of telaprevir. Another group of 172 patients (68%) was treated as described above for the T12PR24 group except for the dosages of telaprevir; this group was divided into two groups treated with either 1,500 mg/day (111 patients) or 2,250 mg/day (61 patients) of telaprevir, as selected by the attending physician. Table 1 summarizes the profiles and laboratory data of the entire group of 252 patients at the commencement of treatment. They included 155 males and 97 females 21 to 73 years of age (median, 58 years). At the start of treatment, telaprevir was administered at a median dose of 30.8 mg/kg of body weight (range, 14.1 to 59.2 mg/kg) daily. One hundred thirty-one patients (52%) were treated with 2,250 mg/day of telaprevir, while the other 121 patients (48%) were treated with 1,500 mg/day of telaprevir. PEG-IFN-α2b was injected subcutaneously at a median dose of 1.5 μg/kg (range, 0.7 to 1.8 μg/kg) once a week. Ribavirin was administered at a median dose of 10.9 mg/kg (range, 4.3 to 15.8 mg/kg) daily. Each drug was discontinued or its dose reduced as required per the judgment of the attending physician, in response to a fall in hemoglobin level, leukocyte count, neutrophil count, or platelet count, or the appearance of side effects. The triple therapy was discontinued when the leukocyte count decreased to <1,000/mm^3, the neutrophil count decreased to <500/mm^3, the platelet count decreased to <5.0 × 10^4/mm^3, or when hemoglobin decreased to <8.5 g/dl.

Follow-up. Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. They were performed every week in the initial 12 weeks of treatment. Adverse effects were monitored clinically by careful interviews and a medical examination at least once every month. Compliance with treatment was evaluated by a questionnaire.

TABLE 1 Profile and laboratory data at commencement of telaprevir, peginterferon, and ribavirin triple therapy in patients infected with HCV genotype 1b

Variable	Patient data
Patient demographics	
No. of patients	252
Sex (no. of males/no. of females)	155/97
Median age (yr) (range)	58 (21–73)
Median body mass index (kg/m^2) (range)	22.8 (16.0–36.7)
Laboratory data (median [range])	
Level of viremia (log IU/ml)	6.7 (5.0–7.8)
Aspartate aminotransferase (IU/liter)	37 (15–624)
Alanine aminotransferase (IU/liter)	42 (11–525)
Albumin (g/dl)	3.9 (2.5–4.7)
Gamma-glutamyl transpeptidase (IU/liter)	34 (3–319)
Leukocyte count (/mm^3)	4,700 (2,000–8,400)
Hemoglobin (g/dl)	14.3 (12.1–17.6)
Platelet count (10^4/mm^3)	16.5 (8.5–33.8)
Treatment	
Median PEG-IFN-α2b dose (μg/kg) (range)	1.5 (0.7–1.8)
Median ribavirin dose (mg/kg) (range)	10.9 (4.3–15.8)
Median telaprevir dose (mg/kg) (range)	30.8 (14.1–59.2)
No. of patients with telaprevir dose of 1,500/2,250 mg/day	121/131
No. of patients on T12PR12/T12PR24 treatment regimen	20/232
Response to prior treatment	
No. of treatment-naive patients/no. of patients with relapse to prior treatment/no. of patients with nonresponse to prior treatment (IFN monotherapy/ribavirin combination therapy)/unknown	79/109/63 (16/47)/1
Amino acid substitutions in HCV genotype 1b	
Core aa 70 (arginine/glutamine [histidine]/ND[a])	162/88/2
Core aa 91 (leucine/methionine/ND)	139/111/2
ISDR of NS5A (wild type/non-wild type/ND)	199/24/29
IRRDR of NS5A (≤5/≥6/ND)	180/69/3
V3 of NS5A (≤2/≥3/ND)	64/185/3
IL28B genotype	
rs8099917 genotype (TT/non-TT/ND)	181/69/2
ITPA genotype	
rs112735 genotype (CC/non-CC)	186/65/1
NS3/4A protease inhibitor-resistant variants by direct sequencing[b]	
V36/T54/Q80/R155/A156/D168/V170	1/7/55/1/2/26/0

[a] ND, not determined.

[b] The NS3/4A protease inhibitor-resistant variants detected by direct sequencing included V36A/C/M/L/G, T54A/S, Q80K/R/H/G/L, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, D168A/V/E/G/N/T/Y/H/I, and V170A (19, 20).

Measurement of HCV RNA. The antiviral effects of the triple therapy on HCV were assessed by measuring blood plasma HCV RNA levels. In this study, HCV RNA levels during treatment were evaluated at least once every month before, during, and after therapy. HCV RNA concentrations were determined using the Cobas TaqMan HCV test (Roche Diagnostics). The linear dynamic range of the assay was 1.2 to 7.8 log IU/ml, and undetectable samples were defined as negative.