Detection of Antiviral Drug-Resistant Variants in Chronic Hepatitis C by Deep Sequencing

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Abstract

Due to the recent development of various novel small molecule compounds called direct-acting antiviral (DAA) agents and their dramatic antiviral potency against hepatitis C virus (HCV), more than 90 % of patients can now eradicate HCV with DAA therapy, a drastic improvement over the days of interferon therapy both in terms of efficacy and tolerability. On the other hand, the emergence of resistance-associated variants (RAVs) during DAA-based therapy has become a major problem. Namely, due to their high potency and specificity, there is a risk that patients treated with DAAs might develop resistance to one or more classes of DAA. Because of high cross-resistance among the small number of approved DAAs and the limited number of targets, it is also possible for HCV strains resistant to all current DAAs to emerge. Although it is possible to some extent to predict the emergence of RAVs through screening for the presence of RAVs prior to therapy using direct sequencing or other methods, precise and accurate prediction is still not possible because the presence of RAVs at baseline does not guarantee treatment failure nor does the lack of preexisting RAVs preclude the emergence of resistance through de novo mutation during treatment. In order to determine whether the emergence of RAVs is predictable based on the composition of HCV quasispecies prior to treatment, in this study deep sequencing was used to correlate the presence of RAVs at baseline with the emergence of RAVs during treatment in patients treated with interferon-based DAA triple therapy versus interferon-free DAA therapy.

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5.1 Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide, progressing to liver cirrhosis over the long course of infection and leading to the development of hepatocellular carcinoma at a rate of 8 % per year among patients with cirrhosis [1]. On the other hand, due to the remarkable recent advances in the development of novel direct-acting antivirals (DAAs), the sustained viral response (SVR) rate, defined as undetectable HCV 6 months after the end of therapy, can now be achieved in most HCV-infected patients [2], and the rate of advancement to cirrhosis or hepatocellular carcinoma is expected to be significantly reduced. At the same time, while DAAs are distinguished for their high potency as well as their low frequency of serious adverse events (SAE), a major problem associated with DAA therapy is the emergence of resistance-associated variants (RAVs).

Most DAAs for HCV in clinical use or under development are low-molecular-weight compounds that inhibit viral enzymatic activity by directly binding to viral proteins. Due to the highly targeted nature of DAA characteristics, the appearance of RAVs is a predictable and perhaps inevitable problem since HCV could easily acquire adaptive mutations that facilitate escape from DAA binding due to the error-prone polymerase that lacks proofreading capabilities. In the era of DAA therapy, therefore, clinicians should understand how RAVs emerge during DAA therapy in order to prevent the development of resistance and obtaining the best treatment outcome.

In this chapter, the association between RAVs and the treatment response to DAA therapy is described briefly by introducing our original deep sequencing data.

5.2 Deep Sequencing Analysis of RAVs and the Aim of RAV Analysis

At present, DAAs in clinical use target the replication phase of HCV life cycle and are classified into three types of inhibitor: (1) NS3/4A protease inhibitors, (2) NS5A replication complex inhibitors, and (3) NS5B polymerase inhibitors. NS5B polymerase inhibitors are further classified into nucleoside inhibitors (NIs) and non-nucleoside inhibitors (NNIs). Variants at V36, T54, R155, A156, D168, and V170 of the NS3 protein are known RAV hot spots among NS3/4A protease inhibitors, while variants at L28, R30, L31, P58, and Y93 of the NS5A protein are RAV hot spots among NS5A inhibitors [3, 4]. NS5B S282 substitutions confer resistance to NS5B nucleoside inhibitors (NS5B NIs), while M495 (thumb1), L419, R422,

M423, I482 (thumb2), M414, Y448, G554, S556, and D559 (palm1 and palm2) confer resistance to NS5B non-nucleoside inhibitors (NS5B NNIs) [5]. Although RAVs generally appear after the initiation of DAA therapy, it has also been reported that a subset of RAVs, particularly within the NS5A protein, are present in a substantial number of DAA treatment-naïve patients [6]. DAA-induced RAVs might exhibit cross-resistance to other DAAs of the same class, and, in the event of non-SVR, accumulation of RAVs might contribute to development of HCV strains resistant to all current DAAs used in therapy.

It is known that HCV exists as mixed populations of closely related variant viruses called "quasispecies" [7] and that quasispecies composition changes dynamically upon exposure to anti-HCV therapy. Since RAVs are also considered to appear or disappear as quasispecies, detailed analysis of viral quasispecies using deep sequencing might help to determine how clinically relevant RAVs develop [8]. In this study, deep sequencing was performed in order to explore the dynamics of RAV quasispecies.

5.3 Interferon-Based DAA Therapy

5.3.1 TVR/PEG-IFN/RBV Combination Therapy

In Japan, the first clinically available DAAs were NS3/4A protease inhibitors (PIs) approved for use in combination with pegylated interferon (PEG-IFN) plus ribavirin (RBV) as an extension of the current standard of care interferon therapy. Telaprevir (TVR), a first-generation PI, was approved in 2011, and two second-generation PIs, simeprevir (SMV) and vaniprevir (VPV), were approved in 2013 and 2014, respectively [9, 10].

In order to determine how clinically relevant HCV variants develop under IFN-based TVR/PEG-IFN/RBV combination therapy, we undertook a deep sequencing study and analyzed quasispecies changes over time in 34 patients with TVR/PEG-IFN/RBV combination therapy.

At first, early changes in genetic complexity after initiation of the triple therapy were determined by calculating Shannon's entropy (Sn) and mutation frequency (Mf), indexes of genetic complexity, and the correlation between changes in genetic complexity and the treatment response or genotype of a SNP in the IL28B(IFNL3) locus were investigated. Deep sequencing of the viral NS3 region was performed in all 34 patients for two time points (baseline and 12 h after the start of therapy) to examine early changes in genetic complexity of viral quasispecies (Mf and Sn) after initiation of triple therapy (12 h) (Fig. 5.1) [11]. As shown in Fig. 5.1, genetic complexity tended to decrease in the SVR and IL28B TT groups, while no such differences were observed in the non-SVR and in IL28B TG/GG groups. Since IL28B is a host factor associated with the response to IFN and since the IL28B SNP is associated with the rate of SVR in triple therapy, the decrease in the genetic complexity observed in the SVR/IL28B TT group could result from differences in the IFN

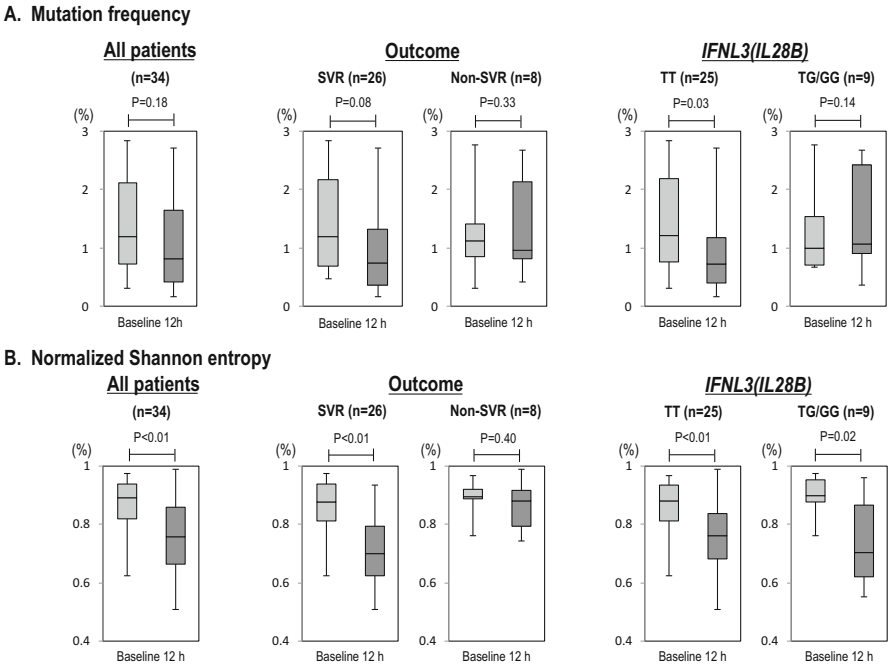

Fig. 5.1 Changes in the genetic complexity of the NS3 region 12 h after the introduction of TVR/PEG-IFN/RBV triple therapy

response between the two groups. It also suggested the possibility that changes in the genetic complexity as early as 12 h after the start of therapy might be used as a biomarker to predict SVR.

Next, we investigated changes in the viral sequence over time in the eight non-SVR patients (Table 5.1). A clinically resistant mutation was observed in five of the eight patients (62.5 %) during treatment and follow-up (V36C, 1; T54A, 2; A156F, 1; and A156S, 1). The same mutation was not observed at baseline even by deep sequencing in three of the five patients, but a T54A RAV was recognized at baseline as a minor population in two patients (Patients 3 and 7). The frequency of TVR-resistant RAVs was 98 % or more when the viral titer rebounded in all five patients, but the RAV frequency decreased in four patients (Patients 3, 4, 6, and 7) and was replaced by wild type as the dominant form during follow-up after the end of treatment.

Changes in the composition of HCV quasispecies over time were investigated in each of the non-SVR patients by constructing phylogenetic trees from all isolates obtained at three time points: baseline, re-elevation of the viral titer, and at the final observation. The isolates obtained at the re-elevation of the viral titer clustered differently from those at baseline (Fig. 5.2). Furthermore, isolates from the last observation point were distinct from those at baseline but seemed to be close to those obtained at the time of re-elevation (Figs. 5.2 and 5.3), demonstrating that the

Table 5.1 Telaprevir-resistant variants responsible for treatment failure in non-SVR patients and their time-dependent changes

Patient	Outcome	Previous response	IFNL3	Resistant variant				Variant rate (%)			
				V36	T54	R155	A156	Baseline	Re-elevation	Last observation (weeks after treatment)	
1	Discontinuation	Relapse	T/G								
2	Discontinuation	Relapse	T/T								
3	Discontinuation	Naïve	T/G		A			0.36	99.79	0.08	(26W)
4	Breakthrough	No response	T/T				F	0	98.19	0	(45W)
5	Relapse	Naïve	T/G								
6	Relapse	No response	T/T				S	0	99.48	1.27	(47W)
7	No response	Naïve	T/G		A			0.28	99.84	0.04	(34W)
8	No response	Naïve	T/G	C				0	98.11	96.05	(25W)

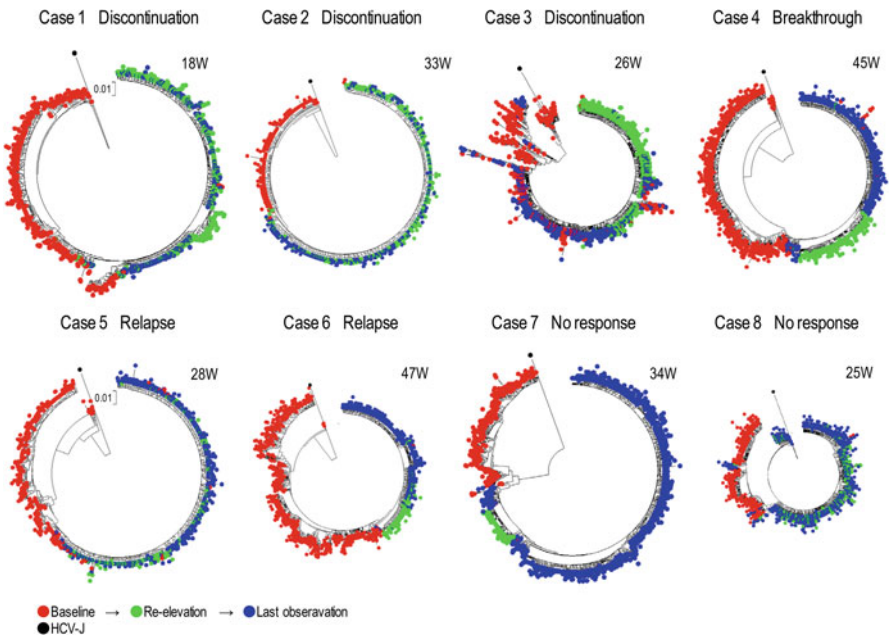

Fig. 5.2 Phylogenetic trees constructed for eight non-SVR patients using isolates at baseline, at re-elevation, and at the last observation. Numbers at the top right of each phylogenic tree indicate the number of weeks after the end of treatment

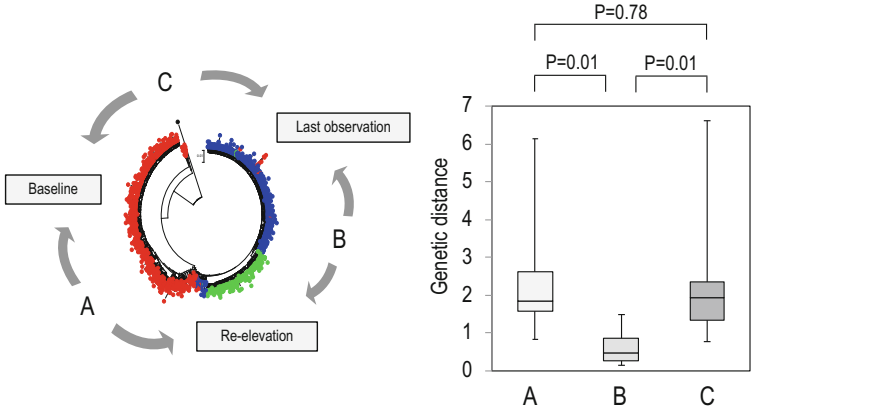

Fig. 5.3 Genetic distances in the NS3 region between baseline and re-elevation, between re-elevation and the last observation, and between the last observation and baseline in eight non-SVR patients. (**a**) Genetic distance between baseline and re-elevation. (**b**) Genetic distance between re-elevation and the last observation. (**c**) Genetic distance between baseline and the last observation

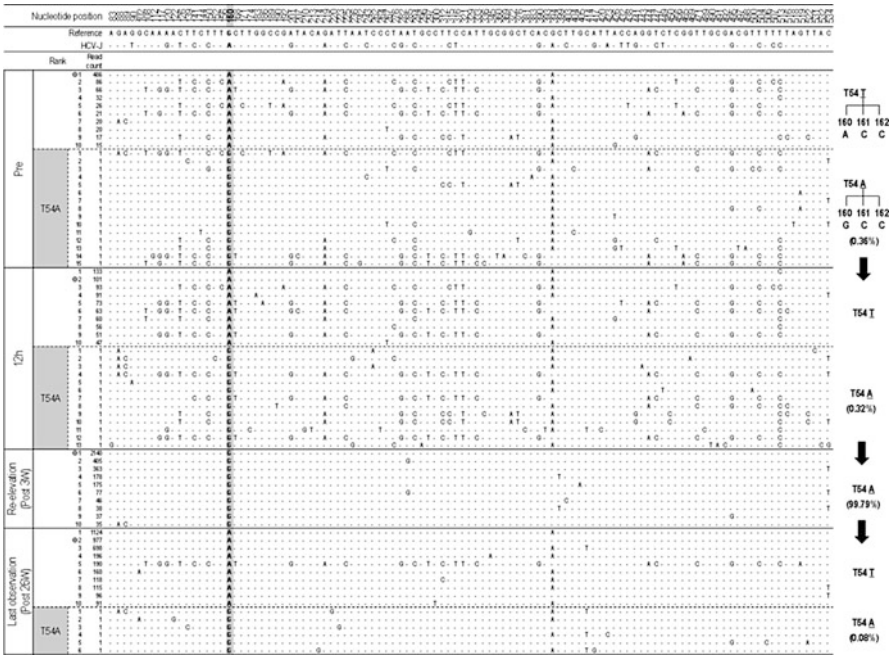

Fig. 5.4 Time-dependent changes in the top ten most populated isolates at baseline, 12 h, re-elevation, and last observation in Patient 3 who had RAV(T54A) at baseline in TVR/PEG-IFN/RBV therapy

changes in population composition induced by the triple therapy were unlikely to return to the pretreatment composition.

Lastly, to investigate which baseline populations developed TVR-resistant mutations, deep sequencing results over time were reanalyzed in patients in whom RAVs appeared (Patients 3, 4, 6, 7, and 8; Table 5.1). Among them, two patients (Patients 3 and 7) had T54A RAVs (0.36 % and 0.28 %, respectively) at baseline. In Patient 3, isolates with T54A accounted for 0.36 % of the population at baseline but had increased to 99.79 % at the time of re-elevation (Fig. 5.4). On the other hand, it was evident from comparison of their sequences that the T54A isolates at baseline were different from the major T54A isolate at the time of re-elevation. The baseline isolate most similar to the major T54A isolate at re-elevation was the wild-type isolate that existed as the dominant population before treatment, and we speculate that the T54A resistance mutation emerged from the wild-type isolate. Likewise, we speculate that the T54A isolate that became the dominant population after treatment was derived from wild type in Patient 7 (data not shown). These results suggest that the preexistence of RAVs does not strongly influence the probability of SVR in IFN-based triple therapy.

5.3.2 SMV/PEG-IFN/RBV Combination Therapy

A second-generation PI, SMV, was approved in Japan in 2013 for use in combination with PEG-IFN plus RBV. We also investigated the correlation between this SMV/ PEG-IFN/RBV triple therapy and the emergence of RAVs. NS3 RAV hot spots for SMV resistance include Q80, R155, A156, D168, and V170, but D168 is the RAV most frequently associated with second-generation PIs, including SMV. In our analysis, 3 out of 26 patients treated with SMV/PEG-IFN/RBV combination therapy failed to achieve SVR, and they each developed D168V. As with TVR, by analyzing the clinical course of these patients, phylogenetic analysis revealed that the composition of viral populations changed significantly and that this change was maintained even after discontinuation of the therapy. Furthermore, the D168V-HCV strains isolated at the time of re-elevation of HCV titer were distinct from the pre-existing D168V-HCVs in all three patients, suggesting that these D168V strains had developed from D168 wild type after acquisition of new mutations. One case with non-SVR is demonstrated in Fig. 5.5.

Considering the results obtained from our analysis for TVR/PEG-IFN/RBV and SMV/PEG-IFN/RBV combination therapy, we speculate the following in the case of PI/PEG-IFN/RBV therapy: (1) antiviral-resistant HCV strains develop not from preexisting minor HCVs with RAVs, but emerge de novo from wild-type HCV after acquisition of new mutations, and (2) the composition of viral populations changes significantly after the development of clinically relevant RAVs, and this change is maintained even after the cessation of triple therapy and reversion to resistance variants to wild type. At present, the impact of the viral compositional change induced during PI/PEG-IFN/RBV therapy on the future of anti-DAA therapy is unknown. However, since this change in viral composition is speculated to be adaptive during exposure to triple therapy, it might weaken the response to future regimens based on PI/PEG-IFN/RBV, and further quasispecies study is needed.

5.4 Interferon-Free DAA Therapy

Recently, DAA therapies are evolving from “IFN-based therapies” to “IFN-free therapies” because of the efficacy, shorter treatment period, simplified administration, and low rate of SAEs. On the other hand, the problem of RAVs affecting the treatment outcome might be more serious in IFN-free therapies for the following reasons: (1) HCV cannot be eradicated by a single DAA agent alone at present, and, therefore, the combination of other classes of DAAs or other drugs such as ribavirin is needed. When two or three different classes of DAAs are combined, the risk in developing multiple DAA-RAVs is elevated. (2) In DAA combination therapy, NS5A inhibitors are frequently used as a backbone. However, it is reported that naturally occurring RAVs for NS5A inhibitors are more frequent than naturally occurring RAVs for PI inhibitors and NS5B inhibitors. (3) It has also been reported that the presence of NS5A RAVs at baseline significantly decreases the SVR rate.

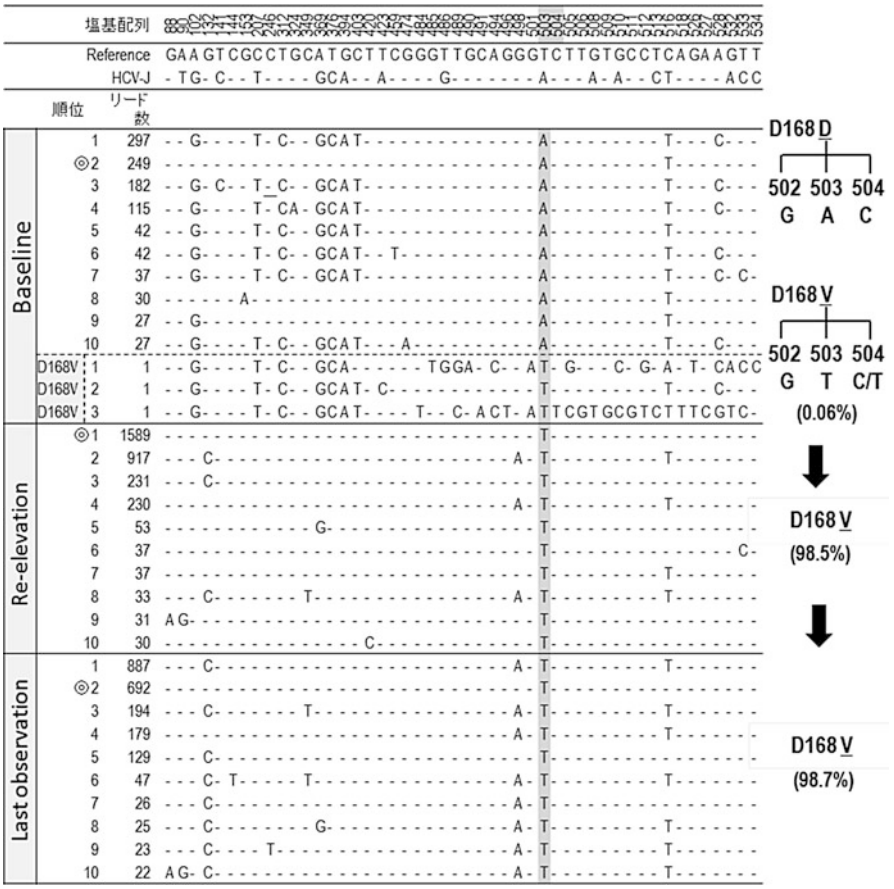

Fig. 5.5 Time-dependent changes in the top ten most populated isolates at baseline, re-elevation, and last observation in a patient who had RAV(D168V) at baseline in SMV/PEG-IFN/RBV therapy

(4) It was reported that RAVs for NS5A inhibitors persist for a long period of time because of their high replication fitness in comparison with wild type. (5) In IFN-containing regimens, the broad antiviral activity of IFN is robust in the presence of most DAA-related RAVs, and therefore, IFN may be useful in treating patients with RAVs.

In Japan, the first-approved interferon-free DAA regimen was the combination therapy of asunaprevir (ASV) and daclatasvir (DCV) in 2014 [12]. In this combination therapy, the presence of NS5A inhibitor resistance RAVs, particularly Y93H and L31M, is known to decrease the SVR rate [13] and may lead to the emergence of triple mutants (e.g., NS3-D168, NS5A-L31, and NS5A-Y93) at the time of treatment failure. HCV strains resistant to both ASV and DCV might develop further cross-resistance to other newer PIs and NS5A inhibitors.

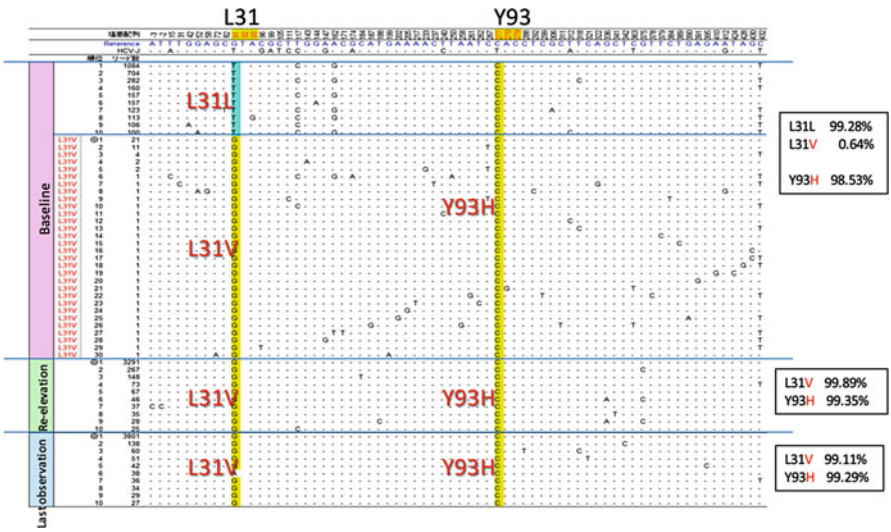

Fig. 5.6 Time-dependent changes in the top ten most populated isolates at baseline, re-elevation, and last observation in a patient who had double RAVs(L31V plus Y93H) as minor populations at baseline in IFN-free ASV/DCV therapy

Against this background, the origin of resistance variants during IFN-free ASV/DCV combination therapy needs to be clarified through deep sequencing and phylogenetic analysis. Our particular concern is to determine whether multidrug-resistant HCV strains emerge by acquisition of new mutations or by selection of preexisting multidrug-resistant strains and to evaluate whether deep sequencing could predict the outcome of treatment. Although analysis of the development of RAVs in ASV/DCV combination therapy is still under way, we encountered a patient for whom a clinically relevant multidrug-resistant HCV strain was considered to have emerged as the result of selection from a preexisting multidrug-resistant strain (Fig. 5.6). Namely, NS5A double mutants (L31M+Y93H) were observed prior to the start of therapy as a minor population, and this population was considered as the origin of the DAA-resistant HCV strain. Although further studies are needed, it is possible that selection for existing variants rather than the new mutations might play the dominant role in the emergence of multidrug-resistant RAVs during IFN-free DAA therapy.

5.5 Conclusions

In this chapter, we briefly described our deep sequencing study coupled with a phylogenetic analysis of RAVs in IFN-based and IFN-free DAA therapies. By applying this strategy, the origin and the role of RAVs in the clinical course could be more evident compared to conventional direct sequencing studies, and in the era of DAA

therapy, understanding the role of RAVs is indispensable for clinical hepatologists to achieve the best treatment outcome.

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