別紙3

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
（総括・分担）研究報告書

次世代シークエンス技術を駆使したウイルスゲノム解析による C型肝炎の病態解明と臨床応用

研究分担者　須田剛生

研究要旨
DAAs 治療前後の患者血清 in vitro の DAAs 耐性ウイルス長期安定培養系を用い、次世代シークエンサー、Molecular dynamics を駆使してその耐性機構を明らかにする。

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A．研究目的
少数存在する耐性ウイルスの存在様式・治療への影響は十分に明らかにされておらず、次世代シークエンサー・in vitro 培養系などを用いた検討が必須である
・DAAs の key drug である NS5A 阻害剤耐性機構は明らかなになっておらず MD による解析と in vitro 耐性ウイルスを用いて耐性化機構を明らかにする

B．研究方法
・DAAs 治療前後の患者血清、DAAs 耐性ウイルス長期安定培養系を用い、次世代シークエンサー、Moleculardynamics を駆使してその耐性機構を明らかにする。

（倫理面への配慮）
肝疾患患者からの試料提供を受ける場合には、試料提供者、その家族、および同様の肝疾患患者の人権、尊厳が保護されるよう充分に配慮する。遺伝子組み換え実験においては「遺伝子組換え生物等の使用等の規制による生物の多様性の確保に関する法律」（平成15年法律第97号）を遵守して実施する。

C．研究結果
MD を用いた耐性化機構の予想モデルの作成
・次世代シークエンスを用いた DAA 耐性ウイルスの IFN 併用療法治への治療効果への評価し治療失敗例では高率に耐性ウイルスが出現する事が明らかとなった。
・in vitro　DAA耐性ウイルス培養系の確立しインターフェロン感受性を評価し野生株と同等以上の感受性を有する可能性が想定された。

D．考察
DAA失敗例では、高率に薬剤耐性ウイルスが出現する事が明らかとなり今後の治療を行ううえでDAA耐性ウイルスを考慮しながら治療最適化を行う必要がある。

E．結論
DAAs 治療前の DAAs 耐性ウイルスの存在様式を明らかにし、治療効果との関連性を評価しえた。薬剤耐性 HCV 培養系を樹立した

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H．知的財産権の出願・登録状況
（予定を含む。）
1．特許取得
なし
2．実用新案登録
なし

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別冊

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Deep Sequencing and Phylogenetic Analysis of Variants Resistant to Interferon-Based Protease Inhibitor Therapy in Chronic Hepatitis Induced by Genotype 1b Hepatitis C Virus

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ABSTRACT

Because of recent advances in deep sequencing technology, detailed analysis of hepatitis C virus (HCV) quasispecies and their dynamic changes in response to direct antiviral agents (DAAs) became possible, although the role of quasispecies is not fully understood. In this study, to clarify the evolution of viral quasispecies and the origin of drug-resistant mutations induced by interferon (IFN)-based protease inhibitor therapy, the nonstructural-3 (NS3) region of genotype 1b HCV in 34 chronic hepatitis patients treated with telaprevir (TVR)/pegylated interferon (PEG-IFN)/ribavirin (RBV) was subjected to a deep sequencing study coupled with phylogenetic analysis. Twenty-six patients (76.5%) achieved a sustained viral response (SVR), while 8 patients did not (non-SVR; 23.5%). When the complexity of the quasispecies was expressed as the mutation frequency or Shannon entropy value, a significant decrease in the *IFNL3* (rs8099917) TT group and a marginal decrease in the SVR group were found soon (12 h) after the introduction of treatment, whereas there was no decrease in the non-SVR group and no significant decrease in mutation frequency in the *IFNL3* TG/GG group. In the analysis of viral quasispecies composition in non-SVR patients, major populations greatly changed, accompanied by the appearance of resistance, and the compositions were unlikely to return to the pretreatment composition even after the end of therapy. Clinically TVR-resistant variants were observed in 5 non-SVR patients (5/8, 62.5%), all of which were suspected to have acquired resistance by mutations through phylogenetic analysis. In conclusion, results of the study have important implications for treatment response and outcome in interferon-based protease inhibitor therapy.

IMPORTANCE

In the host, hepatitis C virus (HCV) consists of a variety of populations (quasispecies), and it is supposed that dynamic changes in quasispecies are closely related to pathogenesis, although this is poorly understood. In this study, recently developed deep sequencing technology was introduced, and changes in quasispecies associated with telaprevir (TVR)/pegylated interferon (PEG-IFN)/ribavirin (RBV) triple therapy and their clinical significance were investigated extensively by phylogenetic tree analysis. Through this study, the associations among treatment response, changes in viral quasispecies complexity in the early stage of treatment, changes in the quasispecies composition, and origin of TVR-resistant variant HCV were elucidated.

Recently, various novel small compounds with potent antiviral effects called direct antiviral agents (DAAs) have been developed for the treatment of chronic hepatitis C (1), and their significant antiviral activity is literally changing the world of anti-hepatitis C virus (HCV) therapy. Among these, nonstructural 3 (NS3) and NS4A protease inhibitors (PIs) were first approved for clinical use, and telaprevir (TVR) and simeprevir (SMV) became available for HCV infection in several countries, including Japan, in combination with pegylated interferon (PEG-IFN)/ribavirin (RBV) (2, 3). In high-titer genotype 1 patients refractory to conventional PEG-IFN/RBV therapy, a markedly higher sustained viral response (SVR) could be obtained with triple therapy by using these PIs combined with PEG-IFN/RBV (3–5).

One of the virological problems underlying DAA treatment is the appearance of drug-resistant HCV. In refractory patients, HCV variants with drug resistance become dominant populations in the host, eventually causing non-SVR. To date, V36, T54, R155, A156, D168, and V170 (6–8) have been identified as hot spot positions for PI resistance mutations, but the process by which HCV variants with resistance mutations appear has not been fully elucidated. On the other hand, it was recently reported that even DAA treatment-naive HCV might naturally have a substantial number of variants resistant to PIs, and this issue has been given

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TABLE 1 Patient characteristics according to response to TVR/PEG-IFN/RBV triple therapy

Characteristic[a]	SVR ($n = 26$)	Non-SVR ($n = 8$)	P value
Age (yrs; mean ± SD)	55.8 ± 7.5	59.4 ± 9.0	0.393
Sex (F/M)	13/13	5/3	0.693
Naive [no. (%)]	16 (61.5)	4 (50)	0.689
Albumin (g/dl; mean ± SD)	4.1 ± 0.4	4.2 ± 0.4	0.403
γ-GTP (IU/liter; mean ± SD)	56.7 ± 61.9	35.3 ± 17.2	0.477
AST (IU/liter; mean ± SD)	67.5 ± 53.5	42.8 ± 23.7	0.155
ALT (IU/liter; mean ± SD)	88.5 ± 75.3	44.8 ± 24.9	0.071
Platelets (10^4/ml; mean ± SD)	15.2 ± 4.9	14.8 ± 5.2	0.745
AFP (ng/ml; mean ± SD)	8.6 ± 9.4	7.6 ± 4.1	0.477
Core aa 70Q [no. (%)]	6 (23.1)	3 (37.5)	0.649
ISDR ≤ 2 [no. (%)]	7 (26.9)	1 (12.5)	0.645
IRRDR ≤ 5 [no. (%)]	11 (42.3)	5 (62.5)	0.438
IFNL3 TG/GG [no. (%)]	4 (15.4)	5 (62.5)	0.017
HCV RNA [log IU/ml; mean (range)]	6.4 (4.7–74)	6.9 (6.2–7.4)	0.080

[a] Abbreviations: AST, aspartate transaminase; ALT, alanine aminotransferase; AFP, α-fetoprotein.

attention (9). However, it was also reported that such naturally resistant HCV did not always exhibit treatment resistance (10, 11).

HCV is present as a mixed populations of closely related variant viruses, called quasispecies, in the host (12–14), and the quasispecies is speculated to modify the treatment response to antiviral agents, although detailed analysis of viral quasispecies has been technically difficult because of the necessity to obtain high-volume multiple viral sequences. Due to the recent development of deep sequencing techniques using next-generation sequencers (15–18), detailed analysis of quasispecies has become possible. Several deep sequencing studies have been undertaken to disclose the origin of DAA-resistant variants through analyzing DAA-resistant variant populations over time (10, 11, 16, 19, 20). On the other hand, previous investigations tended to focus on hot spots for specific mutations but lacked the phylogenetic analysis that is needed to determine the origins of certain viral populations.

To clarify the evolution of viral quasispecies and the origin of drug-resistant mutations induced by PIs combined with PEG-IFN/RBV, we chose patients who were undergoing TVR/PEG-IFN/RBV triple therapy and performed a deep sequencing study, including a phylogenetic analysis. We selected this strategy because TVR-included therapy was the first regimen that included a PI, which enabled us to analyze the influence of PIs on the viral quasispecies over a long period of time.

MATERIALS AND METHODS

Patients. Subjects were 34 HCV genotype 1b patients who consecutively received TVR/PEG-IFN/RBV combination therapy at Yamanashi University Hospital. The 24-week regimen consisted of TVR/PEG-IFN/RBV triple therapy for 12 weeks followed by dual therapy with PEG-IFN and ribavirin for 12 weeks. All patients fulfilled the following criteria: (i) negative for hepatitis B surface antigen; (ii) no other forms of hepatitis, such as primary biliary cirrhosis, autoimmune liver disease, or alcoholic liver disease; (iii) free of coinfection with human immunodeficiency virus. Signed consent was obtained for participation in the study protocol, which had been approved by the Human Ethics Review Committee of Yamanashi University. The clinical backgrounds of the 34 patients are summarized in Table 1.

Deep sequencing. Deep sequencing of part of the viral NS3 region was performed for all 34 patients at 2 time points: baseline and 12 h after the introduction of therapy. For 8 non-SVR patients, deep sequencing was additionally performed at 2 other time points: at viral reelevation and at the last observation (Fig. 1). Briefly, RNA was extracted from stored sera and reverse transcribed to cDNA (21). Then, a 2-step nested PCR was carried out with primers specific for the NS3 region of the HCV genome. This PCR procedure amplified 454 viral nucleotides, including nucleotides 81 to 534 of the NS3 region. The primers for the second-round PCR had bar codes 10 nucleotides in length attached, and these differed for each sample, so that the PCR products from each sample were identifiable (see Table S1 in the supplemental material). After band densities of the PCR products were quantified using a Pico Green double-stranded DNA assay kit (Invitrogen, Tokyo, Japan), concentrations in the samples were adjusted to a common value and pooled samples were prepared.

Libraries were then subjected to emulsion PCR, the enriched DNA beads were loaded onto a picotiter plate, and pyrosequencing was carried out with a Roche GS Junior/454 sequencing system using titanium chemistry (Roche, Branford, CT). The Roche Variant Analyzer version 2.5pl was used for the analysis.

Genetic analysis. The complexity of the quasispecies population obtained by deep sequencing was analyzed at the nucleotide level by 2 different methods: (i) mutation frequency (Mf) and (ii) normalized Shannon entropy (Sn) (22–26). The Mf represents the proportion of mutant nucleotides in a genome population. Briefly, after determining the consensus nucleotide in each nucleotide position at each time point for each

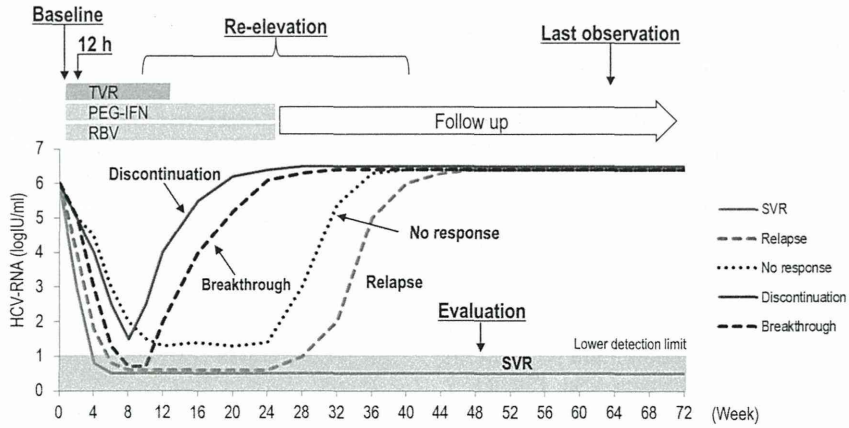

FIG 1 Clinical course of the patients and time points for serum sample collection for deep sequencing. NS3 deep sequence analysis at baseline and at 12 h was performed in all 34 patients, while that at reelevation and at the last observation was additionally performed in 8 non-SVR patients.